Selective H_2O_2 Electrodes for Biosensor Applications

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Hydrogen peroxide was detected amperometrically with gold electrodes modified with thin layers of ruthenium and rhodium. The ruthenium layer was radio frequency (r.f.) magnetron sputtered and rhodium layers were made by vacuum evaporation. Using the cathodic reduction at a potential of -100 mV vs. Ag/AgCl/0.1 M KCl between 2 μM and 10 mM H_2O_2 was detected under FIA (Flow Injection Analysis) conditions. By a combination of these electrodes with a glucose oxidase modified membrane, glucose can be detected in the range between 2 μM and 10 mM which is a good result for new glucose sensor improvements.
The electrodes showed high operational stability and high selectivity against many electroactive substances as well.

For the development and application of amperometric biosensors the selective and sensitive detection of hydrogen peroxide plays a key role. To achieve H_2O_2 detection potentials in the electrochemically optimum range between -100 and +50 mV [1], peroxidases of the protoporphyrine IX type were immobilized on different electrode materials with [2-7] and without redox mediators [8-11]. Except the electrodes proposed by Heller *et al.* [3, 4], all these electrodes showed relatively low operational stability especially under flow conditions.
Gorton and Svensson [12] investigated carbon electrodes modified with sputtered thin layers of palladium/gold and achieved a considerable decrease of the potentials both for the anodic and the cathodic detection of hydrogen peroxide with especially encouraging results were obtained at potentials more negative than 0 mV vs. Ag/AgCl reference electrodes. Thereafter Wang *et al.* [13-16] proposed the use of carbon electrodes modified with particles of rhodium [15], ruthenium [14, 16] and platinum [13] and achieved very low potentials for the detection of hydrogen peroxide by electrochemical reduction. Recently, O'Connell [17] used the cathodic deposition of ruthenium and rhodium layers on glassy carbon electrodes in order to achieve similarly low detection potentials. They gave little or no information about the ratio of the signal to background current.
This paper presents a first investigation of Rh/Ru modified gold electrodes prepared by plasma vacuum deposition. The aim was to achieve low potentials for the detection of H_2O_2, combined with high operational stability and minimized background currents. Hydrogen peroxide is one of the typical products of the flavine-dependent reductase enzymes catalized reactions. There is still a great demand for amperometric sensors for both H_2O_2 and glucose with an improved selectivity. Glucose oxidase was immobilised on highly porous membrane and thereafter everything was placed on a selected Rh/Ru modified gold electrode to construct a glucose electrode. The main sensor parameters were evaluated under FIA conditions.

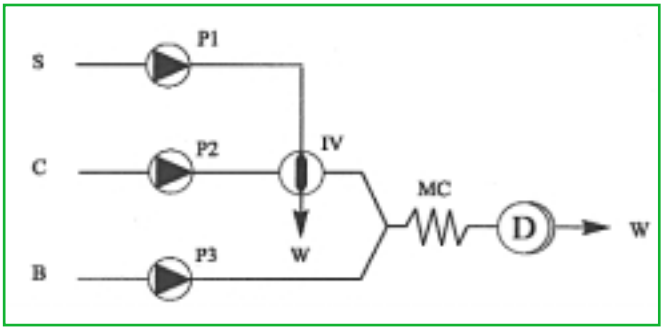

Figure 1 - FIA set-up: P1 peristaltic pump, P2/P3 piston pumps, IV injection valve, MC mixing coil, D flow detector cell, S sample solution, B and C buffer solutions, W waste

Materials and Methods

Chemicals and reagents
Ascorbic acid, uric acid, paracetamol, β-NADH, glucose, glycine, sodium sulfite, EDTA, lithium-potassium acetyl phosphate, cysteine, sarcosine and glucose oxidase (GlucOD) from *Aspergillus niger* (cat. no. G-9010, approx. 1,600 U ml^{-1}) were purchased from Sigma (Deisenhofen, Germany) and used without further purification. All other chemicals were of analyti-

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Figure 2 - Picture of the detection flow cell used for hydrogen peroxide and glucose determinations

cal grade and from Merck (Darmstadt, Germany). 0.1 M potassium phosphate buffer solutions containing 1 mM EDTA were prepared from bidistilled water and used as the carrier solution.

Measuring set-up

Figure 1 shows the FIA (Flow Injection Analysis) set-up, which was used to investigate the H_2O_2 and the glucose detection. The sample solutions were injected into the carrier solution C by a pneumatically actuated injection valve IV (Rheodyne 9010, Cotati, USA) with a sample loop of 20 µl. The carrier was mixed with the buffer solution B in a tightly knotted PTFE tube with a inner diameter of 0.5 mm and a length of 20 cm. The amperometric flow detector D, which is diplayed in Figure 2, consists of two plexiglas plates with a groove (depth 0.1 mm, width 2 mm and length 10 mm) in one side of them. The Rh/Ru modified foil electrode was inclined by an angle of 45° to prevent the fixation of gas bubbles. The effective geometric area for the indicator electrode was 20 mm^2. The enzyme modified membranes were layered on the indicator electrode, whereas a Ag/AgCl/0.1 M KCl electrode served as the reference electrode. The counter electrode was a stainless steel capillary (inner diameter of 0.5 mm, length of 7.4 mm) mounted at the outlet of the detector cell. The cyclic voltammograms were recorded with the potentiostat/galvanostat model 263A (Princeton Applied Research).
Hydrodynamic voltammograms were recorded with the potentiostat CPE-1 (Institut für Technische Biochemie e.V., Halle, Germany) under FIA conditions.

Preparation of the electrodes and enzyme immobilization

To prepare the indicator electrodes, thin gold foils (purity of 99.995% and thickness of 0.1 mm) were covered with two different metallic layer. At first the ruthenium layer was deposited on gold foil by r.f. magnetron (13.56 MHz) sputtering procedure. The Ru deposition was carried out with argon as carrier gas, with a power of 150 W and at a residual pressure of 1 Pa. The thickness of the Ru layer was adjusted by the deposition time and measured by the quartz oscillation method. Ruthenium thin layers with a thickness of 12 nm were used for the following amperometric measurements. By means of TEM observations reported elsewhere [18] it was assumed that the ruthenium forms a continuous layer on the gold surface.
Additional non-continuous rhodium layers with various thickness were deposited by thermal evaporation at high vacuum conditions (<10^{-3} Pa) on the ruthenium. The discontinuous structure do permit to give the thickness information only as apparent values. However it could be expected that both ruthenium and rhodium are able to catalyze the amperometric detection of H_2O_2. In order to investigate the vertical structure of these ruthenium/rhodium modified gold electrodes, also cross-sectional TEM investigations were carried out. The focused ion beam (FIB) technology was applied for the high-precision cross-sectional preparation. More details about the FIB preparation technique and the plasma polymerization are given in [19] and [20], respectively. Cross sectional TEM analysis showed that the ruthenium layer has a thickness of 80 nm and is substantially thicker as used for the sensor experiments. The rhodium layer (thickness 10 nm) was showed on top of the ruthenium layer to cover it completely. At lower effective thickness of the rhodium layer, the ruthenium layer is not completely covered. GlucOD was immobilized covalently onto a preactivated nylon membrane (Immunodyne, Pall, Dreieich, Germany, mean pore size 0.45 µm) by the following procedure: 400 µl of enzyme solution were purified by ultrafiltration, dissolved in 400 µl of 0.1 M potassium phosphate buffer, pH 8.0, and concentrated up to 100 µl. Thereafter 25 µl were dropped onto the membrane and dried.

Results and Discussion

Detection of hydrogen peroxide

Figure 3 shows a hydrodynamic voltammogram (A) and the signal to background current ratio (B) recorded for the H_2O_2 detection at a flow rate of 1 ml min^{-1} in the detector cell. The signal current shows a slightly inclined plateau in the potential

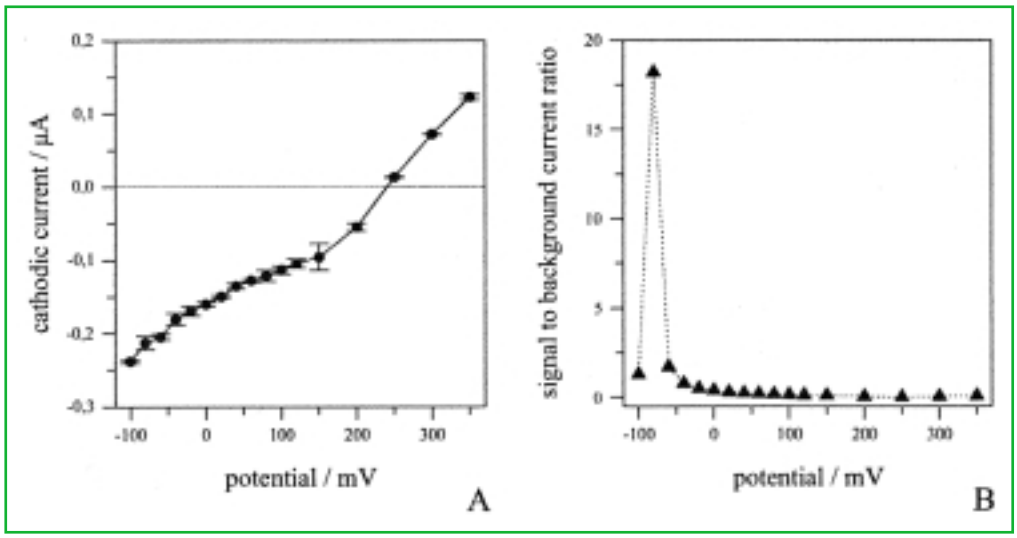

Figure 3 - (A) Signal current and (B) ratio of signal current to background current as a function of the potential; 1 mM H_2O_2 in 0.1 M potassium phosphate buffer and 1 mM EDTA, pH 7.0, v_D=1 ml min^{-1}

range from 0 to +100 mV, whereas the highest signal to background ratio was measured between -100 and -50 mV. Between +200 and +300 mV the direction of the signal current is reversed. To achieve maximum selectivity against easily reducible substances, such as ascorbate, the following experiments were performed at potential E=-100 mV.
The background current increased by decreasing the carrier buffer pH because of the thermodynamic promotion of the cathodic reduction of dissolved oxygen. The signal to background ratio increased with increasing pH values, reaching a maximum in the pH range 8.0-9.0. Taking into consideration the combination of H_2O_2 detection with the glucose oxidase catalyzed analyte conversion the further investigations were performed at pH 8.0.
According to Figure 4 the signal current is increasing only slightly with increasing flow rate under continuous flow conditions. The peak current h, recorded under FIA conditions, is decreasing with the flow rate, indicated as v_D, according to Eq. (1). It can be assumed that the decrease of h is predominately caused by increasing dispersion D, which was defined by Ruzicka and Hansen [21]:

$$h = const v_D^{-0.5} \quad (1)$$

At D=1 the electrode response self seems to be predominantly controlled by diffusion to the electrode surface.
According to Figure 5 a double logarithmic calibration graph was measured between 2 μM and 10 mM H_2O_2.
In the range between 5 μM and 2 mM the graph can be described by Eq. (2):

$$lg(h/\mu A) = (0.891 \pm 0.010\ lg(c/M) + (2.53 \pm 0.04) \quad (2)$$

with r^2=0.999, α=0.05, the number of standard concentrations m=10, the number of replicates n=4, the peak height h and the analyte concentration c. A detection limit of 2 μM was achieved at a signal to noise ratio of 3 under FIA conditions.
The long term operational stability was tested by sequential injection of 1 mM H_2O_2 at a flow rate of v_D=0.2 ml min^{-1} and a frequency of 20 injections per hour. After a period of 2.5 hours, during which the electrode surface has been stabilized, a relatively stable peak signal was achieved for the next 25 hours resulting in an operational stability better than 90% related to the initial peak height.
After more than two or three days of continuous use under these conditions the rhodium/ruthenium layer was sometimes stripped off by corrosion.
The selectivity of many amperometric biosensors is restricted by the anodic oxidation and sometimes also by the cathodic reduction of interferents. The resulting side reactions can also cause electrode fouling and poisoning. To verify the selectivity of the proposed indicator electrode, the influence of electrochemically active compounds was investigated in the presence and the absence of H_2O_2. To this purpose a 1 mM H_2O_2 solution was mixed continuously with 0.1 M phosphate buffer at pH 8.5 containing 2 mM of the corresponding or no interferent at a flow rate ratio of 1 just before the injection valve. The Table summarizes the results.
The measured peak heights were related to the peak height measured for 1 mM H_2O_2 in the absence of any interferent. With the exception of ascorbic acid, cysteine and sulfite the tested interferents had no significant influence on the H_2O_2 detection.
Cysteine and after a longer contact also sulfite caused a significant loss of sensitivity for the following H_2O_2 detection indicating a poisoning of the electrode surface. It should be noted that in comparison to earlier described H_2O_2 indicator electrodes, ascorbic acid had a relatively small effect on the response. Paracetamol did not show any significant effect both in presence and absence of H_2O_2.

Detection of glucose

The determination of glucose was performed under the same conditions as for the H_2O_2 detection but at v_D=0.3 ml min^{-1}: a detection limit of 2 μM was achieved. The double logarithmic re-

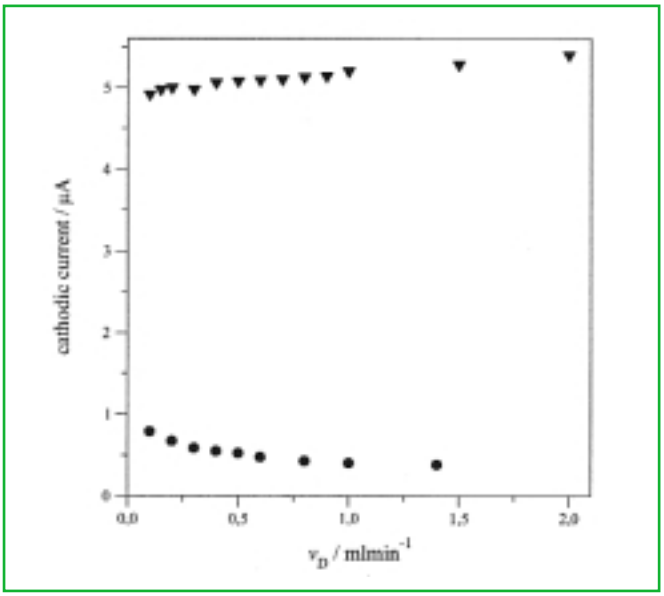

Figure 4 - Dependence of the H_2O_2 signal current for the Ru/Rh modified gold electrode on the flow rate under continuous flow (▼) and FIA (●) conditions; [H_2O_2]=1 mM, pH=8.0, E=-100 mV

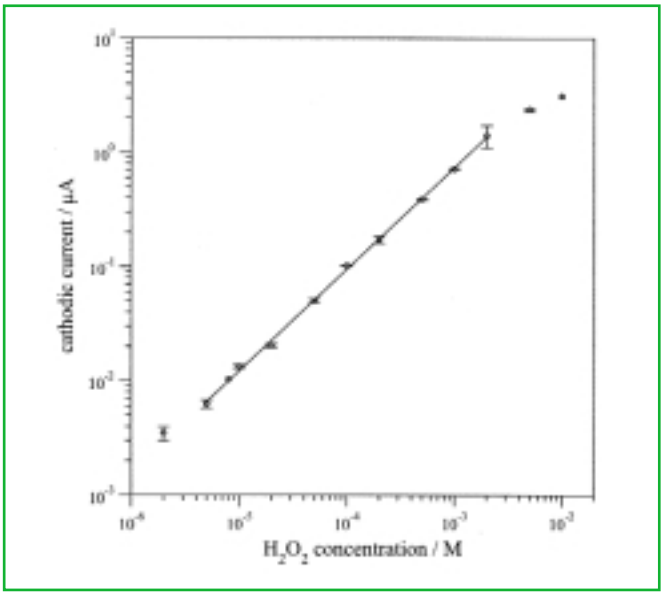

Figure 5 - Calibration curve of the amperometric hydrogen peroxide detection under flow injection conditions; v_D=0.2 ml min^{-1}, pH=8.0, E=-100 mV

Table - Selectivity of the amperometric hydrogen peroxide detection

Interferent (1 mM)	*Relative peak height n the presence of H_2O_2 (%)*	*Relative peak height in the absence of H_2O_2 (%)*	*Relative decrease of the H_2O_2 signal after injection of interferent (%)*
Without	100.0±1.1; n=5	0.0±0.0; n=3	
Ascorbic acid	85.0±1.7; n=6	-10.8±0.4; n=11	0.0±2.0; n=7
NADH	96.8±0.6; n=6	0.0±0.0; n=6	3.0±0.5; n=6
Glucose	99.7±0.3; n=10	0.0±0.0; n=6	1.9±0.4; n=6
Glycine	95.6±0.6; n=5	0.0±0.0; n=5	0.0±0.8; n=4
Formaldehyde	96.6±0.7; n=4	0.0±0.0; n=4	0.7±1.4; n=6
Sarcosine	97.3±0.8; n=5	0.0±0.0; n=5	0.0±0.8; n=5
Paracetamol	100.4±0.4; n=5	0.0±0.0; n=5	1.2±0.4; n=5
Acetyl phosphate	99.4±0.4; n=4	0.0±0.0; n=4	0.0±1.1; n=3
Ethanol	99.8±0.7; n=5	0.0±0.0; n=5	1.0±3.0; n=5
Methanol	99.4±1.6; n=4	0.0±0.0; n=4	0.0±2.0; n=5
Cysteine	57.1±1.0; n=6	-8.5±0.7; n=4	36.0±1.0; n=6
Sulfite	91.8±0.9; n=6	-5.1±0.5; n=6	0.0±0.9; n=7

n=number of repeated measurement; $[H_2O_2]$=0.5 mM; v_D=0.2 ml min^{-1}

gression function can be described by equation (3) in the range from 0.01 to 1 mM glucose (r^2=0.998, α=0.05, m=7, n=4):

$$lg(h/\mu A) = (0.962\pm0.019)lg(c/M) + (2.16\pm0.06) \quad (3)$$

An operational stability better than 90% was obtained during a period of more than 20 hours under FIA conditions at a frequency of 18 injections of 0.1 mM glucose per hour.
The selectivity of the glucose was investigated under the same conditions as those of the H_2O_2 detection by mixing continuously 1 mM glucose solution with interferent solutions of different concentrations: only cysteine caused a significant negative bias and a poisoning effect.

Conclusions

Gold electrodes with a thin layer of ruthenium and thereupon a porous layer of rhodium was used for the first time to detect hydrogen peroxide amperometrically at potentials in the range between -100 and 0 mV vs. Ag/ AgCl/0.1 M KCl by using the cathodic reduction of H_2O_2. Both a high operational stability and a high selectivity against many electroactive substances are achievable. The electrode has been used as an indicator electrode behind oxidase modified membrane layers for the selective detection of glucose. A relatively good linear detection of glucose has been achieved along with a high operational stability and a high selectivity under flow conditions.

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