Amperometric Glucose Biosensor for an Undiluted Whole-Blood Analysis

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The design of an enzymatic biosensor based on the use of immobilized glucose oxidase is described for the determination of glucose in blood serum and undiluted whole blood. Measurements in whole blood with RSD=0.3% are possible due to the use of an outer polypropylene membrane wetted with a mixture of various polyoxyethylene ethers (Triton X-100).

Keywords Glucose, amperometric biosensor, whole-blood analysis

The determination of glucose in whole blood is of great importance in clinical analysis for at least three reasons. Firstly, it allows a shortening of the entire analytical procedure for the determination due to an elimination of the time-consuming separation of blood cells and plasma. Secondly, there is a well-known need for glucose monitoring of critically ill and surgical patients.1,2 Thirdly, the design of such a sensor could lead directly to the development of an implantable sensor for in vivo use with closed-loop insulin infusion systems.

Glucose determination in whole blood represents numerous additional difficulties compared to a determination in blood serum. It has been pointed out in several papers that the glucose content measured in whole blood is 12 to 20% lower than that in serum.3–6 Another difficulty is a rapid decrease in the glucose concentration in upreserved blood samples, because of the glycolytic action of erythrocytes and leukocytes. Hence, some protective components are added, such as fluoride7,8 or sodium iodoacetate.8 In order to protect against coagulation it is common to add oxalate9, or to collect samples into heparinized tubes.7–9 Some influence can also be expected from any differences in the viscosity of whole-blood samples. The elimination of chemical interference from some species present in natural samples (ascorbate, uric acid, glutathione etc.) can be achieved by selecting an appropriate protective membrane.

An amperometric detection of glucose based on measuring the anodic current of the oxidation of hydrogen peroxide exhibits a much better sensitivity of determination than does a differential measurement of the oxygen consumption, and diminishes the effect of the partial pressure of oxygen within the solution in contact with the electrode. The amperometric biosensors described in earlier reports were used for glucose determination in whole blood under both stationary5,11–13 and flow9,10,14 conditions.

In all previous studies much attention was paid to optimizing the outer membrane of the biosensor for use in whole-blood analysis. The use of the perfluorinated ionomer Nafton as an outer dialytic membrane produced a much better sensor than that using cellulose dialysis membranes.11 For a sensor with a thin rubber membrane, satisfactory performance in whole blood was also observed; however, its response time was a few minutes and the lifetime was only several days.12 Good results have also been reported for the use of polycarbonate membranes.9,14,15 When microporous polycarbonate membranes were treated with liquid isopropyl myristate, the linearity of the response to glucose was extended well above the substrate physiological range.13

The aim of this study was to optimize the design of a multimembrane amperometric sensor developed earlier for a flow-injection glucose determination, and based on glucose oxidase16 for a discrete determination of glucose in undiluted whole blood.

Experimental

Apparatus

Voltammetric measurements were carried out using voltammograph-type CV-37 from Bioanalytical Systems (West Lafayette, IN, USA) connected to strip chart recorders (Potentiograph E436 or Labograph E586 from Metrohm (Herisau, Switzerland).

The design of the biosensor measuring cell is shown in Fig. 1. It comprised two perspex blocks mounted together using 4 screws. In part A there was a silver disk
electrode (1) covered with silver chloride, which served as a reference electrode. In the same part, the inlet hole (6) for sample introduction using an automatic pipette and an outlet stainless-steel capillary (2), which served as an auxiliary electrode, were made. In part B, a platinum disk of 2.0 mm diameter (5) was placed as well as a silver/silver chloride ring of 1 mm wide and 7 mm diameter (4), which can be used as alternative reference electrode.

The preparation of the detector for measurements was carried out as follows. The surface of a Pt disk electrode was polished with three different grades of alumina powder (1, 0.5 and 0.03 µm); it was washed with distilled water and methanol and then cleaned for 10 min electrochemically in a hot alkaline phosphate bath by alternate polarization between +5 and -5 V. After careful washing and drying at room temperature, 8 µl of a Nafion solution was placed on the electrode surface, and left for 1 h for evaporation. The Nafion layer was covered with a polyester membrane with immobilized GOD and an outer protective membrane. Glucose measurements were carried out at +0.65 V vs. Ag/AgCl reference electrode.

Reagents

In all measurements, various lots of glucose oxidase (GOD) type X-S cat. No. G-7141 from Sigma (St. Louis, MO, USA)) having specific activities ranging from 117 to 138 units/mg were used. For immobilization of the enzyme on a polyester membrane (PE 04UM from Nucleopore) having a pore size of 0.4 µm a 50% aqueous solution of glutaraldehyde (from Fluka (Buchs, Switzerland)) was applied. The immobilization procedure was the same as that described earlier.16

A stock 20 mM glucose solution in a phosphate buffer of pH 7.1. For preservation, 50 mg of sodium azide was added. The stock solution was filtered using a 0.45 µm nylon filter, and stored in a dark glass bottle at 4°C. The stock solution was prepared at least 24 h prior the use. The standard solutions used for measurements were freshly prepared by appropriate dilution of the stock solution with a 0.05 M phosphate buffer of pH 7.1 containing 8.19 g/l of sodium chloride.

A stock solution of a 0.5 M phosphate buffer was prepared by dissolving 78.0 g of NaH2PO4·2H2O in 700 ml of distilled water. After a pH adjustment to pH 7.1, distilled water was added to 1 l, and the solution was filtered. For use in measurements the stock solution was diluted with distilled water, as required.

The following commercial control sera were used: Seronorm and Pathonorm from Nycomed (Oslo, Norway), Precinorm from Boehringer (Mannheim, Germany) and Serachem from Fischer (Orangenburg, NY, USA).

Procedure for glucose determination in whole blood

Human blood samples were collected in fluorinated and heparinized microtubes. Amperometric measurements were performed using a 100 µl sample volume not later than 45 min, since samples were collected from patients. Prior to sample introduction the detector was washed with as 0.5 ml of a 50 mM phosphate buffer of pH 7.1 containing 0.14 M sodium chloride. A part of each sample was centrifuged, and glucose in the obtained serum was determined using the commercial clinical analyzers described below.

Results and Discussion

Glucose determination in blood serum

The design of an integrated glucose biosensor with a platinum disk used as the working electrode and a three-layer membrane was optimized earlier for flow-injection measurements.16 The design of the biosensor detector used in this study, having the same structure of the sensing part, is shown in Fig. 1. In preliminary measurements for the determination of glucose in blood serum a polyester membrane from Nucleopore (pore size, 0.4 µm) was used as an outer protective membrane. For a glucose concentration range of up to 5 mM with a detector having a 50 µl dead volume of the measuring compartment, a steady signal was obtained after 1.5 min.

The stability of the functioning of the developed biosensor observed earlier in the flow-injection measurements was also confirmed in discrete batch measurements. It was tested for either storage of the detector between measurements in a refrigerator, or for its constant use and storage at room temperature. Table 1 shows the results obtained during a 10-week test, in which the entire detector was stored at +4°C between measurements. During that testing procedure the detector was used for determining glucose in blood-serum samples diluted 1:10 with a phosphate buffer.
Small fluctuations in the observed signal value can be at least partly attributed to the fact that all of the measurements were carried out under non-thermostated conditions. The detector stored in room temperature showed a decrease in the signal magnitude of about 10% after two weeks.

A study concerning the precision of discrete glucose measurements was carried out for control serum samples diluted 1:10 with a phosphate buffer. The obtained RSD values (from 2.1 to 2.3%) were worse than those observed in an automated flow-injection measurement, which can certainly be attributed to the fact that all measurements were carried out manually.

For practical measurements, 1:10 dilution of the serum samples with a 0.1 M phosphate buffer of pH 7.1 was applied, and the signal reading was recorded after 90 s. Prior to the introduction of each sample, the measuring compartment of the detector was washed with 0.5 ml of a diluent. Fresh serum samples were simultaneously analyzed using a Beckman Glucose Analyzer 2 or a Kone Progress Analyzer. The obtained correlation plots for two series of natural samples used for a comparison with commercial analyzers are shown in Fig. 2. The correlation coefficient values were 0.955 and 0.997 for the Beckman and Kone analyzers, respectively.

Optimization of the outer membrane for undiluted whole-blood measurements

The importance of using an outer protective membrane in the design of a biosensor for the determination of glucose in whole blood has already been stressed above. Cutting off macromolecular species and reducing the transport rate of oxidizable interference improves the accuracy of both the determination and lifetime of the biosensors. Thus, such a protection method is often employed in biosensor technology.

The use of the polyester macroporous membrane Nucleopore PE 04UM yields satisfactory results for the determination of glucose in serum in either flow-injection or non-flow measurements. However, because of the much complex matrix of whole blood for that specimen, several different membranes were examined. In these tests a comparison of the results obtained for undiluted whole blood with those obtained for the same sample of whole blood diluted at a different ratio with a phosphate buffer was made. The profile of signal changes in time, and the results of a determination in the correlation with determination results in serum obtained with commercial glucose analyzers were examined.

A biosensor having an outer polyester membrane, regarded as being a satisfactory configuration for serum analysis, exhibits less-favorable signal changes when applied to whole-blood analysis (Fig. 3). Compared with serum analysis, for both undiluted and diluted whole blood, the observed signal changes exhibit a certain maximum of the signal magnitude 10 to 20 s after sample introduction, and then shows a decrease in the signal magnitude (curves A–C in Fig. 3). The nature of overshoots in the current shown in Figs. 3 and 4 when a standard or sample solution was changed to a washing buffer was not investigated, since it did not interfere with the estimation of analytical result. Rather, it seems to have been caused by the charging and discharging

<table>
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<th>Signal magnitude/ nA</th>
<th>Correlation coefficient b</th>
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<td>71</td>
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a. For 1.6 mM glucose solution.
b. For calibration plot.

**Fig. 2** Correlation plots obtained for a glucose determination in blood serum samples vs. Beckman Glucose Analyzer 2 (A) and Kone Progress Analyzer (B).
processes, as reported earlier\textsuperscript{17}, than by an oxidation-reduction of the electroactive surface functional groups suggested for a glassy carbon electrode.\textsuperscript{18} The smaller is the dilution ratio, the faster and larger is the decrease in the signal value. Hence, the glucose determinations in blood samples were performed using 5- and 10-fold dilution of whole blood. In both series of measurements the signal readings were obtained 90 s after sample introduction. For 10-fold diluted samples, no decrease in the signal magnitude was observed. Quantitative results were also satisfactory for 1:5 diluted whole-blood samples, where the correlation plot was \( y = 0.945x - 0.091 \), and the correlation coefficient was 0.945. A systematic negative shift for whole blood results compared to that of serum has also been observed by other authors, as mentioned above.

In this study numerous other membranes were also examined as outer protective membranes in terms of the recorded signal shape, stability of response and accuracy of the glucose determination. Several membranes which are non-permeable for glucose molecules, such as polyaramide membranes covered with glycerol, dialytic polyethane membranes, polycarbonate membranes covered with silicone and polypropylene hydrophobic membranes, can not be utilized for glucose biosensor.

A disadvantageous behavior was observed in measurements of a whole-blood sample for two polyethylene terephthalate membranes having different pore sizes of 0.1 and 0.2 µm. Although the shape of signals recorded for aqueous glucose solutions was regular and the response was linear up to 2.5 mM of glucose, for undiluted whole blood the response was unsatisfactory. As for a polyester membrane after increasing part of the response curve, instead of the plateau, a rapid decrease in the signal was observed. Similarly to a biosensor with a polyester membrane, after several measurements in undiluted whole-blood samples, the sensitivity of the response decrease. Whole-blood samples diluted 1:2 with a phosphate buffer have not exhibited that behavior. Very similar results were obtained for cellulose nitrate and cellulose acetate membranes from Schleicher and Schuell, for nylon membranes (0.4 µm pore size) from the same manufacturer and from Supelco, and for polycarbonate membranes (pore sizes of 0.015, 0.2, 0.4 and 10 µm) obtained from Nucleopore.

A different behavior was found for polypropylene Celgard membranes from Celanese. Among the membranes examined were hydrophobic membranes of 25 µm thickness and pore sizes of 20 Å (Celgard 2400) and 40 Å (Celgard 2500), asymmetric thicker membranes covered on one side with a polypropylene fiber (Celgard 5411 and 5511) and hydrophilic membranes obtained by wetting the hydrophobic membranes mentioned above with an appropriate surfactant.

The use of asymmetric hydrophobic membrane (Celgard 5511) as an outer membrane has significantly improved the response of the biosensor to undiluted whole blood, although the initial rate of the signal increase is much smaller than that for the membranes mentioned above; some decrease in the signal was observed for consecutive measurements of the same sample of undiluted whole blood.

Hydrophobic polypropylene membranes (Celgard 2400 and 2500) were tested as outer membranes after wetting with 5% solutions of a non-ionic detergent (Brij-35), ethylene glycol, glycerin and a surfactant (Triton X-100, from Sigma). The hydrophobic membranes were soaked 10 min in these solutions, rinsed with distilled water and dried with tissue. A membrane pretreatment with Brij-35 and glycerin was not effective, and practically no analytical signal was observed. Very satisfactory results were obtained for membranes wetted with Triton X-100, with a similar sensitivity of response for both Celgard polypropylene membranes. Although a biosensor with a Celgard 2400 membrane exhibits a slightly slower response, its response to consecutive injections of the same undiluted whole-blood sample is more stable than that for the Celgard 2500 membrane.
The signal shape observed for a wetted Celgard 2400 membrane is much more regular than that for any other tested membranes. After a series of measurements of undiluted whole-blood samples the membrane remains white without any trace of adsorbed red blood cells. This outer membrane was assumed to be optimal, and was used for further investigations.

Recordings of measurements in undiluted whole-blood samples are shown in Fig. 4. The smaller recorded current values are due to the use of a Pt disk working electrode of 1.0 mm diameter, smaller than that used for the measurements shown in Fig. 3, and due to a different composition of the multicomponent layer covering the surface of the working electrode. A decrease in the sensitivity from 8.1 µA cm⁻²/mM (Fig. 3) to 0.62 µA cm⁻²/mM (Fig. 4) of glucose was observed. The correlation plot for a comparison with the glucose determination in corresponding blood serum samples using the Beckman Glucose Analyzer 2 shows a constant deviation in the slope, and a satisfactory correlation coefficient value of 0.987 (n=10). Although for a longer control of the response a decrease in the signal value is observed, the stability of the signal in one series of measurements and for measurements carried out within few days was evaluated as being satisfactory. Within a two-week interval the signal magnitude obtained for aqueous glucose solutions did not show any marked decrease. Between measurements the detector was stored in a phosphate buffer at 4°C. The reproducibility of the signal magnitude for six electrodes having the same diameter prepared in the same optimized way (expressed by the RSD value of the current for a 3.7 mM glucose standard solution) was 18%.

**Non steady-response of biosensor**

All of the measurements of glucose mentioned above were based on the steady-state signal of the detector usually obtained after 1 to 2 min. In order to examine the possibility of shortening that time interval and increasing the sampling frequency, several tests were performed with a signal readout after a much shorter time following sample introduction into the detector. For the signal recordings shown in Fig. 5, the current values were measured after 8, 16 and 24 s; the corre-
sponding calibration plots are shown in Fig. 6. In the sensor used for this experiment an additional dialytic Cuprophan membrane was employed between a polyester membrane with immobilized GOD and an outer protective membrane in order to extend the diffusion layer for hydrogen peroxide and to reduce the rate of the signal rise. In all cases, good linearity of the response was observed. The correlation plot for results of glucose determination in undiluted blood obtained with the readout at 12 s following sample introduction (Fig. 7) gives a correlation coefficient value of 0.932, and a smaller deviation from the results obtained in serum using the routine clinical analyzer.

**Stability of biosensor response**

Stability tests for the optimized detector with a glucose biosensor were performed in the solution delivery system of a Microlyte-7 Clinical Analyser from Kone. For 60 introductions of 5 mM standard glucose solutions an RDS of 0.26% was obtained, whereas for 20 introductions of undiluted whole blood samples an RSD of 0.31% was found.

During an 11 day experiment, in which the daily samples were introduced in 2 min intervals during 8 h, no distinct loss in the detection sensitivity was observed. The signal magnitude after overnight storage differed within several percent. Between measurements the detector was stored at room temperature.

In this study two basic goals have been achieved, enabling a successful application of a multimembrane glucose biosensor to a discrete glucose determination in whole undiluted blood. The choice of the appropriate material for the outer protective membrane and its hydrophobization prevents any accumulation of red blood cells and the macromolecular constituents of blood on the sensor surface. This allows a stable biosensor response to be obtained during an almost two-week testing period. It was also found that the introduction of an additional Cuprophan dialytic membrane as a spacer slows down the transport of glucose to the inner layers of the detector, which can be utilized for the use of transient signals for analytical measurements. The 0.31% precision obtained in the mechanized measuring system indicates the satisfactory functioning behavior of the developed system.

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**References**


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