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Recessed microelectrode array for a micro flow-through system allowing on-line multianalyte determination in vivo

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Abstract Using CMOS-compatible processes, a microelectrode system for use in a micro flow-through cell was manufactured. The electrode was specially designed to enable multianalyte determination with immobilized oxidase enzymes and combines minimal flow dependency with a very small dead volume (< 1 μL) of the cell. This allows biomedical applications like measurements of glucose and lactate in interstitial fluid, which can be collected by ultrafiltration. Besides a 3-electrode system with 4 individually addressable platinum working electrodes, the sensor contains 2 electrodes that measure the conductivity of the sample as well as a Pt thermoresistor to measure the temperature. The temperature dependence in enzyme reactions can thus be controlled during on-line measurements. The 4 working electrodes comprise multielectrode arrays, each comprising 192 micro-holes with a diameter of 3.6 μm. They are arranged symmetrically around the central counter electrode, which is surrounded by a circular Ag/AgCl reference electrode. Between the array and the reference electrode are the loops of the Pt thermoresistor. The thermoresistor is electrically insulated from the measurement solution by a Si_3N_4 layer.

A method for the pretreatment of platinum thin-film electrodes that increases the reversibility of the electrode process is described. The chemical modification of the working electrodes by electropolymerization of a resorcinol/1,3-diaminobenzene mixture enables interference-free measurement in blood and plasma as well as protection against electrode fouling.

Dedicated to Professor Dr. Karl Cammann on the occasion of his 60th birthday

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1 Introduction

Microelectronic fabrication processes allow the production of microsensors that have a high density of different sensors in one spot. Such microsensors facilitate the simultaneous measurement of physical parameters such as temperature and conductivity and several biomedical parameters (e.g., glucose and lactate concentration) with one device [1]. Miniaturized thin-film electrodes making use of the unique properties of microelectrodes, such as their higher current density through hemispherical diffusion and their favorable ratio of Faradaic to capacitive current [2], have already been produced for various purposes and studies have clearly shown their advantages [3, 4].

Great interest in the implantation of micro glucose measurement devices has recently been expressed [5], but there are considerable risks that accompany such a procedure (biocompatibility problems, movement of the device inside the body, encapsulation problems). We are investigating methods for the measurement of glucose and lactate in the interstitial, extracellular fluid using microdialysis for sample collection. Research has shown that there is a strong correlation between glucose levels in the blood and in the interstitial fluid and the literature strongly indicates the feasibility and advantages of this method [6–8]. Continuous monitoring of body fluids through minimally invasive procedures creates a need for a flow-through device with a very small volume and the ability to measure several substances at the same time without interference. This goal in mind, we have been constructing a microelectrode chip that will be part of a flow-through chamber with a volume in the nanoliter range, which will enable the use of flow rates of about 200 nL/min, a rate at which complete recovery can be achieved [9, 10].

CMOS-compatible processes allow the mass-fabrication of electrodes, lowering production costs while giving

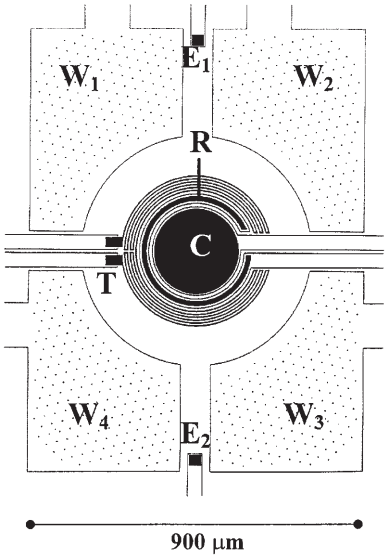

Fig. 1 Electrode layout. W_1–W_4: Working electrode arrays. *R*: Ag/AgCl reference electrode. *C*: Counter electrode. *T*: Pt-thermoresistor. E_1, E_2: Electrodes for conductivity measurements

a high reproducibility. We have been constructing a special electrode system to meet the needs mentioned above (Fig. 1.). On a single 3 × 8 mm chip, we positioned an electrode in an area of 900 × 1200 µm, which will later be surrounded by the seal of a flow-through cell. This area contains a Pt thermoresistor, electrodes for conductivity measurements and a 3 electrode system with 4 working electrodes to allow either the measurement of 2 analytes with a bipotentiostat (2 unmodified electrodes measuring and deducting the background current) or 4 analytes simultaneously. The single electrodes of the working electrode are 27 µm apart, preventing the overlapping of the single diffusion fields. We have exploited the advantages of microelectrode behavior without having the disadvantage of a small current. The single working electrodes were placed in a 3.6 µm deep recess, which reduces the dependence of the electrode signal on the flow speed and leads to a better adhesion of the matrix in which the enzymes will be immobilized. Due to non-linear diffusion at the edges of the electrode, the simple Cottrell equation, which describes the current-time response for macroscopic electrodes, has to be modified with a correction term to describe the current time relation for microelectrodes [11]. This correction term describing the diffusion-limited current, $I_{l,m}$, is dependent on the electrode shape. For an inlaid disc microelectrode it is given by:

$$I_{l,m} = \pi rnFD_0C_\infty^0$$

Where r represents the radius of the electrode, n the number of electrons transferred, F the Faradaic constant, D_0 the diffusion coefficient and C_∞^0 the concentration of the electroactive species in the bulk of the solution. If the disc electrode is recessed within an isolating medium, the diffusion inside the hole must be considered as well, leading to a slight modification of the correction term:

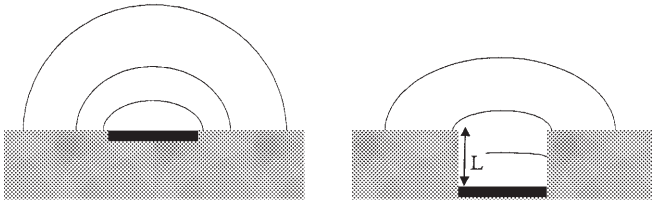

Fig. 2 The graphical presentation of the diffusion at inlaid disc electrodes (*left*) and recessed disc electrodes (*right*)

$$I_{l,m} = AnFD_0 \frac{C_\infty^0}{r + L}$$

In which L stands for the depth of the recession and A for the surface area of the electrode. The mass transport is still enhanced compared to large area substrates, although this advantage may be lost for deep recesses. As a conclusion, a small recess of the electrode is beneficial because this will make it less susceptible to perturbations of the analyte flow like fluctuations of the pump pressure when using flow injection analysis. The diffusion gradients for inlaid disc electrodes and recessed electrodes are shown qualitatively in Fig. 2. An additional difference between arrays of inlaid microelectrodes and arrays of recessed microelectrodes is that after a potential is applied to the electrodes, the diffusion fields of the single electrodes in an array overlap later and to a lesser extent when the electrodes are recessed. The values for the ratio of hole depth to hole diameter and hole distance have been found empirically through extensive research as published previously [12]. The central counter electrode is 4.5 times bigger than the total area of the working electrodes, enough to compensate for the current flow. The Ag/AgCl reference electrode is designed as an arc that is placed around the counter electrode.

When designing the thermoresistor, care had to be taken that the overall resistance of the system was determined by the resistance of the platinum (linear T-dependence) and not by the non-linear temperature dependence of the poly-silicon conducting layer used to provide electrical contact to the bonding pads. To get a sufficiently high resistance, we had to pattern the Pt in 3 µm wide loops, making best use of the space available.

Activation of the Pt electrode surface is a crucial step for microelectrodes since they cannot be polished like macroelectrodes. Additionally, the necessity of using adhesion promoting layers of metals like Ti or Cr creates problems since these metals are soluble in Pt and can diffuse along the grain boundaries to the electrode surface. Once these metals contaminate the surface, the electron transfer becomes irreversible. To prevent this, we used a chemical/electrochemical etching method described elsewhere [13] that removes Ti by complexing it, adapted it for our purposes and tested the reversibility of the electrode process.

Interferences by electroactive species such as acetaminophen, ascorbic acid and uric acid pose a serious problem for the accurate determination of analytes in blood

with oxidase enzymes at the H_2O_2 oxidation potential of +600 mV. Also, electrode fouling occurs when an electrode is being exposed to a biological matrix for a prolonged time. Although ultrafiltration and microdialysis reduce substances that can lead to electrode fouling by 90%, a protective layer on the working electrodes is advantageous for on-line measurements. Electropolymerized films promise to be very effective in this respect and have the advantage of easy and reproducible preparation.

2 Experimental

2.1 Production of the electrodes

The CMOS compatible fabrication process using 4 inch silicon wafers consists of the following steps (Fig. 3.):

(a) A wet oxidation process was used to form an insulating layer. On this layer we deposited 600 nm of poly-Si, which was heavily doped with phosphorus. After removal of the oxide formed during the doping process, a chemical-mechanical polishing (CMP) process was performed to ensure the smoothness of the surface.

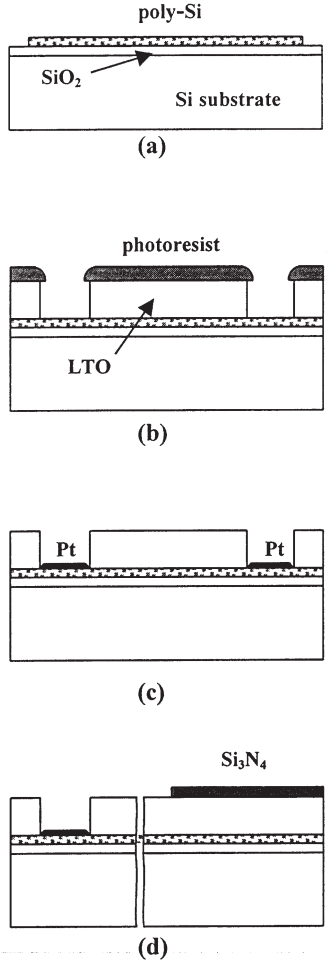

Fig. 3 Cross-sections detailing the production process. (*a*) Forming the poly-Si conductive layer. (*b*) Forming the cavities with SiO_2 reactive ion etching. (*c*) Forming Pt electrodes with the lift-off method. (*d*) Forming the Si_3N_4 passivation layer with the lift-off method

(b) First mask lithography defines the poly-Si conductor layer pattern. After the poly-Si dry etch, a 3.6 μm thick SiO_2 layer is deposited at a low temperature; an annealing step followed.
(c) The second mask lithography defines the opening for the Pt electrodes. In order to obtain the necessary condition for the etching of the recessed holes, a 3 μm thick layer of photoresist (HPR207) is used. The thick silicon dioxide layer is etched with reactive ion etch (RIE). A wet etch is then performed in order to underetch the photoresist, which is required for the lift-off process later.
(d) Sputtering of the metal layers: 50 nm Ti / 50 nm Pd / 150 nm Pt. The lift-off is done in acetone with ultrasonic agitation.
(e) A third lithography step defines the opening for the reference electrode area. The etching conditions are similar to those described under (c). For maximum stability of the reference electrode the following metal layers were deposited by sputtering: 50 nm Ti/ 500 nm Pd/ 1 μm Ag. Lift-off proceeds as in (d).
(f) To cover the thermoresistor and to create a border between the working-electrode arrays and the rest of the electrode (this will facilitate the resolution when deposition of enzymes etc. on their surfaces) a fourth mask is used. After sputtering 150 nm of Si_3N_4 the lift-off of the unwanted Si_3N_4 film is conducted in acetone with ultrasonic agitation.
(g) Chip dicing, packaging and bonding.

2.2 Apparatus

The electrochemical experiments were conducted using a BAS-100B electrochemical detector; the data were recorded with a personal computer using BAS 100 W version 2.0 software. Unless otherwise stated, an Ag/AgCl reference electrode and a Pt counter electrode from Bioanalytical Systems (BAS, West Laffayette, IN, USA) were used.

2.3 Reagents

With the exceptions stated below, all fine chemicals and reagents were obtained from Sigma chemicals (St. Louis, MO, USA) and were used without any further purification. Hexaammineruthenium(3)-chloride 95% was purchased from Aldrich chemical company (Milwaukee, WI, USA) and was dissolved prior to use due to the instability of the compound. Poly(carbamoyl)sulfonate (PCS) hydrogel is a product of SensLab (Leipzig, Germany). Glucose oxidase (EC 1.1.3.4. GOD) from *Aspergillus niger* was obtained from Sigma as lyophilized powder, activity 119.000 u/g.

2.4 Procedures

Pretreatment of the working electrodes. A cleaning solution containing 0.08 M sodium ethylenediamine tetraacetate (EDTA), 5% NH_4OH and 0.03% H_2O_2 was prepared. After immersing the chip the electrodes were pulsed for 100 ms at +2 V and then held at 0 V for 5 s. After 5 min, the electrodes were rinsed with deionized water and then cycled between +0.4 and –0.4 V for 10 min in 1 M KNO_3.

Electropolymerization of anti-fouling film. 3 mM solutions of resorcinol and 1,3-diaminobenzene were prepared and mixed in a 1:1 ratio prior to electropolymerization. For the electropolymerization, the potential was cycled continuously between 0.0 and +0.8 V vs. an external Ag/AgCl reference electrode for 20 h at a scan speed of 2 mV/s. During that time, the current reached a minimum, indicating the complete coverage of the working electrode arrays.

Preparation of a glucose microsensor. 20 μL PCS gel was diluted with 10 μL H_2O. The pH was adjusted with 10 μL 1 % polyethyleneimine (PEI) solution to pH 4. Since PCS gel polymerizes immediately at a pH higher than 7, care had to be taken to avoid a local overconcentration of the PEI. Therefore, vigorous stirring

was necessary. 2 µL of the monomer solution was mixed with 2 µL of a GOD solution in phosphate buffer saline (PBS) with pH 7.0 and with an activity of 10 u/µl. 0.08 µL of this mixture was immediately placed onto a working electrode array. The addition of the enzyme caused a shift to pH 7, leading to almost immediate polymerization. The electrode was left to dry at 278° K for 12 h. Before the measurements, the gel was allowed to swell in a phosphate buffer for one hour.

All measurements were done in 0.01 M PBS buffer at pH 7.0. The electrodes were inserted and a voltage of +600 mV vs. Ag/AgCl (3 M KCl) was applied. After the current became steady, different glucose amounts were added. The concentration of glucose in the vessel was raised by successive addition up to the desired value. After the measurements, the electrode was thoroughly rinsed and placed in a buffer solution until the next measurement.

3 Results and discussion

3.1 Examination of the Pt working electrodes

Although Pd had been used as a diffusion barrier to prevent the diffusion of titanium from the adhesion layer into the platinum along the grain boundaries [14], an electrochemical pretreatment for the etching of Ti-contamination was devised to guarantee the cleanliness of the surface. At the same time, this improved the reversibility of the electron transfer. A cyclovoltammogram of a commercial, cleaned and polished Pt electrode in 0.02 M hexaammineruthenium solution (Fig. 4.) showed a separation of the cathodic and anodic peak potentials of 67 mV, close to the ideal value of 57–60 mV for a reversible electron transfer [15]. The untreated working electrodes showed a distorted sigmoidal shape (Curve a in Fig. 5.) due to different diffusion properties. After the pretreatment (Curve b in Fig. 5.) the peak separation was 65 mV while the redox current was much larger.

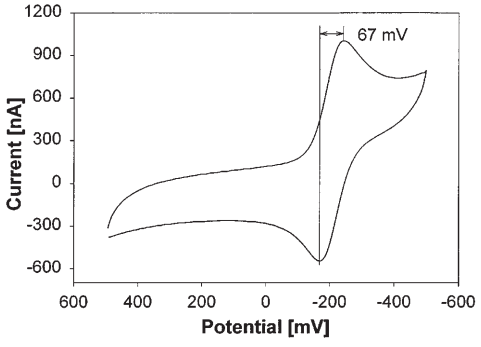

Fig. 4 Cyclic voltammogram of a commercial Pt electrode in 2 mM $Ru(NH_3)_6^{3+}$. The sweep rate was 50 mV/s

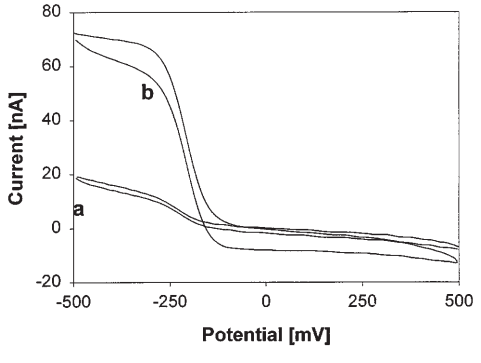

Fig. 5 Cyclic voltammogram of the microelectrode array in 2 mM $Ru(NH_3)_6^{3+}$ before (*a*) and after (*b*) treatment. The sweep rate was 50 mV/s

3.2 Stability of the reference electrode

The stability of the reference electrode potential is essential for an integrated electrode system since a potential shift occurring during the measurement leads to an incorrect signal reading. During an amperometric H_2O_2 measurement at +600 mV in a phosphate buffer with 0.01 M KCl, the potential of the Ag/AgCl reference electrode was monitored against a commercial Ag/AgCl reference electrode (BAS, 3 M KCl). The difference in potential was measured by a pH-meter with high impedance input. During a 10 h long measurement period the potential difference was 150 mV ± 10 mV. This stability is sufficient for our measurements since the oxidation current of H_2O_2 does not vary much between 590 and 610 mV. The theoretical value for the difference in potential calculated according to the Nernst equation is 146.6 mV, which is in good accordance with our measurement considering experimental error.

3.3 Calibration curve for hydrogen peroxide

Since the manufactured system will be used to monitor the H_2O_2 concentration produced by enzymes immobilized on the electrodes, the sensitive response of the electrodes is of vital importance. Figure 6 shows the dependence of the current on the H_2O_2 concentration before and after the pretreatment of the electrode. The current density for the H_2O_2 detection at +600 mV increased from 2.73 mA/cm^{2}µM to 88.86 mA/cm^{2}µM, indicating the success of the treatment.

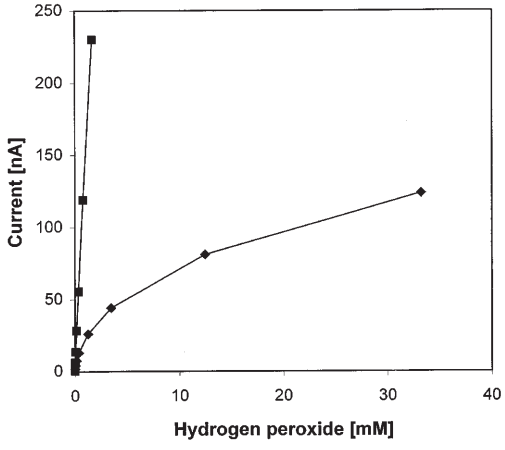

Fig. 6 Calibration curve for hydrogen peroxide in 0.02 M phosphate buffer, before [■]and after [◆] treatment

3.4 Interferences

When measuring glucose in human blood or interstitial fluid, certain substances that are oxidized at the applied potential of +600 mV vs. Ag/AgCl can lead to incorrect results. The most common interfering substances are ascorbic acid, uric acid and acetaminophen. Of these substances, acetaminophen is the most difficult to block [16] and is the most stable in an aqueous solution. Therefore, the response of the electrodes to acetaminophen up to a value 1.5 times the normal physiological value [17] was taken as a reference for the ability of a modified electrode to prevent interference.

For this purpose, a method for the electropolymerization of resorcinol and 1,3 diaminobenzene [18] has been adapted for the use with the microelectrodes. Besides preventing interfering substances from reaching the electrode surface, the electropolymerized film also prevents electrode fouling, which is important when measuring in natural matrices since the electrode should be able to deliver a stable signal for several days. The experiments depicted in Fig. 7 show that the current response to hydrogen peroxide decreased by 2/3 after the electropolymerization. After electropolymerization the current density for the oxidation of hydrogen peroxide decreased from a value of initially 88.86 mA/cm^{2}µM to 23.18 mA/cm^{2}µM. This is still very high compared with a value of 0.0043 mA/cm^{2}µM for a commercial Pt electrode (BAS) with a diameter of 1.6 mm. These high values reflect the unique diffusion characteristics of microelectrodes. Figure 8 shows that the electropolymerized film blocks acetaminophen completely in concentrations higher than physiological values.

3.5 Application as micro glucose sensor

To test the applicability of the sensor to measure glucose, glucose oxidase was immobilized on one working electrode array using PCS gel. The fabricated glucose sensor shows a fast response time, ranging between 1 and 10 s, depending on the concentration of the analyte. The detection limit is 25 µM; the linear range of the calibration plot in Fig. 9 extends from 25 µM to 2 mM glucose.

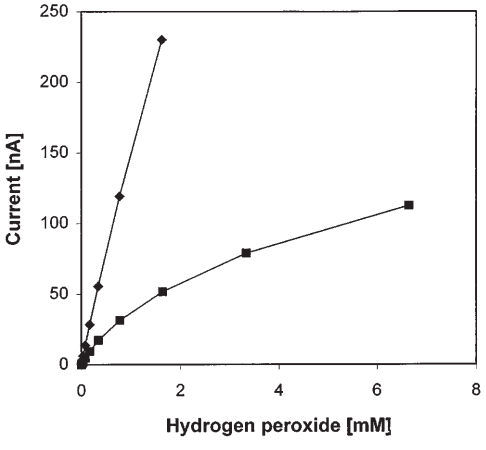

Fig. 7 Calibration curve for hydrogen peroxide using the pretreated electrode system in 0.02 M phosphate buffer before [■] and after [◆] electropolymerization with resorcinol/1,3 diaminobenzene

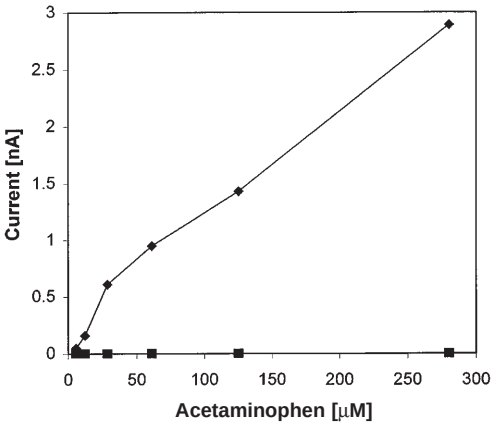

Fig. 8 Response to acetaminophen before [■ and after [◆] electropolymerization of resorcinol/1,3 diaminobenzene

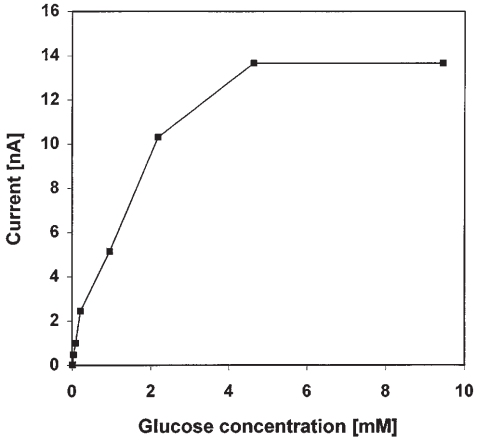

Fig. 9 Calibration curve of the micro glucose sensor at +600 mV vs. Ag/AgCl in 0.02 M pH 7.0 phosphate buffer

4 Conclusions

A microelectrode system designed for the simultaneous measurement of 4 analytes as well as temperature and conductivity of the sample in a flow-through cell has been produced with CMOS compatible processes. The device will later serve as an integral part of a micro flow-through system, enabling the on-line detection in very small volumes in combination with microdialysis and ultrafiltration methods. A chemical/electrochemical etching method has been used for the pretreatment of the Pt electrode surface to increase the catalytic properties for the hydrogen peroxide detection. The current density after this treatment increased significantly, showing that the electrode surface may have been contaminated during the production process.

In order to prevent interfering substances from oxidation at the electrode, a resorcinol/1,3-diaminobenzene layer has been electropolymerized. This layer was able to block physiological concentrations of acetaminophen completely. As a first test, glucose oxidase was immobilized on one of the 4 working electrodes of the sensor to detect glucose. Further experiments are necessary to modify all working electrodes with different oxidase enzymes and to study the cross talk between the sensors. Although the immobilization procedure and enzyme concentration has yet to be optimized, the results show the viability of this approach.

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