Micro-biosensors for Clinical and Food Analyses

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ABSTRACT

Micro-biosensors for glucose and fish freshness were constructed by
using silicon fabrication technology. A micro-H_2O_2 electrode was used
to make a micro-glucose sensor. Glucose oxidase was immobilized on an
organic membrane, prepared by vapor deposition of γ-aminopropyl-
triethoxysilane (γ-APTES) and glutaraldehyde, and used for glucose
determination in the range from 0.1 to 10 mg $\cdot$ dl^{-1}. The micro-oxygen
electrode consists of two gold electrodes and agarose gel containing
electrolyte covered with a gas permeable membrane. A negative
photoresist was used as the gas permeable membrane which was directly
cast onto the gel and submitted photochemical reaction. A 90% response
time of the micro-oxygen electrode took approximately 3 minutes. A
linear relationship was obtained between the response of the micro-
oxygen electrode and that of a convinient Galvanic oxygen electrode.
This sensor responsed almost linearly for glucose concentrations
between 0.2 and 2 mM. The amorphous silicon ion sernsitive field
effect transistor(a-ISFET) made by radio frequency plasma discharge
was used as a pH-sensitive device. The size of the channel of the a-

ISFET is 10 μm long and 500 μm wide. The pH sensitive layer was silicon oxide evaporated over the amorphous silicon nitride layer. The pH sensitivity was about 46 mV/pH at 18°C in the range pH 5-10. The response times of this device to pH change are very rapid, being less than 30 sec to reach a steady state value. The fish freshness sensor was constructed by using an immobilized xanthine oxidase membrane and a-ISFET. Hypoxanthine was detected in the range 0.02-0.1 mM.

1. INTRODUCTION

Methods for the selective determination of organic compounds in biological fluids, such as blood, are very important in clinical and food analyses. Most analyses of organic compounds can be performed by spectrophotometric methods based on specific enzyme-catalysed reactions. These methods, however, involve complicated and delicate procedures and the assay times are rather long because of the several reactions involved. The spectrophotometric methods cannot be applied directly to colored samples or biological fluids.

Alternative systems based on electrochemical sensors have been developed. Electrochemical sensors employing immobilized biocatalysts have definite advantages. Namely, an enzyme sensor possesses excellent selectivity for biological substrates and can directly determine single compounds in a complicated mixture without need for a prior separation step[1, 2]. The development of a simple, inexpensive assay is therefore of interest and the miniaturization of enzyme sensors is especially important for clinical analysis. Semiconductor fabrication technology has permitted the development of ion selective field effect transistor and micro electrodes which have been utilized as pH and enzyme-based sensors [3,4,5,6].

In this paper, micro-electrodes prepared by silicon fabrication technology, are employed as micro-biosensor transducers. Micro-biosensors for glucose and fish freshness constructed from micro-

transducers and immobilized enzyme thin membranes, are detailed and their characteristics are discussed.

2. MICRO-GLUCOSE SENSOR BASED ON H_2O_2 DETECTION

The determination of glucose in blood samples is important in the clinical field, and the development of bioelectrochemical devices considerably help routine laboratory work. Many enzyme sensors have been developed and some sensors are used for clinical analyses [2]. The development of miniaturized and implantable enzyme sensors employing micro-transducers are required in medical field. Recently, several amperometric micro-glucose sensors, which used wire electrodes [7], have been reported. Although a wire micro glucose sensor can be made small, its fabrication is not suitable for mass production. The reason is that each wire must be individually coated by a glucose oxidase immobilized membrane. On the other hand, a planar micro glucose sensor, which uses planar electrodes [8], will be suitable for mass production, because the glucose oxidase membrane can be deposited on all the individual electrodes simultaneously by using an IC process. Hydrogen peroxide sensor could be used for determination of glucose because glucose oxidase (GOD) catalyzes glucose oxidation according to the following reaction.

Glucose + Oxygen $\longrightarrow$ Gluconolactone + Hydrogen peroxide
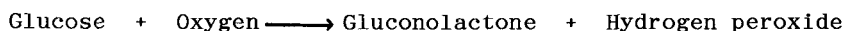
A micro-hydrogen peroxide (H_2O_2) sensor has been developed utilizing the currently available silicone fabrication technology [9]. The structure of micro-H_2O_2 sensor is shown in Fig.1. Micro-Au electrodes were created on the silicon nitride surface using the vapor deposition method and partially insulated by coating with Ta_2O_5. From the cyclic voltammogram shown in Fig.2, this unit works as a H_2O_2 electrode when the potential between both Au electrodes is set at 1.1 V. When the H_2O_2 electrode was placed in a sample solution containing
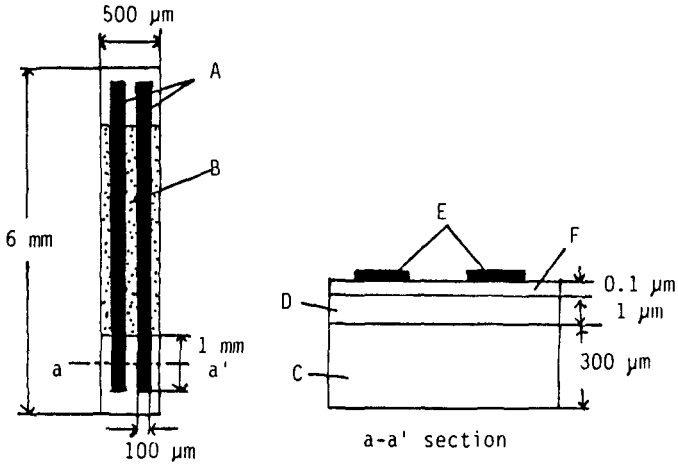
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Figure 1. Schematic diagram of a microelectrode for H_2O_2. (A and E),
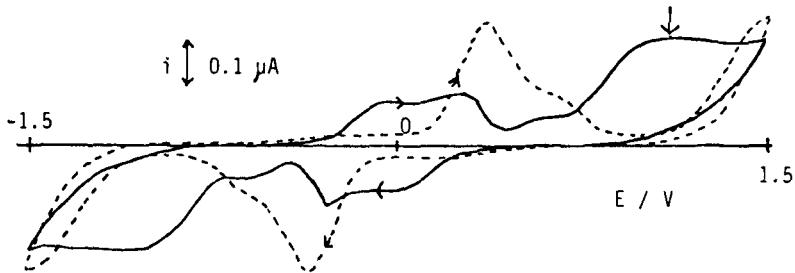Au; (B) Ta_2O_5; (C) Si; (D) SiO_2; (F) Si_3N_4.

Figure 2. Cyclic voltammograms at the microelectrode. Scan rate is
100 mV sec^{-1} and the arrow indicates 1.1 V. (---)Phosphate buffer
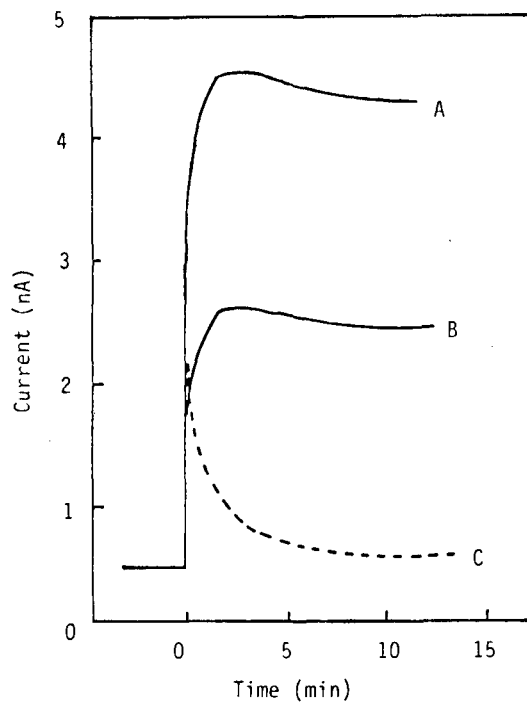solution (——)Hydrogen peroxide solution (0.1 mM)

Figure 3.　Response curves for glucose addition.　Experiments　were done　at　37　C and pH 7.0.(A) 10 mg　dl^{-1} glucose.　(B)5 mg dl^{-1} glucose, and (C) no glucose.

H_2O_2, the output current of the sensor immediately increased and reached a steady-state value within 1 min. A linear relationship was observed between the H_2O_2 concentration, over the range from 1 μM to 1 mM and the steady-state current. This electrode can be used as a transducer for a micro-glucose sensor.

The procedure for GOD immobilization onto the micro-electrode is as follows. Approximately 100 µl of γ-APTES was vaporized at 80°C, 0.5 torr for 30 min onto the electrode surface, followed by 100 µl of 50% glutaraldehyde vaporized under the same conditions. The modified micro-electrodes were then immersed in GOD solution containing bovine serum albumin and glutaraldehyde, the GOD becoming chemically bound to the surface of the micro-electrodes.

Figure 3 shows a typical response curve for the micro-glucose sensor. The output current increased after injection of a sample solution, steady state being reached within 5 min. Figure 4 shows a calibration curve for the micro-glucose sensor.

A linear relationship was obtained between the current increase (the current difference between the initial and steady state currents) of the sensor system and concentration of glucose (0.1-10 mg $\cdot$ dl^{-1}). Examination of the selectivity of the micro-glucose sensor indicated no response to other compounds such as galactose, mannose, fructose and maltose. Therefore, the selectivity of this sensor for glucose is highly satisfactory. The long-term stability of the sensor was examined with a sample solution containing 10 mg dl^{-1} glucose. The output current of the sensor system was almost constant for more 15 days and 150 assays (Fig. 5). Therefore, this micro-glucose sensor possesses both selectivity and good stability, its potential use as a micro-glucose sensor being very good.

2. FABRICATION OF A NOVEL GLUCOSE SENSOR BASED ON MICRO-OXYGEN ELECTRODE

Clark type oxygen sensing electrodes have been applied to various
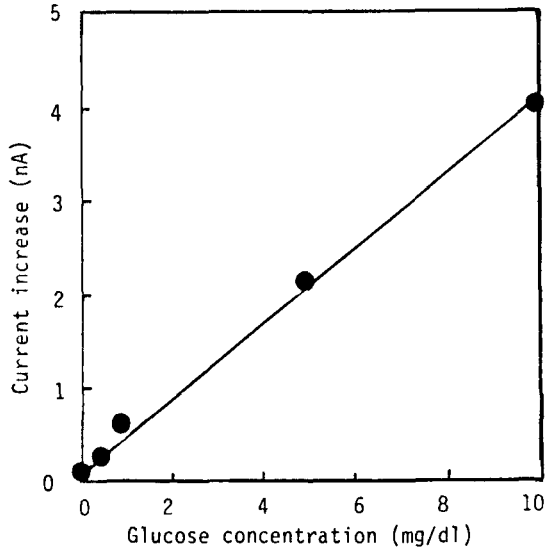
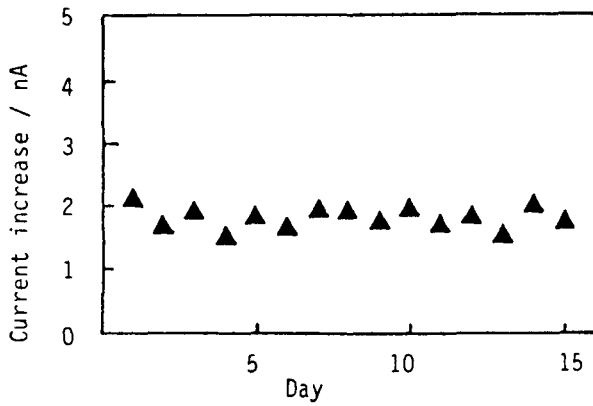Figure 4. Calibration curve for glucose. Experiments were done at 37°C and pH 7.0.

Figure 5. Stability of micro-glucose sensor.

biosensors by immobilizing either enzymes or microorganisms which catalyze the oxidation of biochemical organic compounds. But they have not yet reached the production line because they contain a liquid electrolyte solution, making adhesion of the gas permeable membrane to the substrate. Therefore, it is important to develop a disposable micro-oxygen electrode based on conventional semiconductor fabrication technology and use the micro-oxygen electrode in a biosensor. The key points of improvement were:(1) to use porous material (in this case agarose gel) to support the electrolyte solution, and (2) to use a hydrophobic polymer (in this case negative photoresist) as the gas permeable membrane, and submit it to direct casting over the porous material.

2.1 Construction of the micro-oxygen electrode

Construction of the micro-oxygen electrode is illustrated in Figure 6. The electrode has a U-shaped groove depth of 300 µm, and two gold electrodes over the SiO_2 layer that electrically insulates them. The agarose gel containing 0.1 M potassium chloride aqueous solution was filled in the groove followed by coverage of the gas permeable membrane. Only the pad areas of the two gold electrodes were exposed, while the other parts were covered with the same hydrophobic polymer used for the gas permeable membrane to insulate each electrode when used in a aqueous solution. The areas of the cathode and the anode were the same[10].

2.2 Characteristics of the micro-oxygen sensor

The response curve of the micro-oxygen electrode was examined after adding sodium sulfite to reduce the oxygen concentration. This response is the maximum response when oxygen concentration is reduced from the saturation point to the zero point. Although this curve is that of a micro-oxygen electrode width of 4 mm, the profile is similar regardless of its size. The electrode responded as soon as sodium
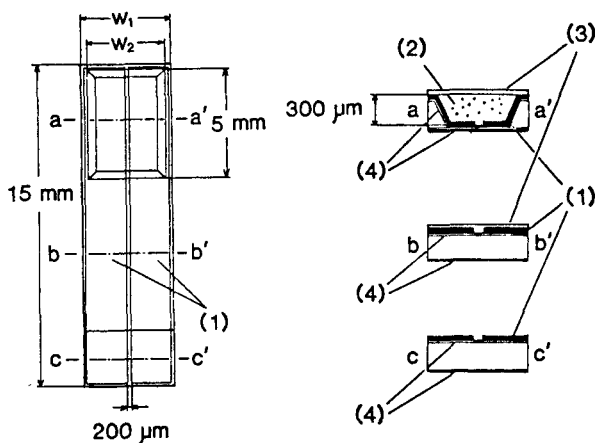
Figure 6. Schematic diagram of oxygen electrode. (1) Au, (2) agarose gel, (3) gas permeable membrane, (4) SiO_2 layer. W_1 is 2,3 or 4 mm. W_2 is 1.4,2.2 or 3 mm in each case. The cross sections on the right side corresponde to a-a', b-b' and c-c'.
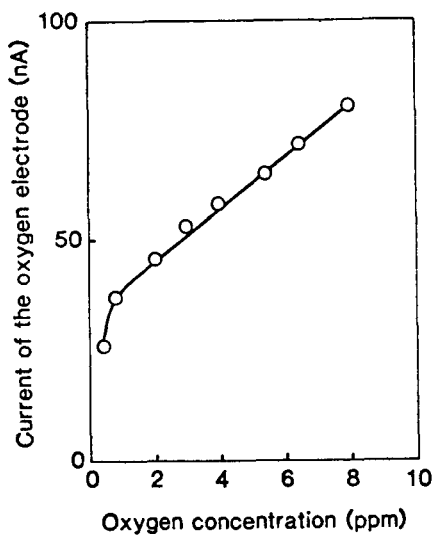

Figure 7. Caribration curve for the 2-mm-wide oxygen electrode. The terminal voltage was 0.8 V.

sulfite was added to the buffer solution, and stabilized 8 - 10 minutes after the addition. A 90% response time of the oxygen electrode took approximately 3 minutes, or three to four times longer than that of conventional oxygen electrodes. The response time was not affected by their cathode area but was considered to be dependent on the distance between the gas permeable membrane and the cathode, since the diffusion of oxygen through the gas permeable membrane and agarose gel seemed to be a decisive factor in the response time. The distance between the cathode and the gas permeable membrane can be shortened. It will be the next step of our improvement.

Output current obtained from a conventional Galvanic oxygen electrode was used as the references since this electrode uniquely responded to dissolved oxygen concentration controlled by adding sodium sulfite. As is shown in Figure 7, a linear relationship was obtained between the responses of the two oxygen electrodes when the terminal voltage between the two gold electrodes was 0.8 V. Therefore, the micro-oxygen electrode was found to work as an oxygen electrode.

Stability of the micro-oxygen electrode was tested using two micro-oxygen electrodes the width of which was 2 mm and 4 mm respectively. When a larger micro-oxygen electrode was used, its response decreased after a few times of successive use in experiments. But if it is stored in phosphate buffer solution or in distilled-deionized water for one day or two with no voltage applied between the two gold electrodes, its sensitivity recovers to the initial level. The smaller oxygen electrode could be stable used for more than 10 times. In this study, the smaller the oxygen electrode, the more stable it was. Bad stability of the larger oxygen electrode was thought to be due to accumulation of reaction products in the vicinity of each of the two gold electrodes.

2.3 Characteristics of the glucose sensor

The glucose sensor was fabricated by immobilizing glucose oxidase on a

sensitive part of the oxygen electrode by cross-linking with bovine serum albumin(BSA) and glutaraldehyde(GA). The enzyme-immobilized membrane was formed by dipping the sensitive part into a mixture containing 2 mg of glucose oxidase, 20 µl of 10 % BSA solution, and 10 µl of 25 % GA solution.

Figure 8 shows a calibration curve for the glucose sensor at 30° C and pH 7.0. As can be seen, the sensor responded almost linearly for glucose concentrations between 0.2 and 2 mM, which is comparable to conventional glucose sensors. The glucose sensor was a little sensitive to glucose at normal blood glucose concentrations(5 mM), but the sensitivity is easily shifted by adjusting the amount of immobilized enzyme. The stability of the glucose sensor was evaluated by performing the same experiments as were done to obtain the response curve. In subsequent experiments at 30° C, its response gradually decreased, but it returned to the initial level when the sensor was stored with no voltage applied, as can be seen to be mainly dependent on the stability of the oxygen electrode used as the transducer.

3. FISH FRESHNESS SENSOR USING AMORPHOUS SILICON ISFET

3.1 Construction of amorphous silicon ISFET

The a-ISFET was mainly made by radio frequency (rf) plasma discharge. The type of grow discharge apparatus is the capasitatively coupled grow discharge deposition system. A 0.05 µm n^+ layer (3000ppm PH_3 in silane) was deposited over an evaporated aluminum layer on a glass substrate in order to ensure ohmic contact between the a-Si:H and aluminum. The evaporating apparatus (ULVAC Co.,TH-500A) was used to form the aluminum layer. The thickness of each layer was measured by tallysurf. After etching, the deposition of the amorphous silicon layer (0.3 µm) and the amorphous silicon nitride layer (0.3 µm) was performed successively in the same capasitatively coupled grow
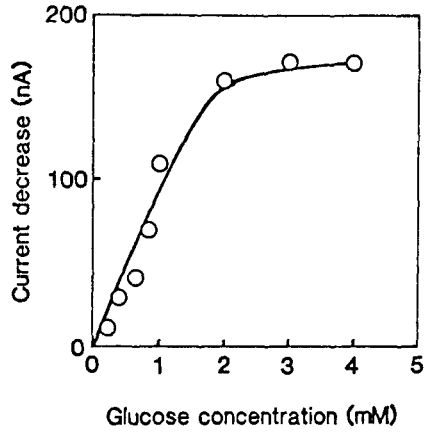
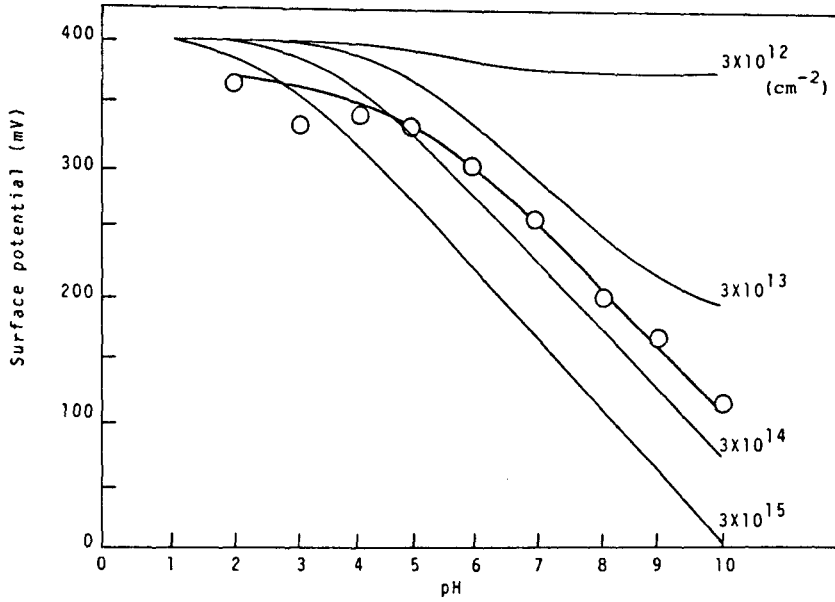Figure 8.   Caribration curve for glucose sensor

Figure 9.   pH characteristics of a-ISFET.   Circles revealed the data

points in 10 mM Tris-HCl buffer solution.

discharge deposition system operating at 13.56 MHz. The amorphous silicon layer was grown from a mixture of silane and hydrogen. The amorphous silicon nitride layer from silane and ammonia. All three layers are deposited at 300°C. The rf power level was 6 W net. Finally, a silicon oxide layer (0.2 μm) was evaporated over the amorphous silicon nitride layer. The size of the channel is 10 μm long and 500 μm wide[11].

3.2 Characteristic as field effect transistor

The characteristic as field effect transistor was measured by curve tracer. The source drain voltage (V_{SD}) / the source drain current(I_{SD}) characteristic of a-ISFET was investigated. The aluminum electrode was used as the gate. The gate voltage(V_{GS}) was applied to this device with two volts step. This device revealed characteristic as field effect transistor. When the gate voltage is zero, the source drain current is zero. The source drain current increase in accordance with the increase of the gate voltage. This device is the field effect transistor of the enhancement mode.

The Ag/AgCl reference electrode was placed in the same solution as the a-ISFET. The surface potential on the silicon oxide insulator of the a-ISFET is affected by the solution's pH, with a concominant change in the gate voltage proportional to the change in surface potential. Therefore, the surface potential change on the silicon oxide insulator of the a-ISFET, caused by a variation in pH, can be measured as the change in the gate voltage. In this case, the voltage between source and drain is held constant at 1.5 V, the current between source and drain also being constant at 0.1 μA.
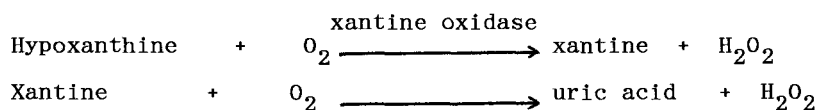
Figure 9 shows the theoretical curve and the pH characteristics of the a-ISFET. Theoretical curves of the relation between the surface potential and pH was calculated with surface dissociated sites. In this figure, it is assumed that pKa is 5 and C_H is 20 μF/cm. The linear Vg/pH characteristic of a-ISFET was obtained over the pH

range 5-10. The pH sensitivity was about 46 mV/pH at 18 ° C. The response times of a-ISFET to pH change are very rapid, being less than 30 sec to reach a steady state value.

3.4 Construction of fish freshness sensor

Hypoxanthine is considered to be a useful and reliable indicator of the fish meat freshness. The hypoxanthine sensor was constructed by using an immobilized xanthine oxidase polyvinylbutyral (PVB) membrane and a-ISFET. The PVB membrane was formed over the gate insulator of the a-ISFET as follows: 0.1 g of PVB and 1 mg of 1.8-diamino-4-aminomethyloctane were dissolved in 10 ml of dichloromethane. This polymer solution was spread over the gate insulator of a-ISFET. The a-ISFET was then immersed in 5 % glutaraldehyde solution at room temperature for 24 h to promote the cross-linking reaction of the amino group of 1,8-diamino-4-aminomethyloctane with glutaraldehyde. Xantine oxidase was covalently immobilized on the cross-linked membrane[12].

This sensor system was applied to the determination of hypoxanthine according to the following reactions. The uric acid formed was detected by this system.

$$\text{Hypoxanthine} \quad + \quad O_2 \xrightarrow{\quad \text{xantine oxidase} \quad} \text{xantine} \quad + \quad H_2O_2$$
$$\text{Xantine} \quad + \quad O_2 \xrightarrow{\hspace{3cm}} \text{uric acid} \quad + \quad H_2O_2$$

The characteristics of the sensor system were investigated. The optimum pH was approximately 7.0 at 37°C. The response reached a steady state for three minutes. A linear relationship was obtained in the range 0.02-0.1 mM between the initial rate of the gate output voltage change after hypoxanthine injection and the logarithmic value of hypoxanthine concentration. The sensor system revealed a response to 0.1 mM of hypoxanthine for more than 1 week. Over this period, the sensitivity decreased by about 30 %.

(Reference)

1) I.Karube and S.Suzuki. Amperometric and potentiometric determinations with immobilized enzymes and microorganisms. Ion-Selective Electrode Review 6,(1984) 15-58

2) I.Karube and S.Suzuki. Immobilized enzymes for clinical analysis, In Enzymes and immobilized cells in biotechnology (ed. A.I.Laskin) (1985) pp.209-226. Benjamin/Cumming Publishing,London.

3) S.Caras, J.Janata. Field effect transistor sensitive to penicillin. Anal. Chem. 52,(1980) 1935-1937.

4) I.Karube, E.Tamiya, J.M.Dicks and M.Gotoh. A microsensor for urea based on an ion-selective field effect transistor. Anal.Chim.Acta, 185,(1986) 195-200

5) I.Karube, E.Tamiya, M.Gotoh and Y.Kagawa. A microsensor for adenosine-5'-triphosphate pH-sensitive field effect transistor. Anal.Chim.Acta, 187, (1986) 287-291

6) I.Karube, E.Tamiya, and M.Gotoh. Polyvinylbutyral resin membrane for enzyme immobilization to an ISFET microbiosensor. J. Mol. Catalyst, 37,(1986) 133-139

7) S.J.Churchouse, C.M.Battersby, W.H.Mullen and P.M.Vadgama. Needle enzyme electrodes for biological studies. Biosensors, 2, (1986) 325-342

8) T.Murakami, S.Nakamoto, J.Kimura, T.Kuriyama and I. Karube. A micro planar amperometric glucose sensor using an ISFET as a reference electrode. Anal.Lett, 19(1986) 1973-1986

9) I. Karube and E. Tamiya. Micro-biosensor for clinical analyses. Sensors and Actuators, 15(1988) 199-207

10) H.Suzuki, E.Tamiya and I.Karube. Fabrication of an oxygen

electrode using semiconductor technology. Anal.Chem., 60(1988) 1078-1080

11) M.Gotoh, S.Oda, I.Shimizu, A.Seki, E.Tamiya and I.Karube. Construction of amorphous silicon ISFET. Sensors and Actuators, 16(1989) 55-65.

12) E.Tamiya, A.Seki, I.Karube, M.Gotoh and I.Shimizu. Hypoxanthine sensor based on an amorphous silicon field-effect transistor. Anal.Chim.Acta, 215(1988) 301-305