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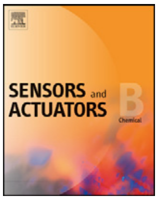

Silica-dispersed glucose oxidase for glucose sensing: In vitro testing in serum and blood and the effect of condensation pH

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ABSTRACT

The objectives of this study were to examine the feasibility of using glucose oxidase (GOx) dispersed in a silica matrix for glucose monitoring in whole blood, and then to assess whether the flexibility of silica sol–gel chemistry could be exploited to enhance glucose sensor performance and stability. Silica-dispersed GOx was deployed on platinized platinum (Pt) wire to form a Clark-type amperometric glucose sensor. Sensors were calibrated using buffered glucose standard solutions, and then tested against glucose spiked human serum and whole blood. All serum and whole blood measurements met the minimum FDA requirement of falling within the “A+B region” of a Clark Error Grid. To our knowledge this is the first report of using silica-dispersed GOx to measure glucose in whole blood. The effect of condensation pH on sensor performance was assessed by dispersing GOx in silica condensed at pH 3, 7, and 12, and then testing the sensor response against glucose calibration standards. The pH 12 silica sensors had statistically faster response time, and higher sensor sensitivity compared to pH 7, pH 3 silica and glutaraldehyde crosslinked sensors. Membranes of the pH 12 silica had statistically higher glucose diffusion coefficient than did the pH 7 and 3 sensors. GOx dispersed in pH 12 silica also had the longest half life. We hypothesize that the gel-like pH 12 silica gels provided reduced barriers to glucose diffusion, and the more aqueous microenvironment provided greater stability for the enzyme.

1. Introduction

Glucose oxidase (GOx) mediated amperometric sensing has been the gold standard for monitoring blood glucose since Leland Clark first designed a H_2O_2 based enzyme electrode to measure the conversion of glucose to gluconic acid. A number of FDA approved glucose monitors continue to utilize modifications of the Clark electrode design, including the Abbott Freestyle Navigator®, DexCom STS®, Medtronic iPro2 CGMS®, and Medtronic Guardian RT®. All of these devices employ GOx immobilized at the surface of the working electrode and covered by a glucose restriction membrane, typically polyurethane.

The most common mode of GOx immobilization is curing with homo-bifunctional glutaraldehyde that covalently crosslinks the adsorbed [1] or chemically tethered enzyme [2–4]. However, glutaraldehyde can also affect catalytic or structural amino acids populating the active site of the enzyme that can compromise enzyme catalytic power and lifetime [5]. Finding an alternative to glutaraldehyde crosslinking of GOx that does not chemically react with the enzyme and that provides greater steric support is attractive. For acute applications such as glucose monitoring of blood droplets, the negative effects of glutaraldehyde crosslinking on enzyme function is minimal; however for longer-term applications these effects could become significant.

Silica serves as a well-established dispersing material for proteins because it is non-toxic, chemically and biologically inert, subject to negligible hydration swelling, hydrophilic, and inexpensive to synthesize [6,7]. Researchers have established silica sol gel encapsulation as an effective means of stabilizing enzymes [8–12]. The improved enzyme stability in silica arises from matrix–protein interactions that constrain the protein conformational state, such as surface hydration [13], hydrophobic interactions [14], electrostatic forces [15,16], surface curvature [17,18], and macromolecular crowding [14]. Constraining the enzyme’s conformational changes also reduces enzyme susceptibility to proteolytic degradation [19,20], an important factor in vivo. A number of researchers have reported the use of silica-dispersed GOx for glucose sensing [8,21–26], only three of which tested their sensor in human serum, and none of which have tested their sensor in either whole blood or intact tissue.

Sol gel synthesis of silica is a two-step chemistry: hydrolysis of the silicon alkoxide precursor and condensation of the silanol intermediates. Control of the buffer pH during the condensation reaction dramatically changes the properties in silica [27]: acidic conditions

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promote cluster growth resulting in branched surface fractals and a dense and brittle solid; basic conditions promote non-fractal particulate sol formation and a less dense and soft gel; neutral pH values produce silica of intermediate density and hardness.

In the current study, a simple three wire Clark-type glucose sensor was produced by deploying silica-dispersed GOx on a platinized Pt working electrode. The sensors were calibrated using standard solutions of buffered glucose, and tested against glucose spiked human serum and whole blood. All serum and whole blood measurements met the minimum FDA requirement of falling within the “A+B region” of a Clark Error Grid [28]. The effect of condensation pH on sensor performance was assessed by encapsulating GOx in silica at pH 3, 7, and 12, and then testing the sensor response against glucose calibration standards. These results were correlated with measurements of glucose diffusion and GOx half life in silica produced at the different condensation pH values.

To our knowledge, this is the first report of using silica-dispersed GOx based sensors to measure glucose in whole blood. Our research utilized a simple Clark-type electrode to allow for proper parity to be drawn against other works but the novelty of our work also hinges on the optimization of the silica gel using varying condensation pHs. The literature lacks studies on this subject and our report would be the first to claim that a higher pH produces a favorable microenvironment for the GOx and favorable mass transport conditions that benefit the sensor performance. It is also the first report that examines whether varying the silica sol–gel chemistry could be exploited to improve glucose sensor performance and stability.

2. Materials and methods

2.1. Reagents

GOx was purchased from Sigma Aldrich (Lot: 040M1349) and EMD Bioscience/Calibiochem (Lot: D00099683). Lead (II) acetate trihydrate (Lot: 03697PJV), chloroplatinic acid. Sulfuric acid was purchased from JT Baker. D-Glucose was purchased from Sigma Aldrich (Lot: 040M6185). Phosphate buffered saline was purchased from EMD Bioscience (Lot: 22706212). Milli-Q water was obtained from university facilities. Tetramethyl orthosilicate was purchased from Alfa Aesar (Lot: USLF001196). The pH meter calibration standards were purchased from VWR International (pH 4 Lot: 2011506, pH 7 Lot: 1008163, pH 10 Lot: 2011584). The storage solution for the Ag/AgCl was purchased from VWR (Lot: OP1). THF was purchased from Sigma Aldrich (Lot: 16996TMV).

2.2. Electrodes

Platinum wire, silver wire, and the potentiostat were purchased from Pine Research Instrumentation (Wave-Now). The 0.5 mm diameter Pt wire for the working electrodes was purchased from Sigma Aldrich. The Ag/AgCl reference electrode and Pt counter electrodes were purchased from CH Instruments. The working electrode was “platinized” by exposing segments of 0.5 mm ID Pt wire to 0.072 M (3.5%) chloroplatinic acid and 1.3×10^{-4} M (0.005%) lead acetate at a current density of 30 mA/cm^2 for 10 min in a potentiostat [29].

2.3. Enzyme–silica matrix

GOx was applied to the working electrode either by encapsulation in a silica matrix or through glutaraldehyde crosslinking as described below. A 2 wt% solution of polyurethane (equal parts Tecoflex and Hydrothane in 50:50 THF and ethanol) was applied to the working electrode and allowed to dry at room temperature. Prepared working electrodes were used for 24 h following fabrication.

Using the technique of Gupta et al. [30], a silica sol gel was prepared by chemical vapor deposition of TMOS into buffered saline (either pH 12, 7, or 3). 4 mg of the resulting silica gel was mixed with 100 μL of GOx creating a paste with 40 mg/mL GOx. 10 μL of the GOx paste was applied to 1–2 cm length of the working electrode creating a GOx coating with 400 μg load of enzyme. Following the protocol of Zoldák [31] or glutaraldehyde crosslinking of GOx, 15 μL of 27 mg/mL GOx in PBS buffer was mixed with 1.7 μL of 8% glutaraldehyde (v/v) in a micro centrifuge tube and allowed to cure for 1 h. 10 μL of the cross-linked GOx was added to a 1–2 cm length of the working electrode creating a GOx coating with 400 μg load of enzyme. In both cases, the applied layer of GOx was allowed to cure for 1 h before the final layer of polyurethane was added.

2.4. Flow cell configuration

Fig. 1A shows the overall sensing configuration. The three wire electrodes were placed in a 1.5 cm × 5.5 cm × 1.2 cm laminar flow cell perpendicular to the direction of fluid flow and held in place by a 1.5 cm thick silicon rubber gasket sandwiched between two polycarbonate plates. The gasket defined a flow cell volume of 10 mL. Approximately 60 cm of a 0.32 cm ID silicone rubber tubing was connected to the flow cell inlet and outlet and to a Master flex® pump. The total volume of the flow loop and flow cell was 15 mL. Test solutions were introduced into the flow loop using a 3-way stopcock and circulated thorough the flow cell at 1.8 mL/min. As per Abdel-Humid et al. [32], the electrodes were connected to the potentiostat at a voltage difference of 0.536 V between the working and counter electrodes was established. This voltage difference was used because of the noted benefits of operating at lower voltages in minimizing the effect of electro-active interfering species. The currents between the working and counter electrode detected by the potentiostat during glucose measurements were in the order of 0–200 μA.

2.5. Sensor calibration and response time

The sensor current was calibrated using a series of glucose in PBS standard solutions (0 mM, 2.3 mM, 6.6 mM, 15 mM, and 25 mM) before each measurement series. Upon the introduction of each glucose standard the sensor current increased rapidly and then leveled off at a new baseline (Fig. 2). The sensor sensitivity was the slope of the baseline sensor current versus glucose concentration plot. The sensor response time was the duration between the introduction of the glucose standard and the establishment of 99% of the next baseline current. The limit of detection (LOD) was estimated using the method of Cunningham [33]

$$\mathrm{LOD} = (k\varepsilon_r) \times [\text{Sensitivity of sensor}]^{-1} \tag{1}$$

where k is the number of standard deviations of separation that constitutes ‘different’ from noise, ε_r is the standard deviation from the amperometric signals ($n = 5$) for 2.3 mM glucose samples.

2.6. Serum and whole blood measurements

Human whole blood was acquired according to Duke University IRB protocol 2257-08-1R17ER. 50 mL of blood was collected in sealed tubes from healthy volunteers and refrigerated, and then used within 24 h. Heparin was utilized as an anticoagulant for the whole blood. Human serum was obtained by spinning 50 mL of whole blood at 1500 × g for 10 min in a 50 mL conical tube. Serum or whole blood was introduced into the flow system by an injection loop (12.7 cm length, 3.175 cm ID silicone tube) and the samples were spiked by injections of glucose solutions into the flow loop. The glucose concentration of each sample (whole blood and serum) was determined using the pH 7 silica glucose sensor in a simple