ELECTROCHEMILUMINESCENT DETECTION OF GLUCOSE IN A FLOW INJECTION ANALYSER

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Abstract: Glucose oxidase was covalently attached to an aminosilanized indium tin oxide coated glass wafer with the hydroxysuccinimide ester of pyridyldithiopropionic acid. The wafer was used as the working electrode in the reaction chamber of a flow injection analyser. When a solution of glucose and luminol was passed over the electrode, glucose was oxidized enzymatically and luminol was oxidized electro-chemically. The products of these reactions generated light in proportion to the amount of glucose in the sample. When glucose was assayed in the range 0–10 mM the detection limit was 0.419 mM with a correlation coefficient of 0.9962.

Keywords: Electrochemiluminescence, glucose, luminol, indium tin oxide, flow injection analysis, glucose oxidase.
INTRODUCTION

The determination of glucose in blood samples from diabetic patients is one of the most common assays carried out in clinical laboratories. It is also important in nutritional analysis and biochemistry. As a result there is a great demand for analytical systems that can measure its concentration rapidly, accurately and inexpensively. Demand for these systems has resulted in a wide range of detection methods being employed. These include colorimetric (Kunst et al., 1984; Wilson, 1991), thermometric (Danielsson et al., 1977; Mandenius et al., 1985), amperometric (Clark and Lyons, 1962; Updike and Hicks, 1967; Cass et al., 1984), potentiometric (Nilsson et al., 1973; Soldatkin et al., 1993) and luminometric (Carter et al., 1982; Cattaneo and Luong, 1993) methods of detection. Most of these systems exploit the ruggedness and specificity of the enzyme glucose oxidase (GOD) (Wilson and Turner, 1992) that produces hydrogen peroxide in the presence of glucose and dissolved oxygen. In the analytical system described here GOD is covalently attached to indium tin oxide (ITO) coated glass wafers that are used as the working electrode in an electrochemiluminescent flow injection analyser. When a potential of one volt relative to a saturated calomel electrode (SCE) is applied to the ITO, luminol is oxidized and the oxidation product generates light when it reacts with hydrogen peroxide produced by GOD as shown in Fig. 1. The light intensity is proportional to the concentration of glucose.
EXPERIMENTAL

Apparatus.
Indium tin oxide coated (ITO) glass was from Balzer Ltd., Buckinghamshire, England. It was cut into wafers that measured 36 mm x 20 mm x 1mm thick. The ITO layer was 20 nm thick and had a resistance of 200 Ω/□. The flow injection analyzer pump, six way valve and injection loop were part of a single unit supplied by Ismatec UK Ltd., Surrey, England. Solutions were pumped at a flow rate of 0.475 ml min⁻¹ to an electrochemical flow cell connected to a potentiostat, both made in-house. Light generated in the flow cell was detected with a type 9558QA photomultiplier tube (PMT) (Thorn EMI, Middlesex, England) connected to a model 475R power supply (Brandenburg, England). The entire system was controlled by a 486/33 PC programmed with Viewdac data acquisition software (Keithley Data Acquisition, Taunton, MA, USA) and fitted with two DAS1602 Data Acquisition Boards (Keithley). All potentials are relative to a SCE.

Reagents and Solutions.
All solutions were prepared in water from a Still Plus purification unit fitted with a deioniser and carbon filter from Purite Ltd., Oxfordshire, England. Reagents of the highest purity available were used. Glucose oxidase (GOD) (EC 1.1.3.4) Type X-S, ovalbumin (grade V), N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) and glucose (HPLC grade) were from the Sigma Chemical Co., England. Luminol was from Fluka Chemical Ltd., England. Glucose solutions were allowed to stand for 48 h before use to allow mutarotation to take place.
Covalent Attachment of Proteins to ITO Coated Wafers.

ITO was silanized as described previously (Wilson and Schiffrin, 1994). Glucose oxidase (50mg) was dissolved in 4.35ml of 0.1 M phosphate buffer, pH 7.5, that contained 0.1 M NaCl. To this was added (dropwise with stirring) 0.65ml of a 12mg ml⁻¹ solution of SPDP in ethanol. The product was allowed to stand for one hour at room temperature. Then it was loaded onto a column packed with Sephadex G-25 and eluted with the same buffer. The absorbance of the eluate at 450nm was monitored and fractions with a high optical density were pooled and stored at -20°C. The dithiol content was determined according to the method of Carlsson (Carlsson et al., 1978). The concentration of GOD was determined from the absorbance at 450nm using an extinction coefficient of 1.41 x 10⁴ mol⁻¹ cm⁻¹ (Swoboda and Massey, 1965). Ovalbumin, at the same concentration, was treated with SPDP in the same way except that the eluate was monitored at 260nm, and protein concentration was determined with Bio-Rad Protein Assay from Bio-Rad, München, Germany, according to the method of Bradford (Bradford, 1976; Spector, 1978). These proteins were attached to ITO coated wafers as described previously for horseradish peroxidase (Wilson and Schiffrin, 1994).

Electrochemiluminescence Measurements.

Derivatised ITO wafers were clamped into the flow cell as shown in Fig. 2. The flow cell was part of the flow injection analyser shown in Fig. 3. When luminol was oxidized at a potential of 1 volt, the oxidation product reacted with hydrogen peroxide from GOD and generated light, which was detected by the
photomultiplier tube (PMT).

The electrochemiluminescent reaction takes place under alkaline conditions, but prolonged exposure to these results in loss of GOD activity. To avoid this 10 mM KCl (running solution) was pumped through the flow cell between assays. The running solution had a pH close to neutral, but no buffering capacity. Therefore enzyme activity was preserved, but the pH could rapidly be made alkaline during an assay. The initial conditions for glucose detection (assay solution) were chosen from a knowledge of the enzyme and electrochemiluminescent reactions. The assay solution was prepared in 10 mM TRIS buffer, pH 8.0, and contained 10 mM KCl, 10 mM glucose and 100 μM luminol. It was injected in 110μl amounts into the running solution. Fig. 4 shows the way light and current varied as the assay solution was pumped through the flow cell.

Optimisation of pH and Luminol Concentration.

The pH was optimised by passing 10 mM TRIS buffer over ITO wafers derivatised with GOD. The TRIS buffer contained 10 mM KCl, 10 mM glucose and 100 μM luminol. The pH was varied between 7.2 and 9.4 and the light intensity recorded. An identical procedure was carried out using ITO wafers derivatised with ovalbumin as a control. The luminol concentration was optimised in the same way except that the pH was fixed at 9.0 and the luminol concentration was varied between 0 and 100 μM. In between measurements at different pH and luminol concentration, glucose was reassayed at pH 8.0 as described previously, to monitor the stability of the
Detection of Glucose.

Glucose solutions in the range 0-10 mM were prepared with pH 9.0 TRIS buffer that contained 10 mM KCl and 100 μM luminol. They were injected in 110μl amounts into the running solution (10 mM KCl). As the glucose solution passed through the flow cell light and current were measured simultaneously.

RESULTS AND DISCUSSION

GOD and ovalbumin treated with SPDP contained 9 and 10 blocked disulphide bonds per mol of protein respectively. No previous knowledge about the stability of ITO coated glass derivatised with GOD was available. To monitor this glucose was redetermined at pH 8.0 at the end of every change in pH or luminol concentration. This showed that the light intensity for a fixed concentration of glucose remained unchanged for the duration of the experiment. The effect of pH and luminol concentration on light intensity is shown in Figs. 5 and 6 respectively. For comparison the way light intensity varied for ITO coated wafers derivatised with ovalbumin is also shown. When the latter values were subtracted from the former optimum conditions of pH 9.0 and 100μM luminol were obtained and these were used in subsequent glucose assays. The value of 9.0 for the optimum pH is a compromise between the optimum pH for the enzyme and for electrochemiluminescence. Immobilized GOD usually has an optimum
pH close to neutral (Liu and Chung, 1994; Koopal and Nolte, 1994) and the electrochemiluminescent reaction has an alkaline pH optimum (Haapakka and Kankare, 1982; Hoshino and Hinze, 1987). Background electrochemiluminescence was generated by wafers derivatised with ovalbumin as shown in Figs. 5 and 6. Systematic elimination of each reagent (including ovalbumin) showed that it was due to glucose. Use of HPLC grade glucose and microbiologically pure water failed to eliminate it, and this problem will be the subject of further investigation.

When glucose solutions of different concentrations were injected into the running solution the light intensity increased linearly from 0-10 mM glucose as shown in Fig. 7. The limit of detection for glucose defined as 2 x S.D. of 21 zero calibrators was 0.419 mM. The correlation coefficient was 0.9962 for 42 different injections. Each measurement took less than three minutes. It was not possible to locate previous examples of the electrochemiluminescent detection of glucose. Therefore it was necessary to compare these results with other methods of detection.

The current through the flow cell gradually increased during the glucose assays. This implied that the current increased as the concentration of glucose increased and suggested that some of the hydrogen peroxide produced by GOD was being detected amperometrically. However, at a potential of 1 V oxidation of hydrogen peroxide at an ITO electrode is inefficient, and in a subsequent experiment it was shown that the electrode slowly
became more active with time, but this had no affect on light intensity, which remained proportional to the concentration of glucose in the sample. This was because the concentration of luminol (100 μM) used in the glucose assays already exceeded the rate limiting concentration of luminol at the start of the experiment, as shown by Fig. 6. This illustrates a major advantage of combining electrochemistry and luminometry. When the product of an electrochemical reaction takes part in a second reaction, the second reaction is unaffected by the electrochemistry, provided the concentration of the first reactant (in this instance luminol) remains in excess of the rate limiting concentration of that reactant throughout the assay. By contrast, passivation or activation of an electrode can interfere with purely electrochemical assays, making it necessary to recalibrate the system or clean the electrode.
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REFERENCES


FIGURE LEGENDS

Figure 1. The Electrochemiluminescence reactions. Luminol is oxidised at the ITO electrode and the product reacts with hydrogen peroxide from GOD in a chemiluminescent reaction.

Figure 2. Flow cell for electrochemiluminescence assays. Light generated in the reaction is transmitted through the ITO coated glass and detected by a PMT positioned above the cell. Key: C.E. = Counter electrode; W.E. = Working electrode. The reference electrode was in the effluent solution.

Figure 3. Flow injection system.

Figure 4. Light and current traces when a 10 mM glucose solution at pH 8.0 was pumped through the flow cell.

Figure 5. Graph showing how the light/current varied with pH.

Figure 6. Graph showing how light/current varied with luminol concentration.

Figure 7. Graph showing how light varied with glucose concentration.
Fig 1

GOD

O₂

GLUCOSE

GLUCONATE

LUMINOL

OXIDIZED LUMINOL

+ H₂O₂

LIGHT

e⁻

ITO

GLASS

TO PMT