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Amperometric detection of D-sorbitol with NAD^+-D-sorbitol dehydrogenase modified carbon paste electrode

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Abstract

A biosensor based on modified carbon paste electrodes for the amperometric detection of D-sorbitol is described. Carbon paste electrodes were modified by D-sorbitol dehydrogenase (SDH) and nicotinamide adenine dinucleotide (NAD^+) and coated with a non-conducting poly(*o*-phenylenediamine) (PPD) film. After partial oxidation of the immobilised NAD^+ by applying a potential step from 0.0 to 1.2 V (SCE), the modified electrode allowed the amperometric detection of the NADH enzymatically obtained at an applied potential of 0.0 V. The resulting biosensor responded rapidly to sorbitol up to 8×10^{-4} M with a detection limit of 4×10^{-5} M. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Sorbitol biosensor; Amperometry; NAD^+ oxidation

1. Introduction

The electrochemical oxidation of reduced nicotinamide adenine dinucleotide (NADH) in aqueous solution has been extensively investigated because it can be used as the transduction reaction for designing amperometric biosensors based on dehydrogenase enzymes [1–5]. The overpotential for NADH oxidation is very high at solid electrodes. This fact causes a loss of selectivity due to the oxidation of other electroactive species present in the sample. The adsorption of nicotinamide adenine dinucleotide (NAD^+) and products obtained in uncontrolled side reactions competing with the NAD^+ regeneration also causes important interference. Many attempts have been made to reduce the high overvoltage of the electrochemical oxidation of NADH. The use of redox mediators constitutes the main strategy to avoid the above mentioned problems [1]. Recently, a new electrocatalytic system for NADH oxidation was described [6]. When carbon paste electrodes are anodically polarised at potentials above ca. 1.20 V (Ag/AgCl) in neutral and mild alkaline solutions containing NAD^+, a reversible redox system with formal potential close to 0.0 V is formed. This system presents high catalytic activity towards NADH oxidation, decreasing the overpotential for more than 400 mV. It has been identified as one of the oxidation products of the adenine moiety in NAD^+ [6]. The same results were obtained for the NAD^+ incorporated into the paste itself [6,7]. This system allows the developing of biosensors for different dehydrogenase substrates in which NAD^+ acts not only as cofactor of the dehydrogenase enzyme, but also as a precursor of the electrocatalytic system for NADH oxidation. The versatility and the simplicity

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of operation of a sensor based on this new approach were already demonstrated by coupling the enzyme glycerol dehydrogenase to the NAD^+-modified electrode to obtain a glycerol biosensor [6].

A reliable and rapid determination of sorbitol is essential in a number of different fields, such as food, pharmaceutical and cosmetic industry. Sorbitol is used as a bulk sweetener, as an agent for preserving moisture and also as a softener. It is found in numerous food products, such as candies, chewing gums, jams and frozen confections. On the other hand, a load of sorbitol may cause a laxative effect and produce abdominal pain in some persons. Standard methods for the determination of sorbitol, such as gas chromatography [8], are laborious and time consuming. An enzymatic method with soluble sorbitol dehydrogenase (SDH) based on the spectrophotometric measurement of the NADH enzymatically produced is used most commonly [9]. Electrochemical biosensors, which combine the inherent specificity of enzymatic reactions with the sensitivity of amperometric detection, have proved to be very useful in detecting a large number of substrates. In this paper, the electrocatalytic oxidation of NADH associated with the products of NAD^+ oxidation is applied to the construction of a sorbitol biosensor. This biosensor was prepared using a carbon paste electrode modified with SDH and NAD^+, immobilised by a layer of a nonconducting poly(*o*-phenylenediamine) (PPD).

2. Experimental

2.1. Reagents and materials

The SDH enzyme (EC 1.1.1.14) from sheep liver (27 units per mg protein), the cofactor (NAD^+) and D-sorbitol were purchased from Sigma. The monomer, *o*-phenylendiamine (Merck) was used without further purification. A sorbitol test kit (Boehringer Manheim) was used for the determination of sorbitol in real samples. Other chemicals employed were of analytical grade.

2.2. Preparation of modified electrode

Unmodified carbon paste was prepared by thorough mixing 1 g of spectroscopy grade graphite powder (Graphite extrapure, Merck) and 360 μl of paraffin oil (Uvasol, Merck). The NAD^+-SDH modified carbon paste was prepared by mixing the unmodified carbon paste with different proportions of the enzyme (SDH) and the cofactor (NAD^+). A portion of the resulting modified carbon paste was packed into the well of the working electrode and the surface was smoothed on a paper over a glass surface. Finally, the electropolymerisation was accomplished by continuous cyclic potential scanning from -0.45 to 0.65 V (10 cycles) at a scan rate of $50\,mV\,s^{-1}$, in a deaerated 0.1 M phosphate buffer solution pH 8 containing 1×10^{-3} M *o*-phenylenediamine. These PPD-modified electrodes were thoroughly rinsed with phosphate solution and they were pretreated in order to generate the electrocatalytic system for NADH oxidation. The oxidative pretreatment consisted in the application of a double potential step from 0.0 to 1.2 V with a pulse width of 15 s. The electrodes thus prepared were used for the amperometric measurements of sorbitol in a phosphate background solution pH 8.

2.3. Apparatus

Voltammetric measurements were performed using a linear voltage sweep generator PAR model 175, a potentiostat–galvanostat PAR model 173 and a HP 4007 B X–Y recorder. Amperometric measurements were done with a Metrohm 641-VA detector. All potentials were measured and referred to a saturated calomel electrode (SCE) and a platinum foil was used as the counter electrode. The NAD^+-SDH modified carbon paste electrode acted as the working electrode. It was constructed using a home-made carbon paste electrode with a disk-shaped active surface of 3 mm diameter. The electrode surface was smoothed against a filter paper. The cell was a $20\,cm^3$ Metrohm measuring cell.

2.4. Analytical procedure

The biosensor prepared as described above was used for the determination of sorbitol in dietetic ice cream and candies. Prior to analysis, weighed samples (ice cream: 9 g or minced candy: 3 g) were dissolved in water, filtered and diluted to a final volume of 100 ml. The accuracy of the biosensor was evaluated by

comparing it to the enzymatic spectrophotometric standard method using Boehringer Manheim Test kit.

During the amperometric measurements, the modified electrode was placed in 20 ml of a stirred 0.1 M phosphate solution pH 8, at an applied potential of 0.0 V. The background current was allowed to decay to a steady value before aliquots of the diluted sample solutions (typically, 500 and 60 μl of ice cream and candy diluted samples, respectively) were added and the steady-state current was recorded. Then, four aliquots (40 μl) of a stock sorbitol solution (5×10^{-2} M) were injected into the electrochemical cell and a new steady-state current was attained after each addition. The results thus obtained were used to construct a standard-addition graph and to calculate the sorbitol concentration in the sample.

3. Results and discussion

3.1. Design of the sorbitol biosensor and optimisation of analytical performance

As it was shown in an earlier work [6], the electrochemical oxidation of the adenine moiety in NAD^+ and other adenine nucleotides at carbon paste electrodes give rise to redox-active products, which strongly adsorb on the electrode surface. The adsorbed products have shown excellent electrocatalytic activity toward NADH oxidation.

The new electrocatalytic system can be obtained by partial oxidation of NAD^+ in carbon paste electrodes modified with NAD^+ and a dehydrogenase enzyme. In this way, a very easy and rapid preparation of amperometric biosensors for several enzymatic substrates could be achieved. Although, high applied potentials are needed for the oxidation of the adenine moiety in NAD^+, it is possible that the new electrocatalytic system can be obtained in conditions that are compatible with the catalytic activity of dehydrogenase enzymes immobilised on the same electrode surface. To demonstrate this possibility, a sorbitol biosensor was designed by coupling the electrocatalytic detection of NADH by an NAD^+-derived electron transfer mediator with the enzymatic oxidation of D-sorbitol to D-fructose catalysed by SDH, according to the reaction scheme outlined in Fig. 1.

The enzyme SDH and the cofactor NAD^+ were dispersed into a carbon paste matrix as it was described in Section 2.

Reactions catalysed by NAD^+ dehydrogenase enzymes have, in general, an equilibrium constant which highly favours the substrate side. A displacement of the equilibrium can be achieved by employing NAD^+ in excess. In addition, NAD^+ is the precursor of the NADH electrocatalytic system. For these reasons, an excess of NAD^+ was used in the electrode design and a cofactor proportion in the carbon paste of 10% was chosen. A 7% of SDH was used in order to achieve high biocatalytic activity. Higher proportions of modifiers could not be used because the carbon paste was difficult to make homogeneous and disintegration occurred.

The immobilisation of enzyme and cofactor dispersed in the carbon paste was achieved by covering the modified carbon paste electrode with an electropolimerised PPD film, as was described in Section 2. The properties of conducting polymer films are,

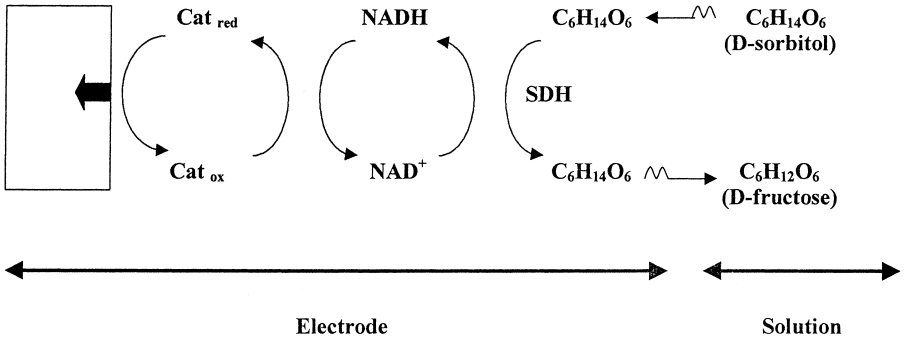

Fig. 1. Reaction scheme for the determination of sorbitol.

in general, influenced by the experimental conditions, which affect the electropolymerisation process. When the PPD electropolymerisation pH is bellow 7, electroactive and electronically conducting films, which have an electrocatalytic effect on NADH oxidation, are obtained [10]. In contrast, at electropolymerisation pH higher than 7, electroinactive films are formed, which are used to prevent interference and electrode fouling [11–13]. In this work, 0.1 M phosphate solution, pH 8 was used as the electropolymerisation medium. The oxidation of *o*-phenylendiamine in this medium showed a completely irreversible oxidation peak at 0.35 V. Continuous scanning resulted in a decrease in the peak current and this behaviour was indicative of a non-conductive polymeric film formation on the electrode surface.

After the electropolymerisation step, the NADH electrocatalyst was generated by partial oxidation of the immobilised NAD^+. The optimum anodic pretreatment was determined in order to obtain the maximum amount of catalyst which leads to highest amperometric response to sorbitol. Double potential steps starting from 0.0 V were applied during the oxidative pretreatment and the effect of the pulse height on the following amperometric response to sorbitol at an applied potential of 0.0 V was analysed. It was observed that no catalytic response is obtained with a pulse height over 1.20 V. Neither an oxidation current was detected if, after the oxidation pretreatment, the potential was set at 0.60 V, a potential at which the direct uncatalysed oxidation of the enzymatically produced NADH takes place. This fact was probably due to the loss of activity of the immobilised enzyme at high anodic overpotentials. Thus, the end potential was set at 1.2 V. This end potential is enough to generate the NADH electrocatalyst from NAD^+ and does not generate an appreciable amount of *o*-quinone groups which could also contribute to the electrocatalytic oxidation of NADH [6].

The effect of the pulse width was also tested. The amperometric response to sorbitol at an applied potential of 0.0 V decreased when pulse widths larger than 15 s were used in the electrocatalyst generation step. This effect may be due to the fact that wider pulses produce not only high amount of the catalytic system, but also a decrease in the amount of enzymatically active cofactor immobilised on the electrode surface [6]. Therefore, the electrolysis time during the preparation step selected for further experiments was 15 s.

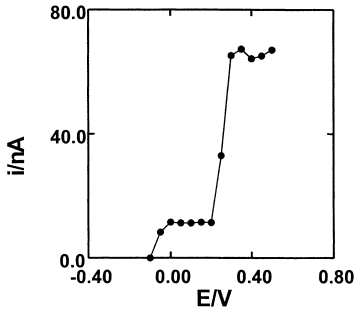

Fig. 2. Hydrodynamic voltammograms for a 10% NAD^+-7% SDH-modified carbon paste electrode covered by PPD and preoxidized. Background electrolyte: 0.1 M phosphate pH 8; sorbitol concentration: 5.0×10^{-4} M.

Modified carbon paste electrodes, prepared under the optimum conditions described, were used for the amperometric detection of sorbitol. The effect of the detection potential on the amperometric response to sorbitol under batch conditions was evaluated over the −0.10–0.50 V range using a 0.1 M phosphate pH 8 solution containing 5×10^{-4} M of sorbitol (Fig. 2). The steady-state current versus detection potential plot shows a plateau between 0.0 and 0.20 V. At potentials higher than 0.25 V, a new increase of current is observed due to the direct uncatalysed oxidation of the enzymatically generated NADH. In order to achieve a good selectivity, the applied potential selected for further experiments should be the less positive potential which guarantee a high catalytic response to sorbitol. A potential of 0.0 V fulfils these conditions and it was selected as the working potential. Carbon paste electrodes modified with the electron transfer mediator, but containing no SDH, show no amperometric response to sorbitol in the assayed potential interval. Nevertheless, a sharp increase in current was obtained after a NADH solution was injected into the cell, even at applied potentials slightly below 0.0 V.

The influence of pH on the amperometric response to sorbitol was examined in a 0.1 M phosphate buffer, over the pH range 5–11. The higher steady-state currents were obtained between pH 8 and 9 (Fig. 3). A 0.1 M phosphate pH 8 was used in the following amperometric measurements. This value is in accordance with the optimum pH reported previously for the enzyme in solution [14]. Higher pH values yielded

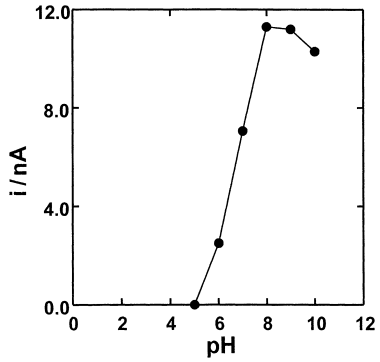

Fig. 3. Amperometric response produced by the 10% NAD^+-7% SDH-modified carbon paste electrode as a function of the background electrolyte pH. Applied potential: 0.0 V; sorbitol concentration: 5.0×10^{-4} M.

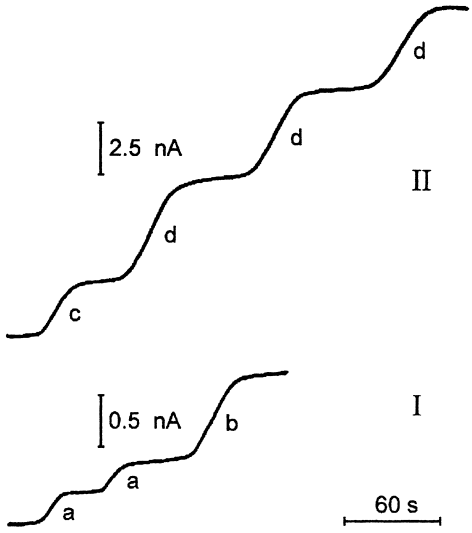

Fig. 4. Amperograms of the biosensor response for successive injections of 20 μl (a) and 60 μl (b) of 0.02 M sorbitol standard solution and 20 μl (c) and 40 μl (d) of 0.1 M sorbitol standard solution. Background electrolyte: 20 ml of 0.1 M phosphate pH 8; applied potential: 0.0 V.

smaller responses and larger background currents and signal-to-noise ratios.

3.2. Response characteristics of the sorbitol biosensor

The amperometric response of the modified carbon paste electrode under optimum conditions to increasing concentrations of sorbitol was investigated using stationary working electrodes in magnetically stirred solutions. Fig. 4 shows amperograms of the background electrolyte to which sorbitol has been added stepwise from 2.0×10^{-5} to 8.0×10^{-4} M. A linear response to sorbitol was obtained up to 8.0×10^{-4} M ($i = (2.05 \pm 0.03) \times 10^4$ [sorbitol] $- (0.3 \pm 0.1)$) ($r = 0.9992$, $n = 7$). The detection limit was estimated as the sorbitol concentration yielding an amperometric signal equal to three times the standard deviation of the intercept, and was found to be 4.0×10^{-5} M. Even though the electrode did not allow sorbitol detection at very low concentration levels, it may be useful from the analytical point of view since high concentrations of sorbitol are normally used in food products.

The reproducibility between different sorbitol biosensors was tested for a set of six recently prepared electrodes. The steady-state current obtained for a sorbitol concentration of 5.0×10^{-4} M was measured at an applied potential of 0.0 V. The resulting mean value was 11.5 nA, with a relative standard deviation of 6.1%. The response time for 95% of the steady-state current was 40 s.

After the biosensor preparation, the slope of the calibration graph remains nearly constant (a decrease of less than 15% was measured over seven successive determinations) over a period of about 48 h. The mean steady-state current measured with the same biosensor in the seven successive determinations at a 5×10^{-4} M level was 10.9 nA, with a relative standard deviation of 5%. After 72 h, the decrease of the slope was found to be 40% of its initial value. Thus, the useful lifetime of the electrode was estimated as 48 h, although it can produce valuable analytical data beyond this time period if recalibration is carried out. The linear interval of the response does not undergo significant changes over the same time period, but the response time was raised to 120 s on the second day after biosensor fabrication.

The stability of the carbon paste modified with NAD^+ and SDH, was checked by making several biosensors from a single batch of modified carbon paste and measuring analytical characteristics of the resulting electrodes. No significant changes in the electrode response were observed within 15 days after the paste preparation. In addition to the very simple and rapid fabrication process, this fact makes the biosensor convenient for routine analytical purposes.

Table 1
Comparison of the amperometric biosensor and the spectrophotometric method for the determination of sorbitol content (% w/w) in food samples

Sample	Biosensor	Spectrophotometric
Ice cream	1.6 ± 0.1 ($n = 3$)	1.51 ± 0.02 ($n = 3$)
Candy	96 ± 3 ($n = 3$)	96.6 ± 0.9 ($n = 3$)

The amperometric response of the proposed biosensor to different polyols was tested in order to check interference with respect to the sorbitol amperometric signal. Xylitol produced practically the same signal as sorbitol at a concentration of 5.0×10^{-4} M, while iditol gives 1.5 times the signal of sorbitol. No response was observed for mannitol and dulcitol.

Sorbitol is not directly oxidised by the electrode because no anodic current was detected when NAD^+ modified carbon paste electrodes interact with sorbitol in the absence of SDH. As the electrode surface was covered by a non-conducting polymeric layer, the access of ascorbate, a common interfering species, to the electrode surface was limited [11–13]. Due to this, no significant interference from ascorbate was observed.

3.3. Determination of sorbitol in food samples

In order to evaluate the application of the biosensor to food samples analysis, the sorbitol content in dietetic ice cream and candy was measured, as it is indicated in Section 2. Table 1 summarises the results obtained for three replicate determinations of sorbitol in each sample. A good agreement is observed between the results from the biosensor and those from the spectrophotometric method.

4. Conclusions

The electrocatalytic system for NADH oxidation based on the products of NAD^+ oxidation was successfully coupled to the sorbitol oxidation by SDH. This system allowed developing a biosensor for sorbitol, which proved to be suitable for the amperometric detection of sorbitol in dietetic products. The simple construction and fast response of the biosensor makes it useful for many practical applications.

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