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Highly stable first-generation biosensor for glucose utilizing latex particles as the enzyme-immobilizing matrix

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The ability of polystyrene latex beads to immobilize glucose oxidase was applied to construct a stable biosensor for glucose. This biosensor measures glucose by detecting the hydrogen peroxide produced by the enzyme. The biosensor performance was studied by amperometry. Glucose concentrations ranging from 1 to 50 mM can be measured with this sensor. The sensor is active over a broad range of pH and is very stable, which makes it suitable for a number of possible applications.

Keywords: Glucose sensor; latex beads; hydrogen peroxide; amperometry

Introduction

In a previous publication1 we described how uniform latex particles (ULPs) can be used to construct a so-called third-generation biosensor. These particles were deposited on an electrode and coated with a thin layer of the conducting polymer polypyrrole. The polypyrrole matrix was found to absorb glucose oxidase, and the resulting enzyme electrode could detect glucose without the need of an additional mediator. In this paper we present evidence that latex particles themselves can also act as a medium for immobilizing enzymes.2-4 Furthermore, we show that they can be applied to construct a first-generation biosensor that competes favorably with other first-generation biosensors reported in the literature.5-9

Latex polystyrene particles with submicron diameter are normally produced by emulsion polymerization in water.2 They are mildly hydrophobic and carry a negative surface charge, because of the presence of sulfonate groups. Enzymes can be adsorbed physically on these particles.10 However, this adsorption is reversible and leakage of enzyme is encountered very frequently.11 Special ULPs with functional groups on the surface have been developed to overcome this problem. These functional groups allow for covalent attachment of the biomolecule to the surface of the latex beads. These functionalized beads, however, are very expensive.

Immobilization of glucose oxidase onto latex beads has been reported before in the literature, and it has been suggested that the enzyme-loaded latex might be used as a biosensor.10 Until now, however, the construction of a mechanically stable first-generation biosensor from these beads has been impossible. We have found that stable membranes of uniform latex particles can be obtained if mixtures of latex and agarose are used to deposit the membrane.12 This finding opens the possibility to develop a stable sensor device, as will be described below. This device relies on the reaction of glucose oxidase with its natural cosubstrate molecular oxygen:

Glucose + O₂ → gluconolactone + H₂O₂ (1)

The enzyme-catalyzed conversion of glucose produces hydrogen peroxide, which can be detected by oxidation at the electrode on which the latex layer is deposited.13

Materials and methods

Materials and equipment

Glucose oxidase (E.C. 1.1.3.4) type II (25,000 Ug⁻¹ from Aspergillus niger was obtained from Sigma. Benzoquinone was obtained from Aldrich (FRG) and was sublimed prior to use. The latex...
suspensions were purchased from Perstorp Analytical and contained beads of 112 or 220 nm. Agarose type VIII was obtained from Sigma. Glucose was purchased from Merck. Solutions of this compound were allowed to mutarotate for at least 24 h before use. All other reagents were of analytical grade.

The electrochemical measurements were performed with a CU-04-AZ electrochemical control unit (Antec Leyden, The Netherlands) or an Autolab potentiostat controlled by an Olivetti M24 Personal Computer and General Purpose Electrochemical System (GPES)-software (Eco Chemie, Utrecht, The Netherlands). The current output was recorded on a Yew 3056 pen recorder. Electron micrographs were made on a CAMSCAN scanning electron microscope (Cambridge Instruments).

**Procedures**

**Preparation of the latex electrodes**

Glassy carbon (GC) disks of 8-mm diameter (Antec Leyden, The Netherlands) were used as the electrode material. The electrodes were polished with Alpha Micropolish Alumina No. 1C (1.0 μm, Buehler LTD., USA). Platinum was applied on the polished surface with an Edwards sputtercoater S150B. A platinum target of 8-cm diameter and 0.5-mm thickness was used as the platinum source. The layer thickness was monitored with an Edwards FTMS unit. Sputtering was continued until the thickness of the platinum layers was 300 nm.

Agarose type VIII was dissolved (0.1 wt%) in boiling distilled water. Freshly made agarose solutions, which were still hot, were used to mix with the latex suspensions. A certain volume of a freshly prepared agarose solution was added to an equal volume of the latex suspension. A 75-μl droplet of the resulting mixture was applied on a polished GC disk or on a freshly sputtered platinum disk. After application, the electrode was put in the refrigerator overnight. The dried latex electrodes were either used as such or put in the oven at 60°C for 1 h.

**Immobilization of glucose oxidase**

All glucose oxidase solutions were prepared in phosphate-buffered saline (PBS) at pH 7.5. Enzyme adsorption was accomplished by agitating the latex-coated electrodes in a solution (3 ml) of 5 mg ml⁻¹ of glucose oxidase at a temperature of 4°C for 4 h (Gyroroty Shaker model G2, New Brunswick Scientific, USA).

Adsorption experiments were also carried out by applying a droplet (50 μl) of a 5 mg ml⁻¹ solution of glucose oxidase onto the latex membranes. Subsequently, the membrane was dried in the refrigerator. After the adsorption procedures, the enzyme membranes were either rinsed and dried or treated further with glutaraldehyde (GA).

Cross-linking of the adsorbed enzyme was effected by placing the membranes in a solution containing 2.5 vol% of GA in PBS for 10 min at room temperature. After this treatment the membranes were rinsed with PBS. When not used directly the sensor electrodes were stored in PBS solution in the refrigerator.

The enzymatic activity of the latex membranes was assayed according to the procedure described by Aubrècle-Lecat et al.¹⁴ The complete biosensor electrode was placed in an electrochemical three-electrode cell and the resulting current at a potential of 0.35V vs. Ag/AgCl was monitored.

**Amperometric biosensor activity measurements**

To perform amperometric measurements, the enzyme membrane electrode was placed as the working electrode in a three-electrode flow cell (Antec Leyden, The Netherlands). Approximately 0.15 cm² of the membrane surface was in contact with the electrolyte solution. Buffer solution was driven through the cell at a speed of 1.75 ml min⁻¹ (Watson Marlowe 101U peristaltic pump). The potential of the membrane was set at the required potential vs. the Ag/AgCl reference electrode. For the measurements the buffer solution was replaced by the glucose solutions in PBS and the current response was monitored.

Automatic assay of the biosensor response was achieved by using a solenoid valve (LFYA 1216032H; The Lee Co., CT, USA), that was controlled by a multifunction timer module (Stock No. 341-395; Mulder-Hardenberg, Haarlem, The Netherlands). This setup allowed for automatic switching between the buffer solution and a solution that contained glucose. The timer module was programmed in such a way that the flow cell measured PBS buffer solution for 180 s and then switched to glucose for 90 s. This cycle was repeated until the measurement was stopped manually.

**Results and discussion**

**Construction of the latex electrodes**

GC electrodes were used as the basic electrode material. On these electrodes a thin layer of platinum was deposited. The latex layers were prepared from 112-F and 220-nm latex particles. A droplet of a 75-μl aqueous latex suspension (0.125 wt% latex and 0.050 wt% agarose) was applied on the electrode surface and dried at 4°C. The dried membranes were either used as such or first heat treated at 60°C for 1 h. This yielded latex layers of approximately 5-μm thickness.

In Figure 1 scanning electron micrographs of the resulting latex membranes are presented. The images show a regular pattern of closed packed polystyrene spheres.

**Immobilization of glucose oxidase**

Different methods were evaluated to prepare latex membranes that showed glucose oxidase activity. In general, membranes that were not thermally treated were unsuitable to immobilize glucose oxidase. The stability of these membranes was not high enough to withstand the enzyme treatment. First, we tried to cast membranes from suspensions of the latex beads, which also contained dissolved glucose oxidase. This procedure yielded very irregular and unstable membranes that could not be used. Secondly, we treated the latex membranes with a small droplet (50 μl) of the enzyme solution. The droplet was allowed to dry in the refrigerator. An enzyme concentration of 1 mg ml⁻¹ gave a stable enzyme-latex membrane. Higher concentrations of enzyme yielded damaged membranes that no longer adhered to the electrode surface. The high amount of enzyme was found to disrupt the latex structure during the drying process. A third procedure, adsorption of glucose oxidase from solution, proved to be the best method for loading the latex. The solution was kept at 4°C and was agitated to promote penetration of the enzyme molecules into the microporous latex structure. The enzyme treatment was carried out for 4 h, after which the membranes were dried. The resulting enzyme-latex membranes remained fully intact. Extreme drying over CaCl₂ after enzyme adsorption had no significant effect on their stability.

Some of the membranes were treated with GA after enzyme adsorption. This bifunctional reagent causes cross-linking of the enzyme molecules and prevents their...
Figure 1 Scanning electron micrographs of polystyrene latex beads on a glassy carbon disk; (a) 112-nm particles; (b) 220-nm particles.

Enzyme activity

The enzyme-latex electrodes were tested for enzymatic activity following a procedure described previously.\textsuperscript{1,14-16} In this procedure the natural cosubstrate for glucose oxidase is replaced by the artificial electron acceptor benzoquinone. The latter molecule is reduced to hydroquinone, which can be detected electrochemically at a rotating disk electrode. The measured current is an indication of the enzymatic activity of the latex membrane electrode.

The various membranes that were investigated are shown in Table 1. The measured enzyme activity and the degree of immobilization are given in a qualitative way. It can be concluded that treatment of the enzyme-loaded latex with GA yields active membranes with a relatively high enzyme loading. Drying the membranes after GA treatment had no additional effect on the degree of immobilization, but the activity of the enzyme was lower.

Membranes that were not treated with GA after enzyme adsorption were active, but the enzyme molecules leaked out of the membrane during the activity assay. Untreated membranes that were rinsed directly after enzyme adsorption and then dried displayed very low activities. These activities further decreased during the assay. First drying and subsequently rinsing slightly increased the activity of the membranes, but their immobilizing ability remained poor. The fact that drying has no substantial effect on the degree of enzyme loading is in contrast with our results found for conducting polymer-modified latex membranes.\textsuperscript{1} These membranes immobilized the enzyme molecules very effectively after drying. As can be seen in Table 1, no large difference in enzyme activity or enzyme loading is observed between latex beads of 112- and 220-nm size.

Figure 2 shows the enzyme activity of a latex membrane composed of 112-nm beads that was treated with glucose oxidase according to the optimized immobilization method of adsorption to a thermally treated membrane, followed by GA cross-linking and rinsing in buffer solution. As can be seen, the current increases immediately upon introduction of the enzyme-latex membrane into the electrochemical cell (Figure 2, point 1). Withdrawal of the membrane makes the slope of the current go back to the value before introduction. This means that the enzyme molecules are properly immobilized and do not leak out of the membrane. Glucose oxidase that was not properly immobilized would have stayed in solution after withdrawal of the membrane and would have caused a higher slope of the line after point 2. Membranes that were not treated with GA actually showed this behavior. Figure 2 shows that repeated introduction and withdrawal of the membrane does not lead to a

<table>
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<td>Activity</td>
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\*See text
\*Size of the latex particles used in the membrane
change in the amount of active, immobilized enzyme present (points 1–4). Calibration of the measured enzyme activity was achieved with a known quantity (5 μg, 0.125 U) of glucose oxidase, which was introduced at point 5 in Figure 2. From a comparison of the slopes of the curves for immobilized and added enzyme, it was concluded that approximately 0.1 U of active glucose oxidase is present in the membrane of Figure 2. Similar values were found for the membranes composed of 220-nm latex beads.

Biosensor activity

The biosensor activity of the latex membrane electrodes was measured in a continuous flow system. Separate experiments were done first to test whether the bare glassy carbon and platinum electrodes showed an electrochemical response to glucose. A glucose solution of 100 mM was offered to these electrodes which were poised at a potential of 0.75 V versus Ag/AgCl. No increase in current could be detected in these cases, indicating that nonspecific electrochemical oxidation of glucose did not occur. The possibility of enzyme adsorption to the bare electrodes was also tested. Glassy carbon and platinum electrodes were treated with glucose oxidase for 24 h, dried, and rinsed in exactly the same way as the latex-modified electrodes. No biosensor activity could be measured for these electrodes. Finally, latex membranes cast on GC and on platinum were tested for glucose sensitivity, without previous enzyme pretreatment. As expected, these electrodes did not respond to glucose.

Hydrogen peroxide in aqueous solution can be oxidized if the anodic potential is sufficiently high. The potential depends on the electrode used. Oxidation of hydrogen peroxide at bare platinum or carbon can be detected at potentials ranging from 0.6 to 1.6 V versus Ag/AgCl. For measurement of the biosensor activity, potentials were chosen that are in accordance with the literature values for other biosensors based on H₂O₂ detection.

The complete latex-membrane enzyme electrode system responded to glucose as shown in Figure 3. The figure shows the current response to 5 mM glucose of a latex membrane sensor composed of 112-nm beads on a platinum electrode that was poised at a potential of 0.75 V vs. Ag/AgCl. All measurements were carried out in air-saturated PBS (10 mM, pH 7.4) solution. Latex membrane sensors based on GC also showed this electrochemical response upon the addition of glucose. However, for these sensors a stable response current could only be obtained when the electrode potential was equal to or higher than 0.75 V (vs. Ag/AgCl). Platinized GC latex-membrane electrodes were found to respond to glucose at a potential as low as 0.15 V (vs. Ag/AgCl). As a low measuring potential is a very important condition for an amperometric biosensor, we decided to test in further experiments only these platinum-covered GC electrodes.

The measuring potential of the electrode was varied from 0.15 to 0.75 V vs. Ag/AgCl and the response to various glucose concentrations (0–20 mM) was evaluated. The results for a sensor based on 220-nm latex beads are shown in Figure 4. It can be seen that the most dynamic response is obtained at a potential of 0.50 V. Increasing this potential further had no effect on the current response. Potentials lower than 0.50 V led to a decrease in sensitivity of the biosensor, especially at higher glucose concentrations (see below).

Figure 4  Plot of the steady-state current of a latex membrane measured at various potentials (vs. Ag/AgCl) as a function of the glucose concentration. Latex membrane composed of 220-nm beads deposited on a platinum-covered GC electrode, heat treated for 1 h, and incubated with a solution (3 ml) of 5 mg ml⁻¹ glucose oxidase for 4 h. Membrane treated after enzyme adsorption with 2.5% GA for 10 min. The measurements were conducted in an air-saturated PBS solution (pH 7.4). (a) 0.15 V; (b) 0.20 V; (c) 0.25 V; (d) 0.35 V; (e) 0.50 V; (f) 0.75 V.

Kinetic evaluation of the biosensor response

It can be deduced from the curves in Figure 4 that the response current of the biosensor becomes limited at 0.50 V or higher. This limitation is probably imposed by the enzyme kinetics and the internal mass transport. At potentials lower than 0.25 V, the kinetics of the electrochemical oxidation of hydrogen peroxide clearly are rate limiting. Further analysis of the data at intermediate potentials (0.25–0.35 V) revealed that under these conditions glucose oxidase immobilized on latex beads obeyed Michaelis-Menten kinetics. This is shown in Figure 5, where the data obtained at 0.35 V vs. Ag/AgCl are fitted to the Michaelis-Menten rate equation. For the first-generation biosensor presented here, a classical two-substrate enzyme kinetics should be used, which has been studied extensively both for glucose oxidase in homogeneous solution and for the enzyme immobilized in different heterogeneous systems. When the data in Figure 4 are plotted in linear form according to the Hanes-Woolf method (Figure 6), it becomes evident that at higher potentials the system suffers from internal transport of substrate as suggested above. For instance, the data at 0.35 V fit very well to a straight line (R = 0.9997), whereas the data obtained at 0.50 V show a relatively poor fit (R = 0.9962). Most likely, oxygen is the rate-limiting substrate in this case. This molecule has a high affinity for glucose oxidase, and its depletion by enzymatic consumption could readily occur in the relatively thick (5 μm) latex membrane. The apparent \( K_M \) for glucose oxidase immobilized in our latex membrane measured at 0.35 V vs. Ag/AgCl amounted to approximately 27 ± 2 mM. This value is in line with the fact that oxygen is the rate-limiting substrate. The \( K'_M \) would have been higher than the true \( K_M \) for glucose (33 mM) if glucose transport had been limiting for the reaction.

Dependence of the biosensor response on pH

The effect of the pH was investigated by measuring the current response due to 0.5 mM glucose in various buffered and air-saturated solutions. The pH values of these solutions were varied between 3 and 10. The resulting pH profile...
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Figure 7. Normalized steady-state current response of a latex membrane electrode to 0.5 mM glucose, measured at different pH values in citrate/phosphate or in borate buffer. The electrode potential was 0.35 V vs. Ag/AgCl. The latex membrane contained 220-nm beads and was deposited on platinum-coated glassy carbon. (•) pH profile of glucose oxidase in an air-saturated solution.25

Figure 8. Normalized steady-state current response of a latex membrane electrode under discontinuous use as a function of time. The latex membrane contained 220-nm beads and was deposited on platinum-coated glassy carbon. The electrode potential was 0.75 V vs. Ag/AgCl.

is presented in Figure 7. For comparison, a pH profile for the enzyme in solution is also shown in this figure.25,30 The free enzyme has an optimum activity at pH 5.5. The enzyme immobilized in our latex-membrane electrode has an optimum at pH 7.5. The anionic character of the latex beads (see Introduction) could explain this shift to higher pH.29-32 The observed optimum activity at pH 7.5 is very advantageous for clinical applications, since this pH is the physiological pH value. Figure 7 furthermore reveals that glucose oxidase immobilized on our latex membrane remains active over a broad pH range. From pH 6 to 9, more than 75% of the activity is retained. This is in contrast with the enzyme in solution, which has a quite sharp pH maximum (Figure 7). Application of the membrane sensor under different pH conditions, therefore, is quite possible.

Stability of the biosensor

The stability of the sensor was tested in PBS (pH 7.4) solutions containing glucose concentrations varying between 5 and 20 mM. The electrode potential was kept at 0.5 V vs. Ag/AgCl. More than 1,000 glucose activity assays were performed automatically over a period of approximately 3 days. Each assay took 4½ min. No decrease in activity was observed during this period. After 1,250 assays, the activity rapidly decreased, probably as a result of enzyme deactivation by hydrogen peroxide.33 This reaction product is probably retained in the microporous latex membrane for a short period of time before it is oxidized at the electrode. This will have a deleterious effect on the enzyme activity.

The stability of the latex-membrane biosensor was also tested under conditions of intermittent use. In these experiments the sensor was held stand-by in the continuous-flow system when not in use. The resulting stability curve is shown in Figure 8. Under these conditions, the response current is fairly stable for a period of 15 days. After this period, the response rapidly decreased, probably for the same reason as mentioned above.

Conclusion

The experiments described show that a stable first-generation biosensor based on glucose oxidase immobilized on latex membranes can be constructed. The sensor activity is high over a broad pH range. The optimum pH value of 7.5 is favorable for a number of possible applications. The present biosensor can compete very well with other first-generation biosensors for glucose that have been described in the literature.

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