The development of an improved glucose biosensor using recombinant carbohydrate oxidase from Microdochium nivale

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Biosensors containing recombinant carbohydrate oxidase from Microdochium nivale (rMnO) were developed using either a chemically modified carbon paste or a graphite electrode. 1-(N,N-dimethylamine)-4-(4-morpholine)benzene (AMB) and 1,1′-dimethylferrocene (DMFc) were used as the mediators. The biosensors showed a linear calibration graph up to 0.018 mol dm−3 of glucose when operated at 0.04–0.36 V vs. SCE. Almost no change was detected in the sensitivity of the biosensors at pH 7.2–8.1. The biosensors responded to a range of α-aldoses, but maximal sensitivity of the biosensor was with α-glucose. The biosensors gave no response to polyhydroxylic compounds such as α-mannitol, α-sorbitol and inositol. The advantage of the biosensor in comparison to the biosensor based on Aspergillus niger glucose oxidase is a wide linear range, low sensitivity to oxygen and (in some cases) broad specificity.

During the last two decades both academia and industry have put forth huge efforts to develop biosensors for glucose determination.1 Although many different configurations of glucose biosensors have been proposed, most are based on either: (i) flavoprotein glucose oxidase; or (ii) NAD(P) dependent glucose dehydrogenase; or (iii) oxygen-independent oxidoreductase.2–4 Flavoprotein glucose oxidase from Aspergillus niger is unquestionably the most widely used enzyme in the field of biosensors.2 The A. niger glucose oxidase gene has been cloned, the primary and three-dimensional structures were determined and the active center was established.5–7 The disadvantage of flavoprotein glucose oxidase is its sensitivity to interference by oxygen and a narrow linear range. These problems have been circumvented by using artificial acceptors.8 The principal disadvantage of glucose dehydrogenase is the requirement of NAD(P).9 Glucose dehydrogenase using pyrroloquinoline quinone (PQQ) as primary electron acceptor is an advantage of the biosensor in comparison to the biosensor based on Aspergillus niger glucose oxidase is a wide linear range, low sensitivity to oxygen and (in some cases) broad specificity.

Reagents

Recombinant carbohydrate oxidase from Microdochium nivale (rMnO) was a product of Novo Nordisk A/S and was used as a solution (15 g dm−3) in 0.02 mol dm−3 TRIS-HCl buffer of pH 7.2 containing 0.1 mol dm−3 NaCl. The concentration of the enzyme was determined by using the absorbance of flavin adenine dinucleotide (1.4 × 10−3 mol dm−3 cm−1) at 450 nm.10 Recombinant Coprinus cinereus peroxidase (rCIF) and catalase from A. niger were supplied by Novo Nordisk A/S. The reduction of rMnO in spectral measurements was performed under anaerobic conditions with 0.17 mol dm−3 glucose in 0.05 mol dm−3 phosphate buffer (pH 6.0). Glucose oxidase (GO) from A. niger (type VII; the product of Sigma, St Louis, MO, USA) was used without further purification. 1-(N,N-dimethylamine)-4-(4-morpholine)benzene (AMB) was prepared as described previously.11 The cation radical of AMB was prepared by addition of 2 × 10−3 dm3 of rCIF (10−7 mol dm−3) and 5 × 10−3 dm3 of hydrogen peroxide (0.002 mol dm−3) to 2 × 10−3 dm3 solution of AMB in phosphate buffer of pH 6.0. The reaction was stopped after 5–8 min by addition of 10−7 mol dm−3 catalase (132 000 CIU ml−1). 1,1′-dimethylferrocene (DMFc) was a product from Aldrich (Milwaukee, WI, USA). Graphite powder and liquid paraffin was a product of Merck (Darmstadt, Germany). Spectrographical graphite rods (diameter 6 mm) were a product of Reachim (Moscow, Russia). D-(−)-Fructose was from Fluka (Buchs, Switzerland), β-D-thioglucose from Chemapol, (Prague, Czechoslovakia) and other carbohydrates were obtained from Reachim. A solution of glucose (1.0 mol dm−3) was prepared 24 h before use. Solutions

Experimental

Apparatus

The biosensor current was measured in the thermostated glass cell (10−2 dm3) by means of a three-electrode system at 25 °C. A saturated calomel electrode was used as reference. A platinum plate electrode served as an auxiliary electrode. The current was measured by using a computerized self-made potentiostat.11 The resolution of the A/D converter was 16 bits and the integration time of sampling was 20 ms.

Kinetic curves and absorption spectra were registered by a computer assisted spectrophotometer (Gilford Instrument 2600, Gilford Instrument Laboratories Inc., Oberlin, OH, USA). The kinetics of rMnO-catalyzed oxygen reduction was performed by using a membrane covered oxygen electrode and a computerized system as described previously.11

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of other sugars were kept for 10 h to allow them to mutarotate.

**Biosensor preparation and parameter determination**

Two types of biosensor were prepared. One type was based on a carbon paste electrode (CPE), the other on a graphite electrode. In order to prepare the CPE biosensor a stick (diameter 8 mm, length 70 mm) was made from organic glass. A pocket (diameter 5 mm, 4 mm depth) was drilled in one end of the stick and filled with carbon paste (CP). The CP was prepared by mixing 530 mg of graphite with 260 mg of paraffin oil containing 2.2 mg of AMB. The electric contact of the CP was achieved by inserting a platinum wire into the CP. The surface of the CP was polished with emery paper to form a semisphere. The enzyme solution (5 × 10⁻⁶ dm³⁻) was added onto the surface of the CPE. The electrode was covered with a dialysis membrane (Spectra/por, MWCO 12 000-14 000, Spectrum Laboratories, Inc., Houston, TX, USA). The membrane was fixed with a rubber ring.

The biosensor based on the graphite electrode was prepared by means of a chemically modified graphite rod. The working surface of the electrode was polished with emery paper to form a spherical shape. One part of the graphite rod was isolated by using polyethylene adhesive tape. AMB solution (10⁻⁵ dm³⁻) in methanol (10 g dm⁻³) was dripped five times onto the graphite electrode surface and allowed to dry at room temperature. With DMFc as modifier a 2 × 10⁻³ dm³⁻ solution of DMFc in toluene (5.3 g dm⁻³) was dripped onto the graphite electrode surface and allowed to dry. Then (5–10) × 10⁻⁶ dm³⁻ of enzyme solution (15 g dm⁻³⁻) was placed on top of the chemically modified graphite electrode and the dialysis membrane was tightly attached to the electrode by means of an O-ring.

The biosensors were calibrated by placing them in a thermostated (25 °C) buffer solution and applying a fixed potential until a steady-state background current was established. The solution of the compound was introduced into the cell. A difference between the electrode steady-state current in the presence of the compound and in the blank buffer solution was accepted as a response. The biosensors were stored in buffer solution at 4 °C.

The biosensor sensitivity was calculated as a slope of the linear dependence of the current on the substrate concentration. The calculated sensitivity ± standard error and correlation coefficient is depicted in Tables 1 and 2.

**Results and discussion**

**Parameters of the biosensors based on CPE and AMB**

The response of the biosensor based on CPE is shown in Fig. 1. It can be seen that 96% of the steady-state current was generated almost linear even at low glucose concentrations. The maximal biosensor sensitivity was recorded at the beginning of the experiment. The sensitivity had decreased by 33% on the second day. After 5 d the sensitivity had decreased 34 times (Table 1).

For comparison the calibration of the biosensor containing GO is shown in the same figure (Fig. 2). It may be noticed that the biosensors containing rMnO demonstrated less expressed lag-concentration and a large linear calibration scale.

The biosensor operation can be described by the reaction of oxidized enzyme with substrate (eqn. 1) and oxidation of reduced oxidase by oxidized mediator (M⁺) (eqn. 2):

**Oxidase(ox) + Substrate → Oxidase(red) + Product**  (1)  
**Oxidase(red) + 2 M⁻ → Oxidase(ox) + 2M**  (2)  
**The current is generated during electrochemical oxidation of the mediator (M) (eqn. 3):**  

M → M⁺ + e  (3)

**Table 1** The parameters of calibration of rMnO–AMB–CPE based biosensor in 0.05 mol dm⁻³ phosphate buffer solution of pH 6.6 at 25 °C. Electrode potential 0.04 V vs. SCE

<table>
<thead>
<tr>
<th>Glucose (mol dm⁻³)</th>
<th>Slope × 10⁴</th>
<th>Intercept × 10⁴</th>
<th>Correlation coefficient</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0018</td>
<td>1.90 ± 0.04</td>
<td>-0.9 ± 0.4</td>
<td>0.9982</td>
<td>First day</td>
</tr>
<tr>
<td>0.0014</td>
<td>1.27 ± 0.01</td>
<td>-0.6 ± 0.13</td>
<td>0.9997</td>
<td>Second day</td>
</tr>
<tr>
<td>0.0011</td>
<td>0.955 ± 0.03</td>
<td>-0.06 ± 0.02</td>
<td>0.9893</td>
<td>Fifth day</td>
</tr>
</tbody>
</table>

**Table 2** The relative biosensor response calculated as a ratio of the sensitivities of the carbohydrate and glucose. Phosphate buffer solution (0.05 mol dm⁻³) of pH 6.6, 25 °C and the electrode potential 0.04 V vs. SCE

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Relative response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-(+)-Glucose</td>
<td>100.0 ± 0.8</td>
</tr>
<tr>
<td>d-(+)-Maltose</td>
<td>72.0 ± 2.1</td>
</tr>
<tr>
<td>β-d-thioglucone</td>
<td>71.0 ± 5.8</td>
</tr>
<tr>
<td>d-(+)-Cellobiose</td>
<td>55.0 ± 8.3</td>
</tr>
<tr>
<td>d-(+)-Xylose</td>
<td>46.0 ± 2.7</td>
</tr>
<tr>
<td>d-(+)-Lactose</td>
<td>42.0 ± 1.1</td>
</tr>
<tr>
<td>d-(+)-Galactose</td>
<td>37.0 ± 0.7</td>
</tr>
<tr>
<td>N-acetyl-d-glucosamine</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>L-(+)-Arabinose</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>L-(+)-Rhamnose</td>
<td>0.1 ± 0.8</td>
</tr>
<tr>
<td>d-(−)-Fructose</td>
<td>0.1 ± 0.8</td>
</tr>
</tbody>
</table>

Fig. 1 The biosensor response at pH 6.6 and 25 °C. Electrode potential 0.08 V vs. SCE; each step corresponds to addition of 0.002 mol dm⁻³ of glucose.

Fig. 2 The calibration of the biosensors based on CPE at pH 6.6 and 25 °C. Electrode potential 0.04 V vs. SCE.
The preliminary kinetic measurements showed that the apparent bimolecular constant ($k_{mm}$) of the cation radical of AMB was $4.0 \times 10^6$ mol$^{-1}$ dm$^3$ s$^{-1}$ at pH 6.0 and 25 °C.² The constant value was $1.3 \times 10^3$ mol$^{-1}$ dm$^3$ s$^{-1}$ for the interaction of the enzyme with oxygen (eqn. 4).

Oxidase$_{(\text{red})}$ + O$_2$ $\rightarrow$ Oxidase$_{(\text{ox})}$ + H$_2$O$_2$  
(4)

The corresponding constants for GO were $7.6 \times 10^6$ mol$^{-1}$ dm$^3$ s$^{-1}$ and $1.5 \times 10^6$ mol$^{-1}$ dm$^3$ s$^{-1}$ at 25 °C and pH 7.0, 13,14

The reaction of oxygen with reduced enzyme induced lag-concentration in the calibration graph. Due to the low rate of rMnO reaction with oxygen the lag-concentration was insignificant (Fig. 2). In the case of GO the ratio between the constants for AMB and oxygen was less. Therefore the biosensor based on this enzyme showed more expressed lag-concentration at the same electrode potential (Fig. 2).

The linearity of the biosensor calibration depends on the affinity of the enzyme, measured by $K_{m_{app}}$, as well as on diffusion parameters.² For native rMnO the calculated $K_{m_{app}}$ for glucose was 0.081 mol dm$^{-3}$ at $10^{-4}$ mol dm$^{-3}$ of AMB$^+$ and if $k_{cat} = 45$ s$^{-1}$ and $k_{cat} = 500$ mol$^{-1}$ dm$^3$ s$^{-1}$ were used. A high value of $K_{m_{app}}$ determined a high linear range of the calibration graph (Fig. 2).

The dependence of the sensitivity of the biosensor on the electrode potential was investigated in the range −0.08 to 0.360 mV vs. SCE. The biosensors responded at an electrode potential of more than −0.05 V vs. SCE (Fig. 3). Only a minor change was observed in sensitivity from 80 to 350 mV, whereas background increased at a higher potential. The largest signal to background ratio was established at the electrode potential 0.04 V.

The potential of the biosensor action depended on the formal redox potential of the mediator. For the pair AMB$^+$/AMB the formal potential is 0.15 V vs. SCE.¹³ The dependence of background on the electrode potential (Fig. 3) correlated with the AMB conversion on CPE, whereas generation of the biocatalytical current at lower potential is associated with a catalytic process.

The effect of pH on the sensitivity of the biosensor was explored in the range of pH 6.0–8.1 (Fig. 4). The sensitivity did not change between pH 7.2–8.1. The sensitivity decrease at a pH less than 7.2 can be characterized by $pK_a$ 5.8 ± 0.5. The investigations of kinetics of the cation radical reduction in solution gave $pK_a$ 6.4 ± 0.1. Therefore, the decreased biosensor response in weak acid solution may be attributed to decreasing activity of the enzyme.

Eleven carbohydrates were used for testing the selectivity of the biosensors. The highest sensitivity of the biosensor was towards D-glucose (Table 2). The biosensor also responded to other D-aldoses. A limited biosensor response was noticed for L-arabinose as well. The sensitivity of the biosensor to β-D-thioglucose was found to be 71% compared to D-glucose. The biosensor did not respond to polyhydroxylic compounds such as α-mannitol, α-sorbitol and inositol.

The biosensor selectivity depended on the specificity of rMnO and mass transport effects. Preliminary experiments showed that in a homogeneous solution, the enzyme catalyzed the oxidation of a broad spectrum of carbohydrates showing higher activity towards di- and oligosaccharides than towards the monosaccharide glucose. The activity with D-glucose corresponded to 69% of the activity with D-cellobiose. However, the highest sensitivity of the biosensor was recorded for D-glucose (Table 2) which can be associated with a larger glucose distribution coefficient and penetration rate through the cellulose membrane of the biosensor.

### Parameters of the biosensors based on graphite electrodes

The biosensors based on graphite were produced by means of rMnO using AMB or DMFc as the mediator. The biosensor containing AMB showed a linear calibration graph up to 0.014 mol dm$^{-3}$ (Fig. 5). The sensitivity was approximately three times lower than the sensitivity of the CPE based biosensor. However, the graphite based biosensor showed high stability as the response of the biosensor was almost unchanged after seven days (Fig. 5).

The biosensors based on DMFc as mediator responded to glucose at electrode potentials above 0.04 V (Fig. 6). The biosensors showed highest sensitivity at electrode potentials of 0.12–0.16 V. At these potentials the biosensor background was also rather high (Fig. 6). In all potential ranges the calibration graph was linear up to 0.01 mol dm$^{-3}$ of glucose (Fig. 6). The sensitivity of the DMFc based biosensor at 0.12 V was similar to the AMB modified graphite electrode at 0.04 V (Figs. 5 and 6). The reactivity of oxidized DMFc with reduced enzyme has not been measured, but it was estimated to be high, as the
calibration graph remains linear even at low electrode potentials, when the concentration of oxidized mediator was low (Fig. 6). The biosensor responded at higher electrode potentials since the formal redox potential of DMFc is higher, i.e., 0.19 V vs. SCE.8

The stability of the biosensors’ response depended on both the stability of the enzyme and the stability of the chemically modified electrodes (ChME). The biosensors produced by using a graphite electrode showed the highest stability of the response (Fig. 5). Since the stability of the biosensors depends on the type of the electrode and the mediator used, the main factor determining long term response of the biosensor might be the stability of ChME. Leakage of mediator has been documented15 for ChME, especially CPE modified with AMB. The decrease in the amount of mediator reduced the response of the biosensor and the linearity of the calibration.12

References