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Amperometric needle-type glucose sensor based on a modified platinum electrode with diminished response to interfering materials

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Abstract

A needle-type glucose sensor that can be used to determine glucose in serum and whole blood samples was developed Platinum wire was used as the working electrode and a disposable hypodermic stainless-steel needle electroplated with platinum was used as the counter and reference electrode A method involving both photocross-linking of PVA–SbQ and cross-linking with glutaraldehyde was used to immobilize the enzyme [PVA–SbQ is a poly(vinyl alcohol) bearing stilbazolium groups] Nafion and cellulose triacetate membranes were used to prevent inaccuracy from interfering materials and to increase the dynamic range of the sensor, respectively The response, reproducibility and long-term stability of the sensor and the effects of temperature, pH and metal ions on the response were investigated Owing to the effective method for enzyme immobilization, the large surface area of the counter electrode and the relative inactivity of the counter electrode to chemical reactions, the sensor showed good response, stability and reproducibility The sensor did not respond to ascorbate and urate at the concentrations normally found in blood Data obtained from the sensor for glucose in serum and whole blood samples showed a good correlation ($r > 0\ 95$) with a clinical laboratory automated analyser

Keywords Amperometry, Biosensors, Enzymatic methods, Blood, Glucose, Serum

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Biosensors for glucose have been studied for a long time and many kinds of materials, including enzymes [1,2] and microbial cells [3] have been used to construct them Enzymes have been used most frequently because of their specificity to the substrate [4] Many methods for enzyme immobilization [5–11] and monitoring of enzymatic reactions [12–14] have been developed for constructing such sensors

Needle-type glucose sensors seem attractive because of their small size, simple construction and the possibility of inserting them directly into a fruit or the vascular tissue of a living organism Many studies concerning the construction of needle-type glucose sensors have been reported [15–17] However, most of them suffer from instability, a low response and the inaccuracy induced by interfering materials [18]

This paper describes the construction of a needle-type glucose sensor suitable for determining glucose in blood The method combines photocross-linking of PVA–SbQ [19] and cross-linking with glutaraldehyde to immobilize the enzyme PVA–SbQ is a poly(vinyl alcohol) bearing stilbazolium groups [20] This polymer is photocross-linkable with light of wavelength shorter than 460 nm Biomaterials including enzymes [21,22] and organelles [23] have been immobilized in this polymer

Nafion was used to diminish the responses to ascorbate and urate [23], which are the major interfering materials in blood [18] The responses to ascorbate and urate at their normal concentrations in blood were eliminated after coating a Nafion membrane on the working electrode Nafion is a fluorine-containing material and therefore it is difficult ot make another membrane adhere to it The high viscosity of PVA–SbQ, however, made it possible to encapsulate the tip of the electrode with an immobilized enzyme membrane

Silver is most popularly used as a counter electrode [24] However when lengths of silver and platinum wires were immersed in phosphate-buffered saline (PBS) solution at room temperature for 3 days, a dark layer formed on the surface of silver but not platinum Electroplated platinum was used as the counter electrode in this study because of its large surface area and comparative inactivity to chemical reactions Both of these properties contributed towards a stable response and good stability of the sensor

The sensor was used to determine glucose in serum and whole blood A good correlation between these results from the sensor and those obtained with an automated analyser confirms the possibility of applying this sensor in clinical analysis

EXPERIMENTAL

Chemicals

Glucose oxidase (GOD) (E C 1 1 3 4) from *Aspergillus niger* and Bis–Tris propane {1,3-bis[tris(hydroxymethyl)methylamino]propane} buffer were obtained from Sigma (St Louis, MO) PVA–SbQ was purchased from Toyo Chemical (Tokyo) Nafion perfluorinated ion-exchange powder (5% solution in a mixture of lower aliphatic alcohols and 10% water) was obtained from Aldrich (Milwaukee, WI) and used as supplied Bovine serum albumin (BSA) was obtained from Wako (Tokyo), cellulose triacetate from Eastman Kodak (Rochester, NY), glutaraldehyde (50% aqueous solution) from Tokyo Kasei (Tokyo) and the electrolyte solution for platinum electroplating (Platanex 3LS) from Japan Electroplating Engineers (Tokyo) A glucose analysis kit based on hexokinase–glucose-6-phosphate dehydrogenase was supplied by Boehringer (Mannheim) and a glucose analysis kit based on glucose oxidase by Wako

Phosphate-buffered saline (PBS) solution was prepared by dissolving 2 754 g of NaCl, 2 081 g of KH_2PO_4 and 0 477 g of NaOH in 1000 ml of distilled water and adjusting the pH to 7 4 with 0 1 M NaOH solution [25] Glucose solutions were prepared in PBS and allowed to stand for at least 24 h before use to equilibrate the α- and β-anomers All other chemicals were of the highest grade available and were used as received

Instrumentation and materials

A potentiostat (BAS LC-4B amperometric detector, Bioanalytical Systems, Lafayette, IN) was used to supply a fixed potential to the electrode

A circulating water-bath (Thermo Minder Mini-80, Taiyo Science, Tokyo) which incorporated a water-jacketed glass reactor was used to control the temperature of the operating system Magnetic stirring was used to maintain homogeneity of the sample solutions in the reactor The response current could be read directly from the digital screen of the potentiostat and recorded simultaneously by a chart recorder (Electronic Polyrecorder EPR-100A, TOA Denpa Kogyo, Tokyo) A schematic diagram of the batch operating system is shown in Fig 1

Another potentiostat–galvanostat (HA 501, Hokuto Denko, Tokyo) was connected with a function generator (HB-107A, Hokuto Denko) to perform cyclic voltammetry and electroplating A multimeter (Digital Multimeter, TR6840, Takeda-Riken, Tokyo) and an Ag/AgCl electrode (HS-907, TOA Electric, Tokyo) were used to measure the potential drift during a determination The clinical analyser used was a Beckman Glucose Analyzer II (Beckman Instrument, Palo Alto, CA) Heat-shrink FEP (fluorinated ethylene–propylene) tubing was obtained from Junkosha (Tokyo) A stainless-steel hypodermic needle (o d 1 2 mm, i d 1 0 mm) was obtained from Terumo (Tokyo) Platinum wire of 0 3 mm diameter was obtained from Tokuriki (Tokyo)

Preparation of the working electrode

Platinum wire (50 mm × 0 3 mm diameter) was soldered to a lead wire (copper wire electroplated with tin and then insulated with Teflon) Heat-shrink FEP tubing was used to insulate the platinum wire The tip of the FEP-encapsulated platinum wire was cut at an 18° angle and successively polished with water-proof sand-papers (No 320 and 1000, Marumoto, Tokyo) and a silicon carbide Paper (No 2400, Struers, Copenhagen) After the electrode had been cleaned by sonica-

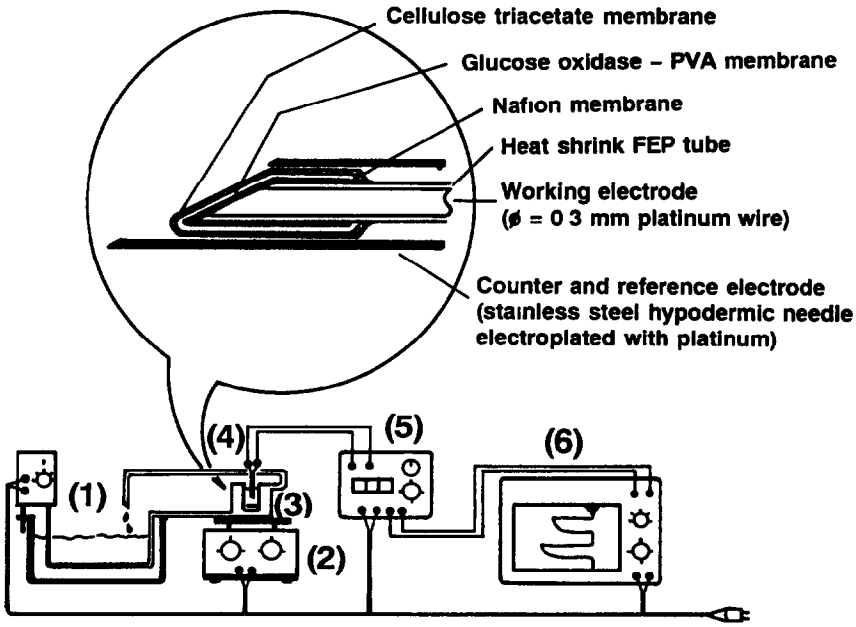

Fig 1 Schematic diagram of the batch-type operating system and the multi-layer membrane system on the tip of the working electrode (1) Thermostated circulating water-bath, (2) magnetic stirrer, (3) water-jacketed glass reactor, (4) electrode, (5) potentiostat, (6) chart recorder

tion for 30 min, the multi-layer membrane system was coated on to the metallic surface of the working electrode

Construction of the multi-layer membrane system

Three membranes made up the multi-layer system Initially the tip of the working electrode was dipped into Nafion solution for 10 s and then dried at room temperature for 30 min The tip of the electrode was then dipped into an enzyme solution composed of 5 mg of GOD (25 U mg^{-1}), 10 mg of BSA, 100 mg of distilled water and 200 mg of PVA–SbQ for 5 s After dipping, the electrode was placed in a sealed, dark box containing glutaraldehyde vapour The box was kept at room temperature for 12 h to complete cross-linking, then the electrode was exposed to a fluorescent lamp for 10 min to induce photocross-linking of PVA–SbQ Finally, the electrode was dipped in a 0 5% (w/v) cellulose triacetate solution in dichloromethane for 3 s and dried for 5 min at room temperature The electrodes were stored dry at 4°C until used The multi-layer membrane system is shown in Fig 1

Preparation of the counter electrode

A stainless-steel hypodermic needle was cleaned with methanol and dichloromethane It was further cleaned with an oxidative acid solution [concentrated sulphuric acid–30% hydrogen peroxide (1 + 1)] and an oxidative alkaline solution [concentrated ammonia solution–30% hydrogen peroxide–water (1 + 1 + 6)] It was thoroughly washed with distilled water after each cleaning step

Nickel and platinum were electroplated successively on to the needle The processes of elec-

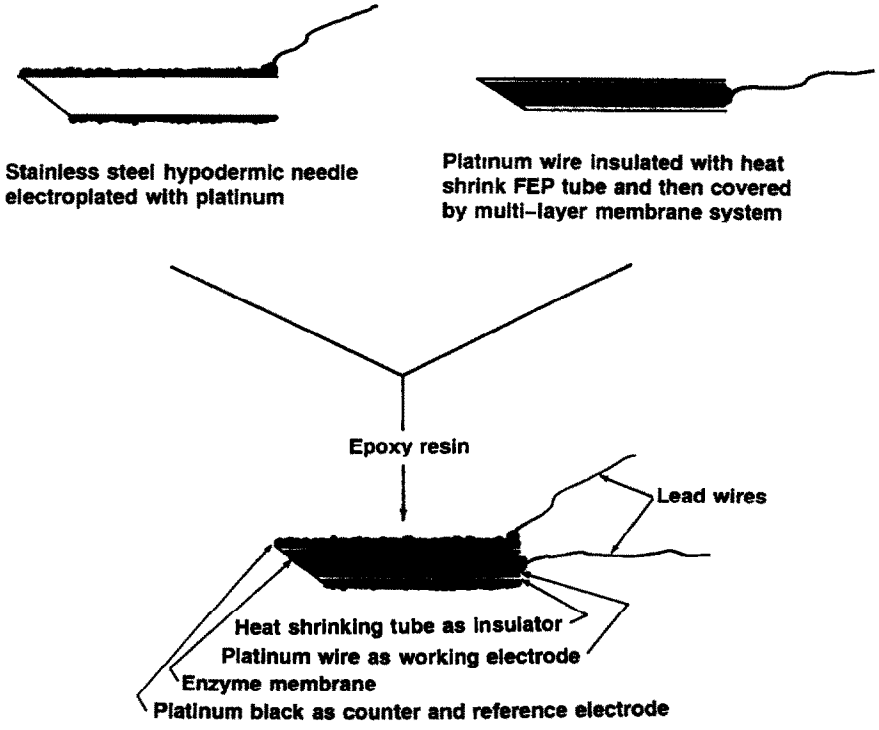

Fig 2 Procedure for constructing needle-type enzyme electrode

troplating were conducted at 80°C The needle and a piece of nickel wire were connected to the potentiostat and immersed in an electrolyte solution prepared by dissolving 60 g of $NiCl_2$ $6H_2O$ in 30 ml of concentrated hydrochloric acid and diluting with distilled water to 250 ml A potential of −4 0 V was applied between the needle and the nickel wire for 30 s After thorough rinsing with distilled water, the needle was transferred into Platanex 3LS platinum electroplating solution A current of −20 mA was applied between the needle and a piece of platinum wire for 5 min The needle was thoroughly rinsed again with distilled water and then stored dry at room temperature until used

Fabrication of glucose sensor

The working electrode was inserted into the hollow interior of the counter electrode Epoxy resin was used to fix the two electrodes in position The procedure for constructing the glucose electrodes is shown in Fig 2

Measurement of potential drift during glucose determination

An Ag/AgCl electrode was inserted in the reaction cell with the glucose sensor During glucose determination the potential between the working electrode and the Ag/AgCl electrode was measured with a voltmeter and recorded with a chart recorder

Determination of glucose concentration

The electrode was immersed in PBS solution for 1 h to equilibrate the membrane system The copper wires were then connected to the potentiostat and a potential of +650 mV was applied between the working and the counter electrodes The baseline current was measured and then glucose solution was injected into the PBS solution using a microsyringe The response current following injection was recorded with a chart recorder until the second steady state was achieved Magnetic stirring was used during this operation to ensure homogeneity of the solution The difference between the baseline and the second steady-state currents was used to calculate the concentration of glucose in the sample according to a calibration graph

Another calibration graph was obtained by adding glucose solution to heparinized whole blood that had been incubated at 37°C for 18 h to glycolyse the glucose present [26] The concentration of glucose in serum and whole blood was determined by taking the baseline and the second steady-state currents in the glycolysed whole blood and the sample, respectively, and using them to calculate the glucose concentration according to the calibration graph obtained with glycolysed whole blood The samples were mixed in the sampling tube by gentle shaking before determination but were not stirred during determination

RESULTS AND DISCUSSION

Determination of the applied potential

The cyclic voltammograms of the electrode using platinum wire as the working electrode and platinum black as the counter and reference electrode in PBS with or without hydrogen peroxide are shown in Fig 3 The plateau of this electrode after contacting with hydrogen peroxide appeared from 450 to 750 mV The potential between the working electrode and the Ag/AgCl electrode drifted by −57 mV when determining 400 mg dl^{-1} (22 2 mM) glucose in PBS A potential of +650 mV was chosen after taking the range of the plateau region and the potential drift during glucose determination into consideration This

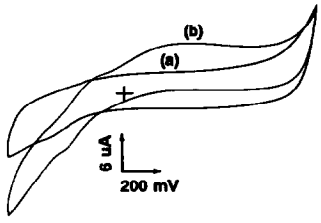

Fig 3 Cyclic voltammogram of the electrode using platinum wire as the working electrode and platinum black as the counter and reference electrode Experiments were done in (a) PBS and (b) 1 mM H_2O_2 in PBS (pH 7 4) at 37°C

ensured that the polarizing voltage of the sensor remained in the plateau region during the determination of glucose

Function and effect of each membrane in the multi-layer membrane system

Three layers were included in the multi-layer membrane system The innermost layer was a Nafion membrane Nafion is a polymer with a negative charge in the pH range of blood and therefore curtails the passage of interfering anions such as ascorbate and urate to the working electrode The middle layer was the immobilized glucose oxidase membrane Enzyme immobilization was achieved by a double cross-linking method that used a photocross-linkable poly(vinyl alcohol) compound (PVA–SbQ) in conjunction with a chemical cross-linking compound (glutaraldehyde) This led to firm entrapment of the enzyme and consequently good stability The outermost layer was a cellulose triacetate membrane This membrane excludes compounds on the basis of molecular size Large molecules were completely excluded and small molecules partly excluded from interaction with underlying layers of the sensor This had two advantages The first function was to act as a protective ultrafiltration membrane and prevent large molecules such as proteins and other enzymes in the sample from fouling the sensor This can prevent the GOD from being damaged by other enzymes such as proteases in the sample solution The second function was to reduce the rate at which glucose permeates into the enzyme layer, which extends the dynamic range of the sensor

The effects of the successive membranes on the response to ascorbic acid, uric acid, hydrogen peroxide and glucose are shown in Fig 4 The response to 2 mg dl^{-1} ascorbic acid is shown in Fig 4A The bare electrode responded strongly to ascorbic acid, but this response was reduced to zero when the metallic surface of the electrode was covered with a Nafion membrane Figure 4B shows the response to 10 mg dl^{-1} uric acid The effect is the same as for ascorbic acid, i e , the bare electrode responded to uric acid, but the response was reduced to zero after spreading a layer of Nafion on the working electrode The

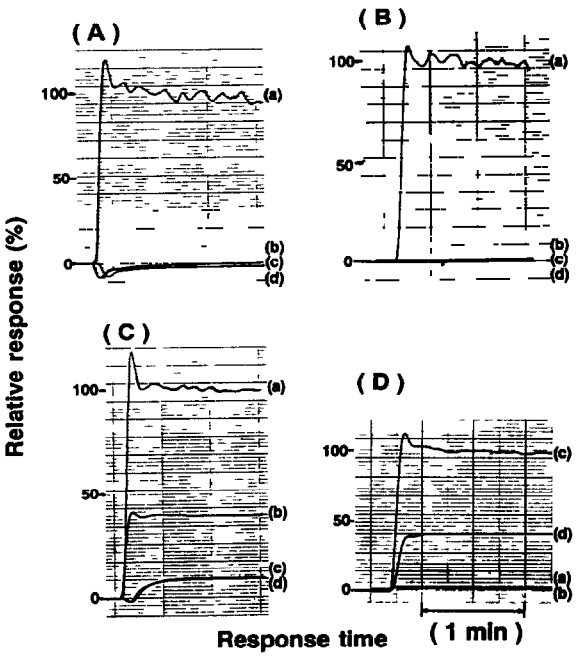

Fig 4 Effects of each kind of membrane on the response of the sensor to (A) 2 mg dl^{-1} ascorbic acid, (B) 10 mg dl^{-1} uric acid, (C) 0 1 mM hydrogen peroxide and (D) 50 mg dl^{-1} glucose Curves (a) are the response of the bare electrode, (b) the electrode coated with Nafion, (c) the electrode coated with Nafion and enzyme–PVA and (d) the electrode coated with Nafion, enzyme–PVA and 0 5% cellulose triacetate Experiments were conducted at 37°C in PBS (pH 7 4)

concentrations of ascorbic acid and uric acid normally found in blood are 0 4–1 5 and 1 5–8 0 mg dl^{-1}, respectively [27] The response to 0 1 mM hydrogen peroxide is shown in the Fig 4C The highest response was from the bare electrode The response was reduced to 40% because of the diffusion barrier induced by coating the electrode with Nafion The immobilized enzyme and cellulose triacetate membranes further reduced the response However, hydrogen peroxide is produced in the PVA membrane during glucose determination and therefore the Nafion membrane alone reduces its response

Figure 4D shows the response to a 50 mg dl^{-1} glucose solution The response to glucose was induced by immobilizing glucose oxidase on the working electrode The response was decreased after coating the electrode with a cellulose triace-

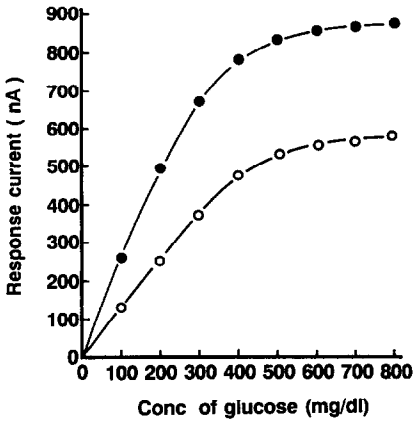

Fig 5 Calibration graphs in (●) PBS and (○) glycolysed whole blood for the sensor dip-coated in cellulose triacetate solution (0 5%, w/v) Experiments were conducted at 37°C

tate membrane Higher cellulose triacetate concentrations lowered the response intensity of the sensor, but increased the dynamic range The concentration of cellulose triacetate used in this work was chosen according to the desired dynamic range of the sensor A decrease in response intensity is one of the disadvantages of using a multi-layer membrane system However, the remaining response was still high enough to monitor the reaction catalysed by the enzyme

Calibration

Figure 5 shows the calibration graphs in PBS and glycolysed whole blood for a sensor dip-coated by immersion in cellulose triacetate solution [0 5% (w/v) in dichloromethane] The dynamic range of this sensor was 0–360 mg dl^{-1} glucose, which is sufficient for measurement of the glucose concentration in the blood of non-diabetic and most diabetic patients This sensor was used to determine the glucose concentration in serum and whole blood samples in this study Experiments to determine the response and calibration graphs using cellulose triacetate membranes of different thickness were also carried out This indicated that it was possible to increase the dynamic range up to 800 mg dl^{-1} glucose by increasing the concentration of the cellulose triacetate solution to 2% (w/v) However, the response was then low (15 nA for 100 mg dl^{-1} glucose) and slow (2–3 min for a 95% response) A 0 5% (w/v) cellulose triacetate solution was adopted in this study because it covers the range of glucose concentrations that are commonly encountered in a clinical laboratory

Effects of temperature, pH and metal ions

The effect of temperature on the response of the sensor is shown in Fig 6 The response increased with increase in temperature from 5 to 50°C, the response at lower temperatures remained unchanged after testing at 50°C These results suggest that the enzyme, glucose oxidase, is stable at fairly high temperatures after being immobilized by the method employed here The response intensity at 37°C was 81% of that at 50°C Figure 6 also shows the response time of the sensor The time required to reach the second steady state was dependent on temperature The lower the temperature, the longer was the response time At 37°C, however, less than 30 s were required to obtain a 95% response

The effect of pH is shown in Fig 7 Four kinds of buffers covering the pH range 4–10 were utilized The response at a given pH was different in different buffers The highest response was obtained at neutral pH in phosphate buffer The optimum pH of GOD in solution is 5 6 and there is a rapid and permanent loss of activity at pH values lower than 2 or higher than 8 [28] The optimum pH of the immobilized GOD was 7 0

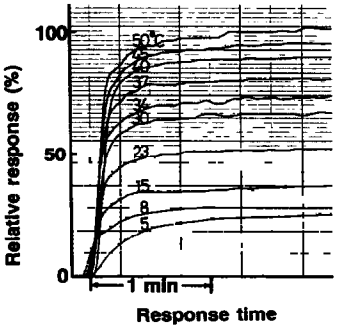

Fig 6 Effect of temperature on the response of the sensor Experiments were conducted in PBS (pH 7 4)

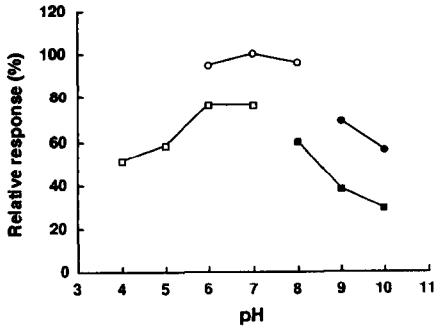

Fig 7 Effect of pH on response of the sensor Experiments were conducted at 37°C in 0 1 M buffers (□) citrate (pH 4–7), (○) phosphate (pH 6–8), (■) borate (pH 8–10), (●) $NaHCO_3$–NaOH (pH 9–10)

However, the activity loss at pH > 8 was similar to that of GOD in solution

The effects of some metal ions are shown in Table 1 Calcium, magnesium, iron and copper are the main metal ions encountered in serum Of these, iron(III) and copper(II) caused a decrease of 2 3% and 8 3%, respectively, in the response of the sensor Silver(I) completely inhibited the response and mercury(II) reduced the response of the sensor to 37% Inhibition of immobilized GOD by silver(I) and mercury(II) has been studied [29] These ions, however, are present only at very low concentrations in biological fluids

TABLE 1

Effect of metal ions on response of the sensor [a]

Metal ion	Salt	Concentration (μM)	Relative response (%)
None	–		100 0
Ca^{2+}	$Ca(NO_3)_2$	2750 [b]	99 0
Mg^{2+}	$Mg(NO_3)_2$ $6H_2O$	1235 [b]	103 9
Fe^{3+}	$Fe(NO_3)_3$	30 [b]	97 7
Cu^{2+}	$CuSO_4$ $5H_2O$	25 [b]	91 7
Pb^{2+}	$Pb(NO_3)_2$	25	103 5
Ag^{+}	$AgNO_3$	25	0 0
Hg^{2+}	$Hg(NO_3)_2$	25	36 9

[a] Experiments were conducted in 30 mM Bis–Tris propane buffer (pH 7 4) at 37°C [b] The concentration is slightly higher than the average value in serum [27]

TABLE 2

Relative response of the sensor and assay kits to various mono- and disaccharides

Sugar	Relative response (%)			
	Sensor [a]	HK–G6PDH	GOD	Reference [b]
Glucose	100	100	100	100
Fructose	3 5	0 7	0 9	0
Galactose	1 7	0 4	0 8	0 14
Maltose	3 6	1 8	1 0	0 19
Mannose	3 1	– [c]	3 7	0 98
Lactose	–	–	–	0
Sucrose	–	–	–	0

[a] The experiments with the sensor were conducted in PBS containing 2 775 mM sugar [b] Data from [30] [c] Dashes indicate undetectable

Selectivity, stability and reproducibility of the sensor

The selectivity of the sensor was tested by determining various mono- and disaccharides at the same concentration as glucose The relative responses of the sensor for various sugars are shown in Table 2 The relative responses to glucose, fructose, galactose, maltose and mannose were 100, 3 5, 1 7, 3 6 and 3 1, respectively The sensor did not respond to lactose and sucrose The specificity of GOD for glucose, galactose, maltose and mannose has been reported as 100, 0 14, 0 19 and 0 98, respectively [30] The same workers reported that it did not react with fructose, lactose and sucrose In order to make sure that the response of the sensor was not due to contamination with glucose, they were assayed for glucose with assay kits based on hexokinase–glucose-6-phosphate dehydrogenase (HK–G6PDH) and glucose oxidase (GOD) The relative responses of the assay kits for various sugars are also shown in Table 2 Both of the assay kits responded to the fructose sample, but did not respond to lactose and sucrose The GOD kit also responded to mannose These results indicate that response of the sensor to fructose was partly due to contamination with glucose GOD reacts, albeit slowly, with galactose, maltose and mannose According to the operating data sheet provided by the supplier, the GOD used in this study also contained maltase, glycogenase, invertase, amylase and galactose oxidase as contami-

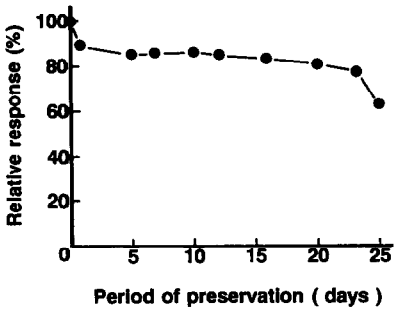

Fig 8 Relative response intensity of the sensor when stored at room temperature in PBS (pH 7 4) Experiments were conducted at 37°C in PBS containing 100 mg dl^{-1} glucose

nants However, sugars other than glucose are present only at very low concentrations in biological fluids and therefore the resulting errors would be negligible

Figure 8 shows the relative response of the sensor over a period of 25 days when stored in buffer solution at room temperature The response declined by 10–15% in the first day but then became stable for more than 3 weeks The decrease in response during the first day could have been caused by escape of enzyme molecules that were not firmly immobilized or by a decrease in the response of the electrode to hydrogen peroxide Because the response of the sensor to 0 1 mM hydrogen peroxide was found to be unchanged after 3 days, it is likely that loss of enzyme was chiefly responsible for the decline Further decrease in response was found on the 25th day The membrane system was found to be folded up when observed under a microscope at that time

The reproducibility of the sensor was tested on the seventh day after the sensor had been fabricated For 24 successive determinations of the same glucose solution, the relative error of the responses was less than 1%

Determination of glucose in serum and whole blood

Serum and whole blood samples were assayed for glucose with both the sensor and the automated clinical analyser Linear correlations were achieved in both instances, i e $Y = 0\,925x + 12\,45$ ($r = 0\,95$, $n = 14$) for serum and $y = 0\,991x + 6\,597$ ($r = 0\,993$, $n = 17$) for blood, where y is the glucose concentration (mg dl^{-1}) measured by the sensor and x that by the clinical analyser

Conclusion

A needle-type glucose sensor had been fabricated Electrodeposition of platinum on to a disposable hypodermic needle allowed a cheap but reliable counter electrode with a large surface area to be constructed Either the large surface area or the relative inactivity of the platinum to chemical reactions contributed to the stable response of the sensor Nafion was coated on to the metallic surface of the working electrode This prevented ascorbate and urate from interfering with the response of the sensor The high viscosity of PVA–SbQ made it possible to coat the enzyme membrane on the surface of the Nafion membrane by dipping it into the enzyme-containing PVA–SbQ solution The enzyme was immobilized by being cross-linked by glutaraldehyde and then entrapped by photocross-linking of PVA–SbQ As a result, GOD was retained firmly in the membrane, and this also contributed to the stability of the sensor A cellulose triacetate membrane was used as the outermost layer to protect the enzyme membrane and to vary the dynamic range over which the sensor responded to glucose The dynamic range of the sensor to glucose could be adjusted from 0–30 to 0–800 mg dl^{-1} depending on the concentration of cellulose triacetate used in constructing the multi-layer membrane system The needle shape of the sensor means that it can be used in a small volume of sample, implanted in a living organism or inserted in a fruit The construction of this sensor was simple and easy to perform Each step of fabrication can be carried out in a laboratory equipped with a potentiostat–galvanostat, chart recorder and water-bath The response, stability and reproducibility of the sensor were good enough to be applied to the determination of glucose in blood samples in a clinical laboratory The batch operating system allowed more than fifteen samples to be assayed per hour Faster operation may be possible if the sensor is connected to an autosampler and flow-injection

system [31–33] It will be necessary to investigate biocompatibility [34] and mediated electron transfer [35] prior to in vivo use of the sensor, and this is in progress

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