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Fe_3O_4-MODIFIED THICK FILM CARBON-BASED AMPEROMETRIC OXIDASE-BIOSENSOR[1]

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An amperometric biosensor was developed from screen printed carbon electrodes modified with magnetite (Fe_3O_4/SPCE) as the transducer element and Nafion-entrapped glucose-oxidase as the bio-recognition layer, cast by drop-evaporation. In a flowing stream (0.2 ml min^{-1}) of phosphate buffer (pH 7.5 / 0.05 M), at a working potential of –125 mV vs. Ag/AgCl with an, injection volume of 100 µl, the biosensor has linear range of 7 – 150 mg l^{-1} glucose (r^2 = 0.999), a sensitivity of 3.8 nA mg^{-1} l cm^2, and a precision of 2% at 10 mg l^{-1}. Uric acid, xanthine and hypoxanthine at 10 and 100 mg l^{-1} do not cause significant error (± 1– 6 %) relative to 100 mg l^{-1} glucose. Ascorbic acid at 100 mg l^{-1} causes 10 – 14

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% deviations. Dilute blood samples showed average recovery rate of 95 %. Thus, this model biosensor is precise, fairly sensitive and accurate.

Introduction

It is known that amperometric biosensors based on the detection of hydrogen peroxide (H_2O_2) at classic metal or carbon electrodes require high operating (working) potentials (> 600 mV) and, thus, suffer interferences from common electro-active components of samples [1,2,3]. A preferred way to avoid such interference is to modify the electrode with substances which can specifically mediate the electron transfer between H_2O_2 and the electrode at reduced over-voltages, specially in the region from +100 to –200mV [3]. For instance, carbon electrodes modified with peroxidases [4], MnO_2 [5], RuO_2 [6], Prussian blue [7], metallophthalocyanins [8], particulate Rh, Ru [3], hexacyanoferrates [9,10] are able to lower the H_2O_2 over-voltages to different extents, including operating potentials of 0 mV [7] like the case of Prussian blue which has been applied to the construction of large number of oxidase enzyme-based biosensors for clinical, environmental and food analysis [11, and references therein]. However, some operating potentials are still not low enough or the modifiers are either not readily available, leaching-prone, toxic, or unstable during long term storage.

Electrocatalysts which are insoluble transition metal oxides are of special interest since they are thermodynamically stable and are available easily and economically. Recently, we found that the electroreduction of H_2O_2 occurs at low cathodic working potentials (0 to –200mV vs Ag/AgCl) at Fe_3O_4 bulk-modified carbon paste and screen printed carbon electrodes (Fe_3O_4/SPCE) which also show promising analytical performance as H_2O_2 sensors [12]. Thus, it should act as a proper transducer of oxidase-based biosensors using the glucose/glucose-oxidase (GOD) model system [13]. Figure 1 depicts the glucose recognition mechanism of a biosensor made of GOD/entrapment-agent layer on this H_2O_2 transducer.

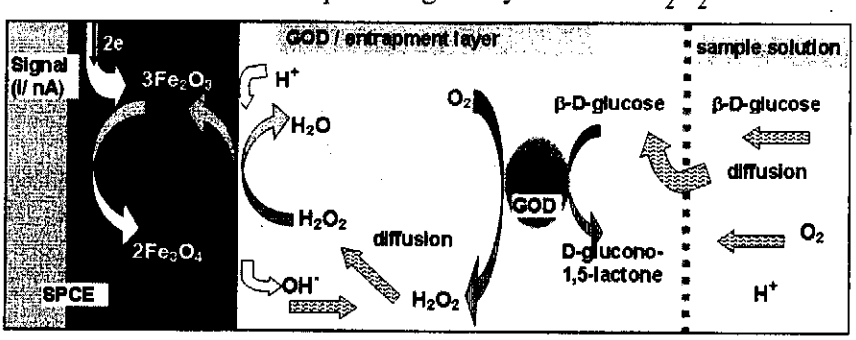

Fig. 1 Possible glucose recognition mechanism and operational principle of a biosensor based on H_2O_2 detection and polymer entrapped glucose oxidase layer

Among the enzyme-entrapment agents available for making amperometric biosensors, Nafion® has become a favorable choice for its multiple effects and properties: selectivity against ascorbate, paracetamol, urate, [14–23], high O_2 storing capacity [24,25], chemical stability and suitable mechanical properties [26, and references therein], biocompatibility and fouling-protection [27, and references therein, 28].

In this paper we report on the development of a new glucose biosensor based on Nafion-entrapped GOD and SPCE transducers modified with Fe_3O_4. The experimental and measurement parameters have been optimized; applications to real samples (blood plasma) are presented and discussed.

Experimental

Chemicals

Ammonia (25 % aq., Fischer), ethanol (99.9 %, absolute, Merck), NaCl (Fluka), $NaH_2PO_4 \cdot H_2O$ (Merck), Na_2HPO_4 (Merck) and $Na_2HPO_4 \cdot 2H_2O$ (Merck) were of analytical grade. Fe_3O_4 (98 %, pulver < 5 micron) was obtained from Aldrich; conductive thick film printing ink (Electrodag 421 SS) from Acheson; D(+)-glucose (bacteriology) was bought from Merck and glucose oxidase from SIGMA (210,000 units g^{-1} solid, ~75 % protein, type X-S from Aspergillus niger). Nafion® was obtained as solution in lower aliphatic alcohols and 15 – 20 % water (5 wt. %, Aldrich).

Reagents and Solutions

Water was de-ionized (18.5 MΩ cm) by a cartridge purification system (Mili-Q plus – Milipore). Phosphate buffers were prepared by mixing equal concentration Na_2HPO_4 and NaH_2PO_4 solutions until the desired pH was obtained. Glucose stock solutions (1% m/v in the buffer) were allowed to equilibrate with mutarotation overnight at room temperature and then always stored at +4 °C. For interference studies, ascorbic acid (1 % m/v), hypoxanthine (0.1 % m/v), uric acid (0.1 % m/v), xanthine (0.1 % m/v) were prepared on the day before use in 10 ml PE tubes using a mixture of appropriate proportions of 0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 so that each stock solution had the same pH as the carrier buffer (pH 7.5). All interference solutions were wrapped with aluminum foil when standing to avoid influence from light. Glucose and interference working solutions were made just before use by dilution in the buffer of interest.

Flow Injection System

The pump was a multisolvent delivery system (Waters 600E) with an injection port (Rheodyne 4-way rotary valve, model 5041; 0.8 mm i.d. PTFE tubing) and injection loop of 100 µl. The detector was a thin layer electrochemical flow cell (CC5, BAS) in a configuration described elsewhere [29]. An Ag/AgCl electrode (3 M NaCl, BAS RE-4, Part # MF-2021) served as the reference (RE). The counter electrode (CE) was the stainless-steel back-plate the cell. A 1 × 4 × 0.635 cm biosensor body was fit into the rectangular slot of a lab-made PTFE block. The spacer between sensor and CE was a polyester gasket (0.19mm, RS 681-407, RS Components) with an oval opening just enough to expose the bio-electroactive surface of the biosensor body.

Electrochemical Measurement

Cyclic voltammetric (CV) and amperometric (single potential time base) runs were performed using the electrochemical workstation BAS 100B and the corresponding software (BAS 100W, version 2). For amperometry, the working potential was held until the baseline became straight (~10 min), followed by pilot injections to check reproducibility of signals. For better sensitivity the biosensor was always electrochemically pretreated once per day by scanning (50 mV s^{-1}) between ±800 mV until steady state (≤30 min) with the last scan towards –800 mV.

Fe_3O_4-Bulk-Modified Screen Printed Carbon Electrode (Fe_3O_4/SPCE)

Iron (II,III) oxide was mixed (5 % m/m) with the carbon printing ink in a glass vial by stirring (10 – 30 min), with glass rod or stainless steel spatula, and sonication (3 – 30 minutes) as described elsewhere [12]. The resulting uniform mixture was immediately screen printed on a ceramic substrate (Coors ceramics GmbH, alumina plate) with an area of the sensor and a thickness of 0.1 mm using a semi automatic screen printing machine (SP-200, MPM) and stainless steel mask. A plate contained 30 sensors and was dried at room temperature overnight or at 90 °C for 30 min.

Biosensor Preparation

The Nafion® stock solution (5 %) was neutralized to ~ pH 7±1 according to previous recommendations [30,31] by drop-wise addition of diluted conc. NH_3 (1

+ 3 with water) and intermittent testing of pH with a pH indicator. The GOD stock solution (50 mg l^{-1} in water) was prepared just before use. To prepare enzyme casting solutions, according to the optimized composition, volumes of ethanol, the NH_3-neutralized Nafion stock solution, water and the GOD stock solution were mixed in the ratio 8 + 2 + 9 + 1 (v/v/v/v), respectively, in a plastic vial (1.5 ml microcentrifuge tubes). First, 10 µl of the casting solution were drop-coated on the electroactive surface of the Fe_3O_4/SPCE and allowed to dry at room temperature. When dry, another 10 ml were coated over the previous one and dried similarly. Electrical contacts were made with a conductive silver ink. The coating and drying was complete in 20 min or less. The bio-recognition layer thus made had an area of 47.5 mm^2. The biosensor was immediately mounted into a flow cell or stored either dry at room temperature or in phosphate buffer of pH 7.5.

Samples (Blood Plasma)

Blood samples (50 µl) were taken consecutively and as fast as possible with sterile PE micropipette tips after pricking a finger tip. Each sample was transferred into a 10 ml sterile tube, immediately diluted 40 times with the FIA-carrier buffer (pH 7.5, 0.05 M sodium phosphate), spiked with 0 µl (native) and 50 µl of glucose standard (1000 mg l^{-1}) at constant total volume, shaken gently and briefly to visual uniformity, centrifuged at 4500 rpm for 10 minutes and finally stored at +4 °C until the measurement. The same samples were also measured using a "GlucoMen® Glyco" pocket glucometer for reference.

Results and Discussion

Nafion Layer Casting Solution

A frequently used commercial formulation of Nafion is a 5 % (WT %) solution in ca. 80 – 85 % lower alcohols and 15 – 20 % water [30, and the references therein]. The activity of Nafion-entrapped GOD is improved if the casting solution is diluted with water and neutralized with NH_3 [30,31].

Studies using ethanol/phosphate buffer mixture as the diluent showed that the resulting enzyme/Nafion layer easily cracks when allowed to dry and stay in air. It is probably the effect of crystallization of the buffer used. The cracking problem can be avoided by replacing the buffer with distilled water, achieving a visually very uniform GOD/Nafion caste layer which stays uncracked for unlimited period of time in air at room temperature.

Figure 2 shows the effect of ethanol content in the casting solution. Variation in sensitivity of up to 60 % is observed when the ethanol composition

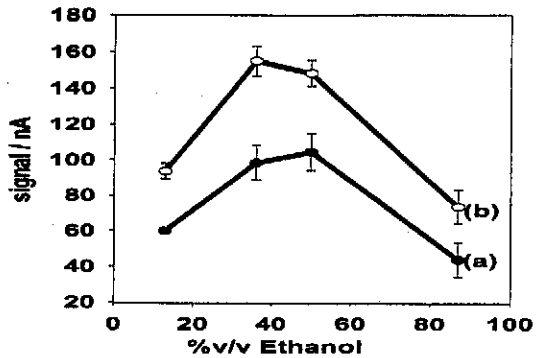

Fig. 2 Effect of ethanol/water ratio (calculated) in casting solution on signal: Curves (a) and (b) are measurements at 200 and 500 mg l^{-1} D(+)-glucose. Casting solution: 0.42 % (m/v) Nafion, 0.25 % GOD: carrier: 0.1M pH 7.5 sodium phosphate; potential: –100 mV; injection volume: 100 µl; flow: 0.10 ml min^{-1}

is changed: an increase from about 15 % to about 50 %, and then a decrease. Thus, the biosensor under study gives the highest sensitivity with about equal proportions of ethanol and water in the casting solution. The physical property of the casting mixture with respect to its easy drop-coating is also suitable at this composition. Too high concentrations of ethanol seem unfavorable probably because the active sites of the enzyme are not optimally unfolded and exposed, whereas high amounts of water probably deteriorate the membrane formation of Nafion.

Apart from the solvent also the absolute amount of Nafion in the casting solution influences the response of the biosensor (Fig. 3). The graph shows that lower amounts of the polymer yield higher signals due to better accessibility to the enzyme and less diffusion processes in the membrane. Additional enzyme immobilization processes such as direct adsorption or covalent binding onto the transducer surface might be involved. This speculation is also supported by a recent report [32] in which nanometric Fe_3O_4 particles were used to immobilize some redox heme proteins on a pyrolytic graphite electrode surface without any cross-linking or coupling agents. But it could also be simply a physical adsorption process [33]. Since too small Nafion load would lower its enzyme holding capacity and perm-selectivity, and shorten the operational life-time of the biosensor, its casting-mixture concentration was set at 0.42 % (84 µg biosensor^{-1}) for subsequent experiments.

Preliminary tests of the effect of enzyme loading showed that the response does not increase appreciably on doubling the enzyme concentration from 50 µg to 100 µg per biosensor. This result is in accordance with that of Kalcher *et al.* Obtained with an MnO_2/SPCE-based glucose biosensor for which even a 10-fold increase in GOD-load did not give significant enhancement of signal [31]. This is probably due to the fact that the number of active centers exposed to the sample

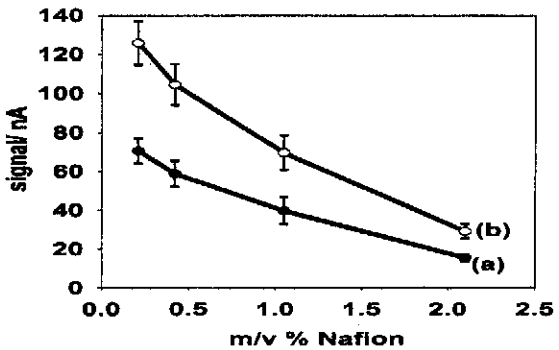

Fig. 3 Effect of Nafion concentration in casting solution (1 + 1 ethanol + H_2O). Curves (a) and (b) measurements at 100 and 200 mg l^{-1} D(+)-glucose; carrier: 0.1 M/pH 7.5 phosphate; potential: –100 mV; injection volume: 100 µl; flow: 0.1 ml min^{-1}

on the surface of the biosensor does not change so much by increasing the amount of enzyme. Even though higher enzyme loads would definitely provide wider dynamic ranges for the biosensor, no attempt was made to find the maximum possible load as the biosensor under study is also a model system in which expensive enzymes would be used later on.

Thus, as the outcome of this section, for subsequent studies the working enzyme-layer casting solution was prepared from the indicated components in the ratio 8 + 2 + 9 + 1 (v/v/v/v) of ethanol (99.6 %), Nafion (5 WT %, NH_3 neutralized), water and GOD (50 mg ml^{-1} in water), respectively, mixed in that order.

Operational Parameters

Working Potential

The working potential is decisive for the selectivity and sensitivity of the biosensor. Figure 4 shows the dependence of FIA signal on the operating potential. The highest signal occurs between –150 mV and –200 mV. However, the maximum signal is accompanied by a high background-to-signal ratio (Fig. 5). Thus, at about –125 mV the biosensor provides a signal not much smaller (–20 %) than the maximum, but much better with respect to the background. Additionally, higher potentials would raise the risk of interference. Potentials lower than –125 mV down to 0 mV might be used, albeit at the expense of sensitivity of the sensor. Thus, –125mV was used for the subsequent experiments.

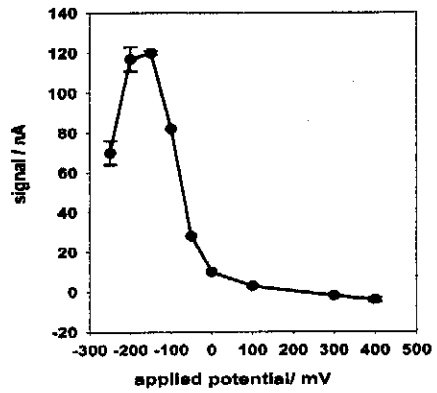

Fig. 4 The hydrodynamic voltammogram. Glucose: 100 mg l^{-1} inj. vol.: 100 µl; flow: 0.2 ml min^{-1}; carrier: 0.1M/pH 7.5 sodium phosphate

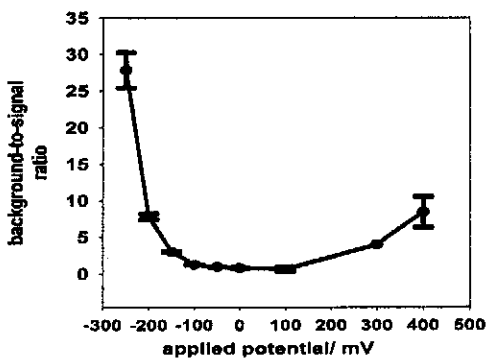

Fig. 5 Effect of potential on background to signal ratio. Glucose: 100 mg l^{-1} inj. vol.: 100 µl; flow: 0.2 ml min^{-1}; carrier: 0.1M/ pH 7.5 sodium phosphate

Flow Rate

When increasing the flow rate, the signal becomes smaller with injected samples due to dispersion of the analyte in the carrier (Fig. 6). Due to experimental reasons (analysis time) a flow rate of 0.2 to 0.4 was usually chosen for analysis yielding a sample frequency of 2 – 4 min^{-1}.

Effect of pH

In the investigated pH range (4.25 to 8.5), the signal somewhat linearly increases with decreasing pH, and levels off between pH 7.0 and pH 6.5 below which it shows a tendency to decrease.

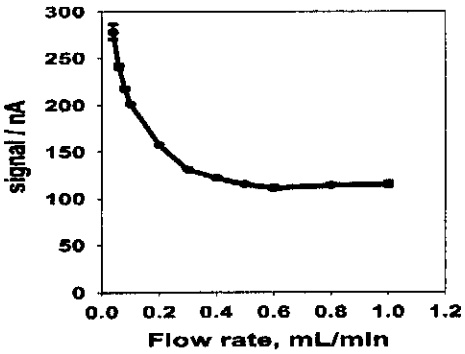

Fig. 6 Influence of flow rate on signal at 100 mg l^{-1} D-glucose; working potential: –125 mV; carrier: pH 7.50, 0.1M Na_2HPO_4/NaH_2PO_4; 100 µl injection volume

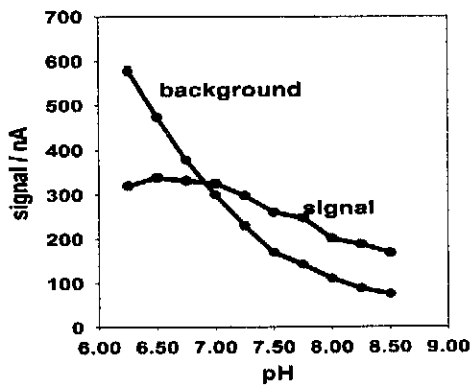

Fig. 7 Influence of pH on signal and background. Glucose: 200 mg l^{-1}; potential: –125mV; flow: 0.2 ml min^{-1}; inj. volume: 100 µl; carrier: 0.1M sodium phosphate

At pH 6.75 and below the background current becomes unfavorably high with a strongly decreasing signal-to-background ratio. Concerning the latter, pH 7.5 – 8.0 is preferential also with regard as a medium for enzymatic reactions.

Buffer Concentration

The sensitivity of the biosensor increases with decreasing the ionic strength (concentration) of the carrier. A possible cause of this trend is the incorporation of phosphate species into the Fe_3O_4 surface lattice [34,35]. Actually, phosphate inhibits the catalytic action of α-FeOOH (s) (oxidation of organic compounds by H_2O_2) because of its preferential adsorption on the surface of the catalyst [36]. Thus, as the concentration of the phosphate buffer is reduced, the surface

concentration of catalytically active iron oxide species increases leading to a rise in sensitivity.

As far as the choice of operating conditions for the biosensor is concerned, it is obvious that too small concentration of the buffer would compromise its buffer capacity. Considering this and other obvious factors, buffer concentrations between 0.05 and 0.1M seem to work fine.

Figures of Merit

Figure 8 shows a typical calibration curve for the biosensor being reported. The biosensor responds linearly from 5 to 100 mg l^{-1} glucose ($r^2 \geq 0.999$) with a sensitivity of 1.78 ± 0.01 nA l mg^{-1}. With a relative error of ±5 %, the calibration graph is quasi-linear from 7 to 150 mg l^{-1} D-glucose. The repeatabilities, for example, at 10 and 100 mg l^{-1} glucose are 2 % and 0.6 %, respectively. Its detection limit is 0.5 mg l^{-1} (3σ, n = 10).

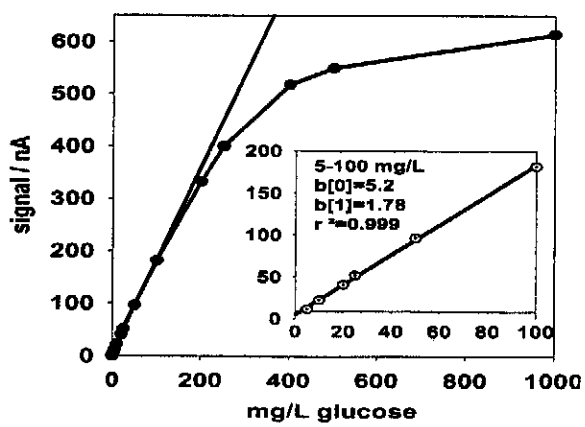

Fig. 8 Calibration curve. Potential: –125 mV; flow rate: 0.2 ml min^{-1}; 100 µl injection volume; carrier: 0.05 M/pH 7.5 sodium phosphate; speed: 15 – 20

A Lineweaver–Burk plot was used to analyze the data of the calibration curve, and an apparent Michaelis–Menton constant ($K_{M,app}$) of 1.6 (±0.3) mM or 280 (±60) mg l^{-1} was found. This value is significantly less than values of GOD in aqueous solution (10 – 110 mM; [37–39]) indicating that the response of the biosensor being reported is not limited by the actual enzyme kinetics [40]; thus, it seems that the rate of diffusion through the enzyme layer limits the biosensor's response, whereas the bare transducer has much higher sensitivity and wider linearity towards H_2O_2 [12].

Stability

No more than 4 % change in signal was observed during continuous FIA measurements using the same biosensors for a whole day.

When the biosensor was left in the flow system (0.2/0.1 ml min^{-1}) for 11 days while measurements were taken each day, its response showed only 6 % variation (RSD) around the mean reading. Thus, it withstands several days of continuous operation under flow.

Shelf Life-Time

Biosensors stored one month in dry room condition or wet in the phosphate buffer at +4 °C did not show any significant change in response. After storage for 3 – 4 months under same conditions responses decreased by 50 – 60 % in sensitivity.

Interferences

Four common clinical indigenous interferences, uric acid, hypoxanthine, xanthine, and ascorbic acid, were tested with the determination of glucose with the new biosensor. None of the interferents disturbs the determination severely (≤ ±10 % relative signal change) up to the concentrations equal to that of glucose. At a 10 fold excess of interferent, significant changes occur with ascorbic acid, xanthine and uric acid. Tests with interferents alone showed that these substances gave signals comparable to the signal change when injected with glucose.

Table I Influence of interferents as relative change (%) of signal in the presence of D(+)glucose (100 mg l^{-1}): flow 0.2 ml min^{-1}, injection volume 100 µl, potential –125 mV, carrier 0.05 M / pH 7.5 sodium phosphate

Interference	10 mg l^{-1}	100 mg l^{-1}	1000 mg l^{-1}
Ascorbic acid	2.4	–10.8	33.9
Hypoxanthin	–1.1	–3.5	–10.0
Xanthine	–6.6	–8.5	–19.8
Uric acid	–2.6	6.3	22.8

For the determination of glucose in blood, the analyte must be diluted some 10 times. Thus, the final concentrations of interferents are below the border of the significant influence [41–43]. Thus, it is obvious that the biosensor can be applied

without interference elimination steps.

Striking is the fact that the signals with Fe_3O_4-mediators are in general much less interfered than MnO_2, which could be primarily because of the difference in their operating potentials [31,38]. After comparison with the results of the study made on the bare Fe_3O_4/SPCE H_2O_2 transducer [12], the success of this biosensor in selectivity can be ascribed to the concerted effects of the cathodic mode of operation, perm-selectivity of the GOD/Nafion layer, and catalytic selectivity of the modifier itself.

Blood Samples

Figure 9 shows the FIA amperogram for the analysis of a 40-times diluted and centrifuged blood sample. Samples from two different volunteers were analyzed, and recoveries rates of 86 % and 105 % compared to a commercial pocket glucometer, GlucoMen® Glycó, as a reference method were found. Thus, one may conclude that the proposed biosensor gives fairly accurate measurements.

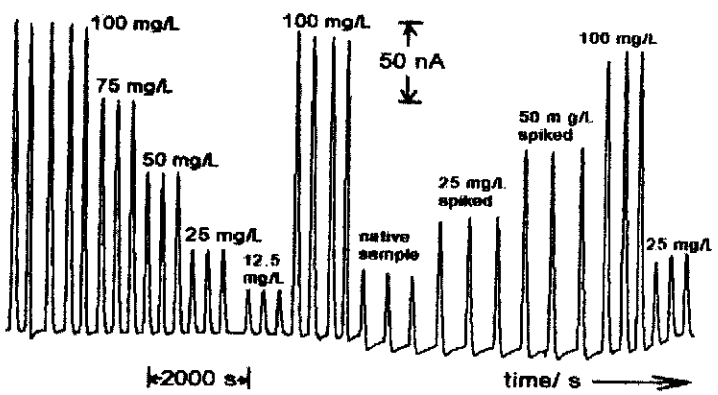

Fig. 9 Amperogram of glucose standards and blood plasma samples (40× diluted & centrifuged: 4500 rpm, 10 min); flow: 0.2 ml min^{-1}; potential: –125mV; inj. vol.:100 µl; carrier: 0.05M/pH 7.5 sodium phosphate; base-line = 189 nA

A few drawbacks could be noticed during blood analysis. The base-line was slightly shifted upon subsequent injections of blood plasma, but recovered after some period to its initial value, prolonging the analysis time. Investigations concerning some improvement of real sample analysis are currently on the way.

Conclusion

This work demonstrates that Fe_3O_4 may be used as a mediator in low-voltage (–125 mV) amperometric oxidase-based biosensors. The enzyme casting procedure is effective, simple, reproducible (3 – 4%), and suitable for working with expensive enzymes. The model biosensor with glucose oxidase gives stable signals for hours of uninterrupted-polarization in a flow system, withstands several days of operation under continuous flow, and practically is not endangered by interference in the context of clinical application. A preliminary application on clinical samples (blood plasma) showed that this model biosensor gives acceptably accurate results.

Acknowledgements

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References

[1] O'Neill R.D., Changb S.-C., Lowryc J.P., McNeil C.J.: Biosens. Bioelectron. **19**, 1521 (2004).
[2] Prodromidis M.I., Karayannis M.I.: Electroanalysis **14**, 241 (2002).
[3] Wang J., Lu F., Angnes L., Liu J., Sakslund H., Chen Q., Pedrero M., Chen L., Hammerich O.: Anal. Chim. Acta **305**, 3 (1995).
[4] Csoeregi E., Joensson-Petterson G., Gorton L.: J. Biotechnology **30**, 315 (1993).
[5] Schachl K., Alemu H., Kalcher K., Ježková J., Švancara I., Vytřas K.: Analyst **122**, 985 (1997).
[6] Douskou M., Efstathiou C.E, Koupparis M.A.: 3rd International conference, Instrumental Methods of Analysis - Modern Treands and Applications, 23 – 27 September 2003, Thessaloniki, Greece, Conference proceedings, pp. 742–745, (2003).
[7] Moscone D., D'Ottavi D., Compagnone D., Palleschi G., Amine A.: Anal. Chem. **73**, 2529 (2001).
[8] Linders C.R., Vincke B.J., Patriarche G.J.: Anal. Lett. **19**, 1831 (1986).
[9] Garjonyte R., Malinauskas A.: Sensor. Actuat. B-Chem. **B46**, 236(1998b).
[10] Lin M.-S., Lai J.-S., Wang J.-S.: Huaxue **60**, 483 (2002).
[11] Ricci F., Palleschi G.: Biosens. Bioelectron. **21**, 389 (2005).
[12] Waryo T.T., Kalcher K.: 2005, manuscript.
[13] Raba J., Mottola H.A.: Crit. Rev. Anal. Chem. **25**, 1 (1995).

[14] Bath B.D., White H.S., Scott E.R.: Anal. Chem. 72, 433 (2000).
[15] Brown R.S., Luong J.H.T.: Anal. Chim. Acta **310**, 417 (1995).
[16] Dunne S.O., Brown F.O., Lowry J.P.: in O'Connor W.T. (Ed.), Proc. Int. Conf. on in Vivo Methods, 9th, Dublin, Ireland, June 16–19, 2001, pp. 55–56 (2000).
[17] Fortier G., Vaillancourt M., Belanger D.: Electroanalysis **4**, 274 (1992).
[18] Garjonyte R., Malinauskas A.: B. Electroche. **19**, 529 (2003).
[19] Ji H., Wang E.: J. Chromatogr. **410**, 111 (1987).
[20] Karyakin A.A., Gitelmacher O.V., Karyakina E.E.: Anal. Chem. **67**, 2419 (1995).
[21] Kenneth W.W., Lawrence B.J., Anderson E., Gerard R., Jean-Claude K., George S.W.: Bioesens. Bioelectron. **17**, 181 (2002).
[22] Mailley P., Cosnier S., Coche-Guerente L.: Anal. Lett. **33**, 1733 (2000).
[23] Pan S., Arnold M.A.: Talanta **43**, 1157 (1996).
[24] Ogumi Z., Takehara Z., Yoshizawa S.: J. Electrochem. Soc. **131**, 769 (1984).
[25] Gottesfeld S., Raistrick I.D., Srinivasan S.: J. Electrochem. Soc. **134**,1455 (1987).
[26] Rikukawa M.: Maku (Japanese) **28**, 14 (2003).
[27] Wisniewski N., Reichert M.: Colloid. Surface. B **18**, 197 (2000).
[28] Turner R.F.B., Sherwood C.S.: ACS Symposium Series **556**, 2111 (1994).
[29] Lunte S.M., Lunte C.E., Kissinger P.T.: in Kissinger P.T., Heineman W.R. (Eds), *Laboratory Techniques in Electroanalytical Chemistry*, 2nd ed., Marcel Dekker, 1996, pp. 813–883.
[30] Karyakin A.A., Kotel'nikova E.A., Lukachova L.V., Karyakina E.E., Wang J.: Anal. Chem. 74, 1597 (2002).
[31] Beyene N.W., Moderegger H., Kalcher K.: South African J. Chem. **57**, 1 (2004).
[32] Cao Dongfang, He Pingli, Hu Naifei: Analyst **128**, 1268 (2003).
[33] Rossi L.M., Quanch A.D., Rosenzweig Z.: Anal. Bioanal. Chem. **380**, 606 (2004).
[34] Balasubramanian R.: Kinzoku Hakubutsukan Kiyo **34**, 64 (2001).
[35] Haenisch M., Otto A.: J. Electroanal. Chem. & Inter. Electrochem. **308**, 113 (1991).
[36] Gurol M.D., Lin S.-S.: J. Adv. Oxi. Technol. **5**, 147 (2002).
[37] Gozia O., Ciopraga J., Schel H.D.: Studii si Cercetari de Biochemie **26**, 135 (1983).
[38] Gibson Q.H., Swoboda B.E.P., Massey V.: J. Biol. Chem. **239**, 3927 (1964).
[39] Karmali K., Karmali A., Teixeira A., Curto M.J.: Anal. Biochem. **333**, 320 (2004).
[40] Bartet P.N., Toh C.-S.: in Cooper J.M., Cass A.E.G. (Eds), *Biosensors, Practical Approach*, 2nd ed., 2004, Oxford Univ.,2003, pp. 59–96.
[41] Schachl K.: *Development of Electrochemical Sensors based on Carbon*

Electrodes with Manganese Dioxide (PhD Thesis), Karl Franzens University of Graz, Austria, 1998.

[42] Curtius H.C., Roth M.: *Clinical Biochemistry*, Vol. 2, Walter de Gryter, Berllin, BRD, 1974; cited in 41.

[43] Harper H.A., Löffler G., Petrides P.E., Weiss L.: *Physiologische Chemie*, Springer Verlag, BRD, 1975; cited in [41].