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Glucose Biosensor Using Glucose Oxidase Immobilized in Polyaniline

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ABSTRACT

A biosensor for glucose utilizing kinetics of glucose oxidase (EC 1.1.3.4.) was developed. The enzyme was immobilized on polyaniline by covalent bonding, using glutaraldehyde as a bifunctional agent. The system showed a linear response up to 2.2 m*M* of glucose with a response time of 2.5–4.0 min. In addition, the immobilized enzyme had a higher activity between pH 6.5 and 7.5. The system retained 50% of its activity after 30 d of daily use. The optical absorption spectra of the polyaniline/glucose oxidase electrode after glucose had been added to the buffer solution showed that the absorption band around 800 nm had changed considerably when glucose was allowed to react with the electrode. This optical variation makes polyaniline a very promising polymer for use as a support in optical sensor for clinical application.

Index Entries: Glucose oxidase; polyaniline; biosensor; oxygen electrode.

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INTRODUCTION

Since the early 1960s, there has been considerable interest in developing sensors for the continuous measurement of glucose in human blood (*1*). Such developments have been partly motivated by the possibility of using such devices in conjunction with automatic feedback control for normoglycemic control, especially for patients with chronic diabetes (*2*). The methods used to assay blood and urine samples for glucose have undergone an evolution from general chemical assays for reducing sugars, through the more specific biochemical approach, to the present multifarious proposals for biosensors (*3*).

An alternative biochemical assay for glucose exploits the reaction catalyzed by the flavoprotein glucose oxidase. This method is cheaper than the others, such as HPLC or fluorometric techniques, and also allows a number of approaches to monitoring the enzyme reaction. Several spectrophotometric and fluorometric techniques are based on the reaction of the hydrogen peroxide formed by glucose oxidized with a suitable chromogen (*4*). The glucose concentration can also be determined by immobilized glucose oxidase on an electrode by measuring the oxygen consumption, hydrogen peroxide formation, or acid production. Other possibilities are the use of an electron acceptor, such as ferrocene (*5*) or benzoquinone (*6*). In this case, glucose is oxidized while the mediator is reduced. Glucose is determined by measuring the current produced by the reoxidation of the reduced mediator at an electrode.

The high specificity of glucose oxidase makes this enzyme one of the most popular enzymes in the field of enzymatic technology. The enzyme has a very low operational stability. Kleppe has demonstrated (*7*) that hydrogen peroxide irreversibly inactivates the reduced form of glucose oxidase. In order to overcome this problem, several methods have been developed to reduce the peroxide concentration (*8*), such as coimmobilization of catalase. Autoinactivation depends on the number of catalytic cycles carried out by the enzyme when oxygen is used as electron acceptor. Fortunately, oxygen can be substituted with other electron acceptors. Bourdillon et al. (*8*) has demonstrated that electrochemical regeneration is a particularly convenient method of introducing chemical energy in the enzymatic reactor without byproduct formation.

The purpose of this article is to describe a biosensor for glucose using glucose oxidase covalently immobilized on a conductive polymer surface. This method permits large immobilization areas to be used. In addition, the redox properties of polymer can work as an electron acceptor for the enzyme reaction. Polyaniline has been chosen because it is a cheap and a air-stable electroconducting and electrochromic polymer that exhibits isolating properties when doped with aqueous protonic acid, or when treated with an oxidizing or reducing agent. Careful control of these properties allows the conductivity changes over 11 orders of magnitude

(*12*). The polymer chain consists mainly of an equal number of amine and imine repeat units where irreversibly chemical reaction with glutaraldehyde at the amine group can take place (*13*). The wide range of associated properties, such as easy protonation reversibility, excellent redox recyclibility, conductivity, and electrochemical and optical properties, coupled with good stability make polyaniline potentially attractive for use as a support for biosensor development.

EXPERIMENTAL SECTION

Reagents

Aniline obtained from Merck was purified by distilling several times until a colorless liquid was obtained. Glucose, glutaraldehyde, and glucose oxidase (GOD; β-D-Glucose: oxygen 1-oxireductase; E.C. 1.1.3.4) from *Aspergillus niger*-type VII-S (9700 U/g) and bovine serum albumin were purchased from Sigma, St. Louis, MO. Solutions were prepared with deionized water and were not deoxygenated prior to use. All the other reagents were analytical grade.

WORKING ELECTRODE

The working electrode was formed by an oxygen electrode used as a transducer (measuring the level of oxygen) and a modified reaction chamber as described by Marques et al. (*9*). The chamber was composed of a methacrylate cuvet where the enzyme was immobilized on the polyaniline film (Fig. 1).

Polyaniline was electrochemically grown from an aqueous solution of nitric acid (1*M*) and aniline (0.5*M*), on a methacrylate cuvet using a steeless plate (attached very closely to the internal cuvet face) as a working electrode and a gold wire as an counterelectrode. The polymerization was carried out in galvanostatic mode with maximum density current of 0.1 mA/cm^2 over a 24-h period. The polymer growing mechanism at nonconductive support will be presented in a forthcoming paper.

The film produced, at nonconductive support, was rinsed with a copious amount of deionized water in order to remove low-molecular-weight species and excess monomer from the polymer surface. The film was treated with 0.1*M* of ammonium hydroxide to convert the polyaniline salt to base form. After exhaustive washing with deionized water, the polymer was allowed to remain in a phosphate buffer, pH 5.8, in order to stabilize at this pH level. Glutaraldehyde (2.5%) was prepared by dilution of the stock solution with phosphate buffer, pH 5.8, and allowed to interact with the polymer for 1 h. Afterward, the modified polymer was washed

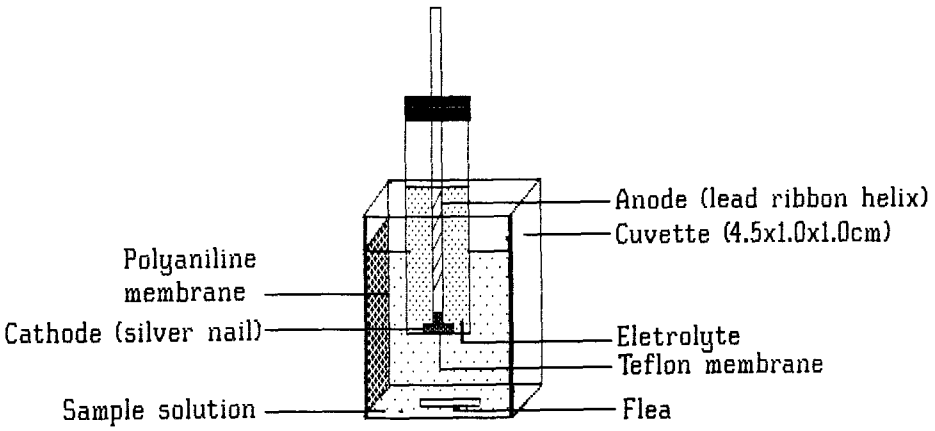

Fig. 1. The glucose biosensor design.

with fresh phosphate buffer, and enzyme immobilization was performed by adding 1 m*M*/mL of enzyme solution (prepared at same buffer) and allowed to react for 1 h.

The final product was rinsed several times with NaCl (1*M*) and phosphate buffer following 2 m*M* glycine. Finally, the polyaniline GOD electrode was stored in potassium phosphate buffer, pH 7, at 4°C when not in use.

RESULTS AND DISCUSSION

Figure 1 shows the modified biosensor system. The transducer is based on a low-cost and easily constructed potentiometric "homemade" oxygen electrode developed by Marques et al. (*9*). It is based on the oxygen consumption by glucose oxidase immobilized in polyaniline attached to the internal cuvet wall in the presence of glucose and oxygen. A second approach is based on the absorbance change of polyaniline at 800 nm caused by polymer reduction following the oxidation of glucose in the presence of oxygen by glucose oxidase. Tuner and Pickup (*3*) have described a similar biosensors system that uses glucose oxidase immobilized on a water-insoluble support directly attached to the electrode. This immobilized enzyme membrane has the disadvantage of decreasing oxygen diffusion to the electrode blocking the oxygen to reach the electrode. To overcome this problem, the immobilization was carried out on the polyaniline film attached directly to the internal wall of a 3-mL methacrylate cuvet. This procedure avoids the low enzyme loading owing to the small surface of the electrode, and it has only one selective membrane. This characteristic allows light to pass through the polyaniline polymer. The absorbance difference resulting from the oxireduction of the film can be used as the transducer. The polyaniline absorbance properties change when it is reduced (becomes yellow) or oxidizes (becomes violet). In this case, it be-

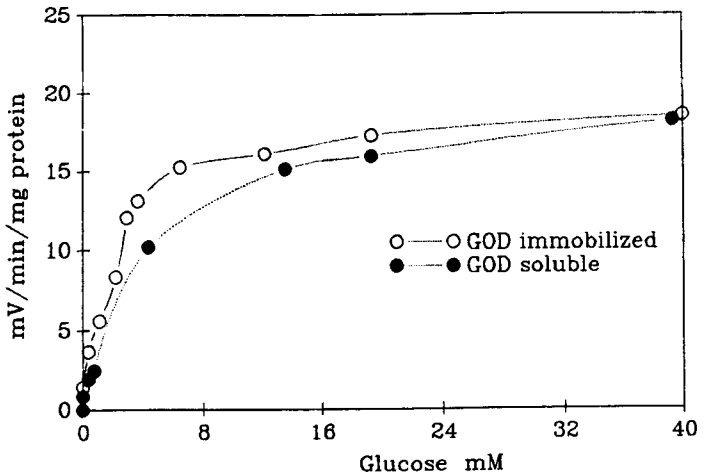

Fig. 2. Response of the oxygen transducer system using soluble and immobilized glucose oxidase on polyaniline, carried out at 28°C and pH 5.8.

came yellow because of glucose oxidase activity in the presence of glucose and oxygen. This characteristic can be related to glucose concentration.

Figure 2 shows the response of the oxygen transducer system, which has a linear range up to 2.2 m*M* of glucose with a response time of 2.5–4 min, which is faster than the system described by Harrison et al. (*10*). They developed a miniature glucose electrode with a glucose oxidase membrane covered by a semipermeable membrane. The system showed a response time between 10 to 20 min using blood samples (*10*). This delay could be the result of the presence of a second membrane on the transducer. Using a system that avoids a second barrier to the oxygen allows a faster response time. The lower linear response of the system as compared to other biosensors (*10*) does not permit detection of glucose over a concentration of 4 m*M* or below 1 m*M* with good precision. On the other hand, it is able to determine concentrations below 0.05 m*M* with reasonable precision.

Figure 4 presents the pH curve of immobilized GOD, showing higher activity between 6.5 and 7.5, but with reasonable activity between 4.5 and 8.5. Similar results were found by Trettnak and Wolfbeis (*11*) using glucose oxidase entrapped at a fiber tip within a semipermeable membrane. This range between pH 4.0 to 8.5 does not significantly influence the sensor response, which is independent of the enzyme.

The half-life of the biosensor is approx 30 d with daily use and with more than 160 readings. The optical absorption spectra of the polyaniline/GOD electrode before and after glucose addition to the buffer solution is shown in Fig. 3. From these results, we can see that the absorption band around 800 nm changed considerably when glucose was allowed to react with the composed electrode.

From the spectroelectrochemical results of Genies and Lapkowski (*14*) on polyaniline as a function of the redox potential, we observe that when

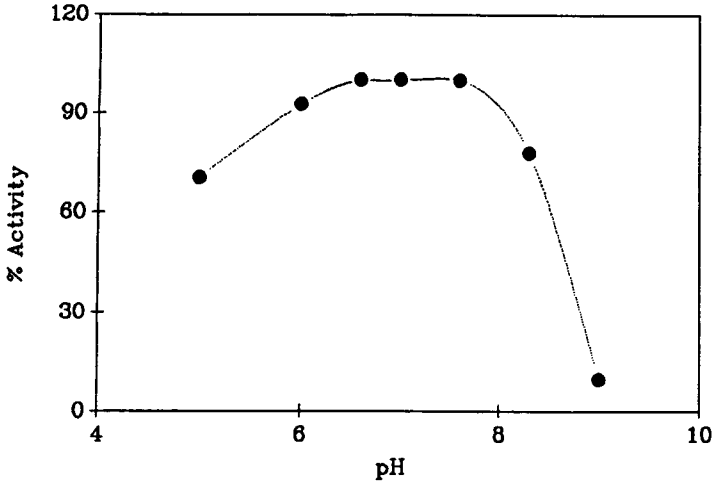

Fig. 3. pH profile of glucose oxidase immobilized on polyaniline.

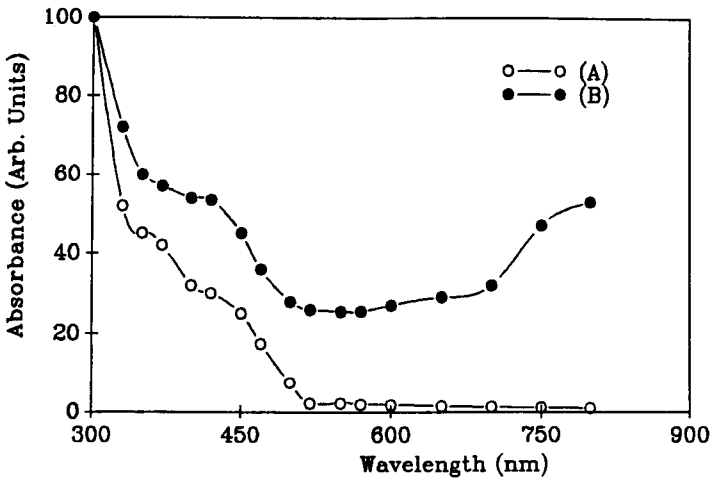

Fig. 4. Absorption spectra of a polyaniline biosensor before (A) and after glucose addition (B).

the potential changes from 0.8 to −0.15 V, the spectral intensity of the 800-nm absorption band decreases. This means that the spectral intensity is directly related to the oxidation state of the polymer film. We can assume that after reaction takes place at the enzyme active center, the product of that interaction is transferred to the polymer surface. The polyaniline changes it oxidation state, passing to the reduced form. This variation is so clear that we are able to recognize it by the naked eye. The transparent polymer color changes from blue-green to yellow. This perceptive variation makes polyaniline a very promising polymer to be used as a support in "clinical" optical sensor devices.

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