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# Biosensor based on an oxygen reducing bilirubin oxidase electrode

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## Abstract

An oxygen reducing electrode made of bilirubin oxidase and multi-walled carbon nanotubes (BOD-MWCNT-Au electrode) is coupled to enzymes catalysing oxygen-consuming reactions such as glucose oxidase (GOD) to result in a membrane-free bienzyme electrode. The feasibility of such a molecularly assembled system stabilized by covalent linkage has been demonstrated. The electrochemical characterisation of the bienzyme electrode reveals sensitivity to the enzyme substrate. The results indicate that the BOD-electrode provides a suitable platform for sensing analytes for which oxidases of high activity are available.

*Keywords:* bienzyme sensor, bilirubin oxidase, carbon nanotubes, membran-free, mediator-less

## 1. Introduction

Bilirubin oxidase (BOD) can be effectively used for bioelectrocatalytic oxygen reduction. This enzyme belongs to the group of ‘blue’ multicopper oxidases containing three different types of copper centres. Other examples are laccase, ascorbate oxidase and nitrite reductase. BOD oxidises bilirubin to biliverdin while the oxygen is reduced to water by electron transfer via the T1 centre and the trinuclear T2/T3 cluster [1]. The relatively high stability [2] and the high oxygen tolerance [3] are reasons for its application as cathodic biocatalyst in several biofuel cells. BOD-electrodes have a high start potential for oxygen reduction at physiological pH [4,5]. Direct electron transfer between the enzyme and an electrode can be established via multi-walled carbon nanotubes (MWCNT) [6]. A BOD-MWCNT-Au electrode with covalently bound BOD can achieve current densities of  $500 \mu\text{A}/\text{cm}^2$  [7].

In the course of development of new biosensors different requirements have to be fulfilled. Besides a high specificity and sensitivity to the analyte also a fast response and a low sensitivity to interfering substances is required. Short response times to changing analyte concentrations can be obtained by construction of membrane-free sensor systems. This can be realised by fixing all parts of the sensing layer directly to the electrode surface.

One problem of the electrochemical detection of different analytes is the appearance of disturbing signals by interfering substances such as ascorbate, uric acid and acetaminophen (paracetamol) in physiological samples. At higher potentials these substances will be oxidised on the working electrode and may distort the measuring signal. This problem can be solved by the application of selective membranes [8]. But these selective barriers are often limiting the diffusion of the substances to be analysed and the response time is increased. To avoid both the

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interfering signals and an increased response time it is possible to work in a potential window where no reactions with potentially interfering substances occur.

Another problem in the construction of biosensors is the electron transfer from the sensing enzyme to the electrode and vice versa. With a direct protein electrode communication the use of mediators can be circumvented. However the direct contact of proteins to a technical surface often changes their structure and disturbs their function. MWCNTs can provide a surface to avoid these negative effects.

By considering all these requirements it seems to be promising to combine the high oxygen sensitivity of a BOD-MWCNT electrode with the specificity of oxygen-consuming enzymes. In such a system the selected oxidase has to recognise and convert the analyte while consuming the co-substrate oxygen. The resulting oxygen depletion near the electrode surface then should be transformed by the BOD-electrode via biocatalytic oxygen reduction and direct electron transfer into a measurable signal.

## 2. Experimental

### 2.1. Electrode preparation

Multi-walled carbon nanotubes with thiol surface groups (NANOCYL S. A., Belgium) were suspended in ethanol and a treated by sonification. The surface of the gold electrode was wet polished with sandpaper. Then an amount of the ethanolic MWCNT suspension was applied onto the polished electrode surface and dried. After this the MWCNT modified gold electrode was incubated in a Sulfo-GMBS-solution (N-[ $\gamma$ -maleimidobutyryloxy]sulfosuccinimid ester) to activate the thiol groups of the nanotubes and then in a BOD-solution for coupling the enzyme. After washing GOD or AOD were applied onto the BOD-MWCNT electrode. Then the crosslinker solution (glutaraldehyde (GA), ethylene glycol bis[sulfosuccinimidylsuccinate] (S-EGS) or disuccinimidyl glutarate (DSG)) were added. Subsequently the electrode was washed in order to remove unbound enzyme and impurities. The resulting electrode assembly is schematically given in fig. 1.

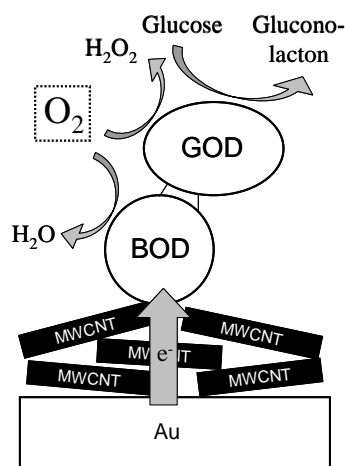


Fig. 1. Assembly and function of the GOD-BOD-MWCNT-Au electrode. By switching on the catalytic activity of GOD in the presence of glucose enhanced oxygen consumption depletes the concentration near the electrode surface. Thus oxygen reduction at the BOD is reduced resulting in a diminished cathodic current.

## 2.2. Electrochemical measurements

The electrochemical characterisation was carried out by linear sweep voltammetry and chronoamperometry in a 1 ml-measuring cell with a 3-electrode arrangement at room temperature. For the measurements an air-saturated citrate-phosphate-buffer was used. A working gold electrode ( $2 \text{ mm}^2$ ), an Ag/AgCl, 1 M KCl reference electrode and a platinum wire as counter electrode were applied. The voltammetric measurements were performed in a range from 0 V to +0.6 V with a scan rate of 10 mV/s and a step potential of 2 mV with a CH instrument (CHI 800, USA) in an unstirred solution. For the amperometric experiments at different potentials a magnetic stirrer with a rotation speed of 2500 rpm was used.

## 3. Results and discussion

After fixing the thiol groups containing MWCNTs by chemisorption onto the gold working electrode, coupling BOD to the MWCNT-surface groups and linking GOD to the BOD-MWCNT electrode the reaction of the assembled bienzyme sensor to different analyte concentrations was examined by means of linear sweep voltammetry. Figure 2 shows the current response to glucose additions in a millimolar concentration range. The voltammetric oxygen reduction starts at a potential of +500 mV vs. Ag/AgCl, 1 M KCl and results in a diminished current in the presence of the GOD substrate. These measurements clearly demonstrate the feasibility of the sensor concept.

In a next step different crosslinkers for an effective coupling of GOD onto the established BOD-MWCNT electrode were tested. Therefore we used the homobifunctional reagents S-EGS, DSG and GA (see experimental). The voltammetric measurements in the presence and absence of glucose suggest that a covalent linkage with GA is the most efficient method to bind a high amount of the GOD for an effective oxygen consumption in front of the oxygen reducing BOD-electrode (fig. 3).

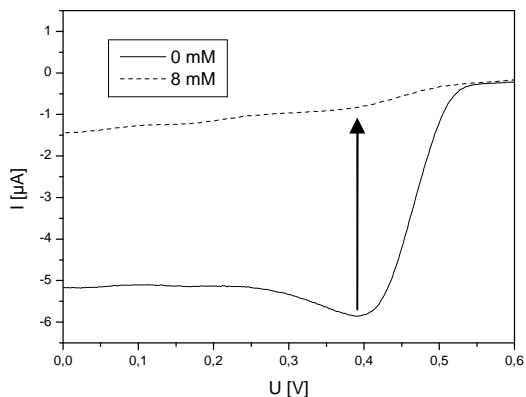


Fig. 2. Voltammograms of the oxygen reduction current of a GOD-BOD-MWCNT-Au electrode at different glucose concentrations.

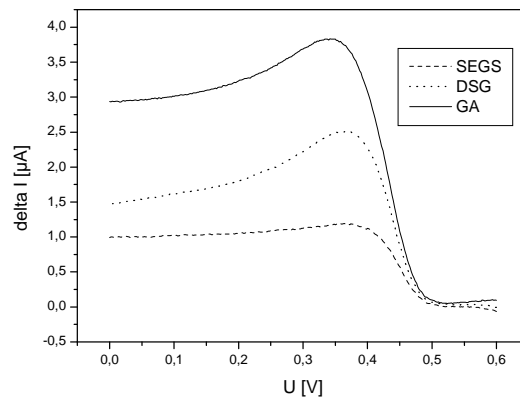


Fig. 3. Voltammograms of three BOD-MWCNT electrodes covalently coupled with GOD by different homobifunctional crosslinkers. The difference voltammograms of 0 mM and 1 mM glucose are shown.

Beside the sensitivity also the pH-dependence of the current response is an important parameter of a biosensor. That's why the GOD-BOD-MWCNT-Au electrode was voltammetrically examined with buffers of different pH-values (citrate-phosphate-mixtures). The sensor measurements (fig. 4) indicate rather high signals in the broad pH-range from 4 to 8.

Furthermore the selectivity of the GOD-BOD-MWCNT-Au electrode was investigated. Therefore the amperometric current responses to several interfering substances at different potentials were recorded. In this study

the focus was on ascorbate, uric acid and acetaminophen which are common interferences in sensorial blood parameter analysis. Fig. 5 represents the data for acetaminophen as an example. The curves clearly indicate that at a potential of 0.1 V the bienzyme sensor shows only a very small interference by acetaminophen.

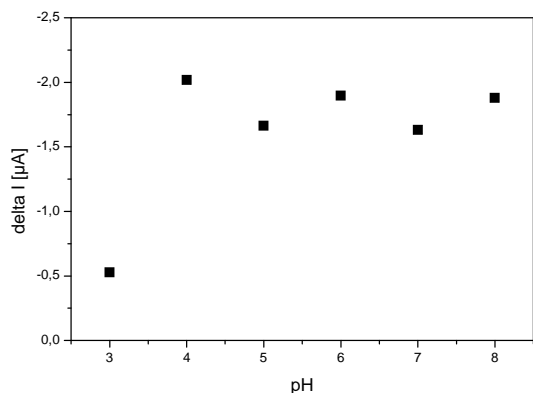


Fig. 4 pH-dependence of the bienzyme sensor signal for an 1 mM glucose concentration change

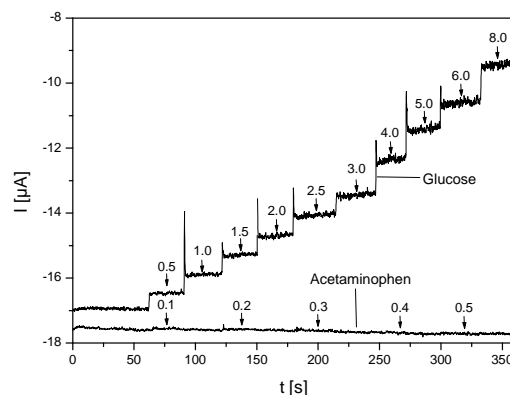


Fig. 5. Comparison of the amperometric current response to a concentration increase of glucose and acetaminophen. The numbers above the arrows indicate the concentration in mM.

Further experiments are directed to extend the applicability of this concept by coupling other oxidases of high catalytic activity to the BOD-MWCNT electrode. As a first example voltammetric measurements of an AOD-BOD-MWCNT-Au electrode can be shown to result in a clear substrate-dependent oxygen current decrease.

#### 4. Conclusions

The feasibility of a bienzyme sensor with a specific oxidase and a transducing BOD-MWCNT-Au electrode is demonstrated with the example of GOD. In an amperometric mode of operation at a potential of 0.1 V the GOD-BOD-MWCNT-Au electrode can detect glucose concentrations down to 250  $\mu\text{M}$ . The bienzyme sensor shows nearly no interferences by acetaminophen, ascorbic and uric acid (up to 500  $\mu\text{M}$ ). Considering its performance in a wide pH-range and the fast response rate it can be stated that with such kind of bienzyme electrode essential demands of a biosensor can be met.

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