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Glucose sensing employing fluorescent pH indicator: 4-[(*p*-*N*,*N*-dimethylamino)benzylidene]-2-phenyloxazole-5-one

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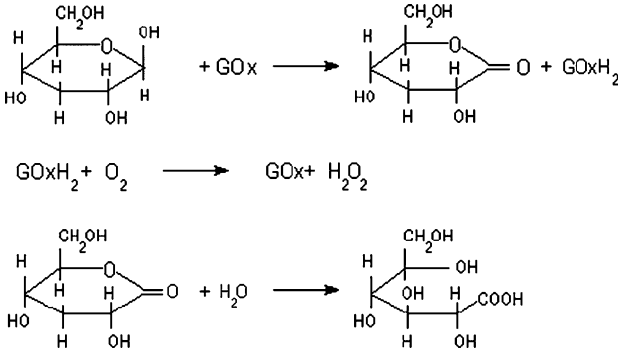
Abstract

The proton sensitive azlactone derivative; 4-[(*p*-*N*,*N*-dimethylamino)benzylidene]-2-phenyloxazole-5-one (DPO) exhibits high quantum yield, excellent photostability, and high molar absorptivity in a sol–gel matrix. In this work, two different DPO containing constructions were tested and evaluated as glucose sensors. In the first, both glucose oxidase (GOx) and azlactone derivative were incorporated into a single tetraethylorthosilicate (TEOS) based sol–gel phase. In the second, TEOS and 3-aminopropyltriethoxysilane (APTEOS) containing dye-doped sol–gel layer was covered with GOx entrapped sol–gel (the two-layer configuration). The monolayer configuration has the fastest response time ($\tau_{90} = 20$ s) but suffers from leaching upon prolonged use. Response time and dynamic working range of the cross-linking agent containing two-layer configuration are 40 s and 0.1–15 mM glucose, respectively. The resulting glucose sensor was characterized by high sensitivity, low interference from acidogenic species, and a working lifetime of at least 90 days.

Keywords: Glucose sensing; Sol–gel; Fluorescence spectroscopy

1. Introduction

Glucose sensing is of major importance in biotechnology as well as in clinical applications. Glucose oxidase (GOx) is by far one of the most intensively studied enzyme and, capable of catalysing the oxidation of D-glucose by molecular oxygen into D-gluconolactone and D-gluconic acid as follows.



The enzymatic reaction can be divided into two steps. In the reductive half reaction, two protons and electrons are transferred from β-D-glucose to the enzyme yielding

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D-gluconolactone. In the oxidative half reaction, the enzyme is oxidized by molecular oxygen yielding hydrogen peroxide. Finally D-gluconolactone can be hydrolysed non-enzymatically to gluconic acid [1].

In order to optically monitor the reaction, the formation of hydrogen peroxide can be followed by optical measurements using a second enzymatic reaction, in which the oxidation of an organic dye is catalysed by a peroxidase such as horseradish peroxidase (HRP), for glucose sensing purposes [2]. Monitoring of oxygen consumption [3–5] or proton formation by spectroscopic techniques [6] are the other alternative methods. Recently, Wolfbeis and co-workers presented a sol–gel based glucose biosensor employing a ruthenium dye together with an enzyme glucose oxidase, whose emission is quenched by oxygen [7]. De Marcos et al. covalently labelled enzyme GOx with a fluorescein derivative and followed the increase in the fluorescence signal as the analytical information [8].

The construction of enzyme sensors requires direct or indirect immobilization of enzymes to proper matrices. First sol–gel encapsulation of biomolecules (enzymes, antibodies and even whole cells) within silica matrices was reported by Braun et al. in 1990 and later by Livage in 1997 [9,10]. They showed that enzymes trapped in a porous matrix retain their biological activities.

Here we present a new planar sol–gel based thin-film glucose biosensor. The sensor is based on the fluorescent pH sensitive azlactone dye entrapped in a TEOS based sol–gel together with enzyme GOx. There is no literature report pointing out the usage of DPO dye in glucose sensing. Additionally, the applied sol–gel preparation protocol overcomes one of the difficulties of sol–gel process providing excellent crack-free monoliths. The protective sol–gel cage contributes to the stabilization of the sensor slides inhibiting bending of the enzyme and providing a rigid microenvironment for the fluorescent dye molecule. The sensor slides were tested and evaluated in terms of response time, dynamic working range, reversibility, cross sensitivity to pH and short term stability.

2. Experimental

2.1. Reagents

The DPO dye was synthesized and purified as described previously [11]. The sol–gel components; tetraethylorthosilicate (tetraethoxysilane, TEOS), and 3-aminopropyltriethoxysilane were supplied from Merck and Fluka, respectively. Glucose oxidase (E.C. 1.1.3.4, IU/mg, from *Aspergillus niger*, 200 U/mg) was from Fluka. Glucose working solutions were prepared by dilution of a 0.1 M glucose stock with BES buffer (pH = 7.0) at 24 °C. Analytical grade hydrochloric acid was from Merck. Acid solutions and buffers used for investigating the pH effects were prepared with high quality pure water obtained from Elga. The surfactant additive sorbitol was obtained from Merck. Regeneration of the sensor was accomplished with titrisol buffer from Merck. Absolute ethanol was used without any treatment.

2.2. Spectroscopic measurements

The absorption spectra of sol–gel glass slides were measured using a Jasco V-530 UV–VIS spectrophotometer. The fluorescence emission spectra were recorded using a Schimadzu (RF-5301) fluorescence spectrophotometer. pH measurements were performed with a pH-meter Jenway 3040 Ion-Analyser calibrated with Merck pH standards of pH = 7.00 (titrisol buffer) and pH = 4.00 at 20 °C. The film thickness of dry sensor slides was measured with Tencor Alpha Step 500 Prophylometer and found as 2.089 μm for monolayer configuration. This result is an average of eight measurements and exhibits a standard deviation of ± 0.0074. In two-layer containing sensor slides, film thickness was approximately 6.230 ± 0.0097 μm.

2.3. Sensor layer preparation protocols

Two different thin film configurations are as follows: In the first composition, both GOx and pH sensitive fluorescent indicator dye were incorporated into a single TEOS based sol–gel phase. This composition was obtained by mixing 1 mL of TEOS, and 1 mL of absolute ethanol. Subsequently 0.5 mL of 0.1 M HCl was added drop by drop and stirred for 24 h in a glass vial. The stopper of the glass vial alternately opened and closed during the stirring. In order to improve the homogeneity of the silica sol–gel and to obtain a crack-free monolith 12.5 mg of sorbitol was added into the composition. Before the addition of the acid sensitive dye (5 mg in 300 μL EtOH), a concentrated phosphate buffer (which has enough buffer capacity, 250 μL, 0.14 M, pH = 6) was added, and the pH of the sol was increased. Ten microlitres of GOx solution (15 mg of GOx in 250 μL of water) was added, stirred properly and used for coating. Sensor layers were stored for 12 h in the refrigerator. In the second composition; 1 mL of TEOS, 125 μL of APTEOS and 1.2 mL of absolute ethanol were mixed properly. Subsequently 0.5 mL of 0.1 M HCl was added and stirred for 24 h in a glass vial. After that, 200 μL of phosphate buffer and dye (3 mg in 300 μL EtOH) were added, mixed for an additional 1 h, and, stored in the refrigerator.

Cross sections of the sensor membranes M-1 and M-2 are shown in Fig. 1. One layer configuration, M-1 was prepared by casting the first composition onto the pre-treated glass supports, which contains GOx and

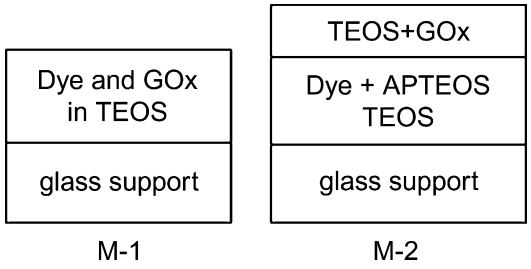

Fig. 1. Schematic cross-sections of the sensor membrane M-1 and M-2.

indicator dye in TEOS based sol–gel phase. The first layer of the two-layer configuration contains TEOS and APTEOS together with indicator dye. This surface was covered with another sol–gel entrapped GOx layer.

Glass slides ($11 \times 40 \times 1$ mm) were used as solid support onto which the sol–gel was cast by manual pipetting technique. Prior to casting, the glass surface was activated by treatment with concentrated HNO_3 for 24 h, washed with distilled water and then with ethanol. After evaporation of the solvents in a desiccator, the glass slides were fixed diagonally in a quartz sample cuvette. The advantage of this kind of a placement was to improve the reproducibility of the measurement.

3. Results and discussion

3.1. Choice of matrix

The sol–gel encapsulation of enzymes, metalloproteins (cytochrome-*c*, myoglobin, hemoglobin) and proteins have no effect on their chemical and biochemical reactivity [9,12,13]. Pandey et al. used glucose oxidase in different composite sol–gel glasses and determined the optimum working conditions [14]. Wolfbeis et al. calculated and interpreted the short- and long-term apparent activities of GOx after entrapment in sol–gel by a photometric assay. They reported satisfactory apparent activities (more than 4 months) if stored at 4 °C [7].

In this work, applied sol–gel preparation protocol contains high levels of ethanol and is unusual. Dilution of TEOS with equal volume of ethanol increases the quality of the matrix providing a more homogeneous acidic-catalysed reaction mechanism; preventing local hydrolysis reactions around the drop, and, a longer and more stable sol phase after addition of the buffer.

However, addition of the enzyme into the alcohol containing sol–gel composition may result in a less sensitive sensor glass, because of the denaturation of glucose oxidase that is avoided by applying long mixing periods (24 h) and allowing evaporation of the excess alcohol prior to the addition of the enzyme. The bio-computable and chemically inert sol–gel matrix also allows the investigation of pH dependent changes in the absorption and emission spectra of the chosen dye by optical detection. Due to the given reasons, in this work, TEOS and the cross-linking agent, 3-aminopropyltriethoxysilane containing TEOS were chosen as the matrix materials.

3.2. Choice of indicator dye

This work is the first application of the fluorescent pH sensitive azlactone derivative: 4-[(*p*-*N*,*N*-dimethylamino)benzylidene]-2-phenyloxazole-5-one (DPO) in glucose sensing.

The absorption and emission maximum values of the dye in the sol–gel matrix are 480 nm and 537 nm, respectively. The DPO exhibits high molar extinction coefficient, ($\varepsilon_{max} = 208{,}000$), quantum yield ($Q_f = 0.296$), and excellent photostability in sol–gel matrix [15]. In order to achieve the highest precision, pK_a value of the indicator should be close to the pH value expected in the transducing system when the sensor is in contact with the sample [16]. In our case, the initial pH is near neutral and the production of gluconic acid was expected to give a pH of 6.5 in the sensing layer which is in accordance with the pK_a of DPO ($pK_a = 5.2$), and, covers the dynamic working range (pH = 3.0–7.0) of the azlactone dye.

The photostability test of DPO dye in sol–gel matrix was performed with a steady state spectrofluorimeter in mode of time-based measurements. The data in Fig. 2 were acquired at 537 nm in sol–gel matrix after 1.5 h of monitoring. The absorption/emission spectra and photostability test results were shown in Fig. 2. The long wavelength absorption and emission characteristics make the pH sensitive dye also Light Emitting Diode (LED) and solid-state optics computable.

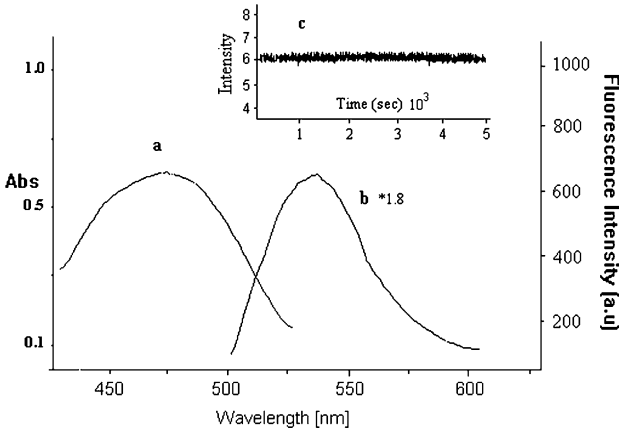

Fig. 2. (a) Absorption spectrum ($\lambda_{max} = 480$ nm), (b) emission spectrum ($\lambda_{max}^{ex} = 460$ nm, $\lambda_{max}^{em} = 537$ nm), (c) photostability test line of azlactone dye in sol–gel matrix after 1.5 h of monitoring.

3.3. Sensor response, reversibility, reproducibility

Sensor slides respond to proton resulting from enzymatic activity by a decrease in absorbance and emission intensity that is used as the analytical signal. Absorption and emission spectra of the structures are expected to be reversibly pH dependent in solid matrices and our further experimental results proved this idea.

Upon exposure to the glucose solutions between concentration ranges of 1.0–15.0 mM, the monolayer composition, M-1 exhibited approximately 40 and 60% relative signal change in direction of decrease in absorbance and emission intensities, respectively. Absorption and emission characteristics of the two-layer composition M-2 were very similar to the M-1 in terms of absorption/emission maximum and signal intensity (see Fig. 3). From the results of the emission based measurements, the dynamic working range of the sensor slides were found between 1.0–15.0 mM and 1.0–12.0 mM glucose in 10^{-3} M BES buffered solutions for M-1 and M-2, respectively. The drop in the dynamic working range of M-2 can be attributed to the accessibility difficulties of two-layer configuration. The glucose induced emission spectra of M-1 and M-2 are given in Fig. 3. Fig. 4 shows the plot of normalized emission intensity (I_0/I) vs. glucose concentration for monolayer configuration within the dynamic working range. Data presented are mean of four sets of measurements all with different fresh sensor slides. The linearized calibration plot of sensor can be described by $y = 0.0754x + 1.0564$ and the correlation coefficient $R^2 = 0.9759$.

As expected, the monolayer configuration has the fastest response time ($\tau_{90} = 20$ s) but suffers from leaching upon prolonged use. Response time of the cross-linking agent containing two-layer configuration was approximately twofold of the M-1, 47 s.

This increase in response time of M-2 can be attributed to the long equilibrium time due to the accessibility difficulties between two layers and increased film thickness (from 2.1 to 6.2 μm).

The reproducibility and reversibility behaviour of the sensor compositions were tested by exposing the glass slides to glucose concentrations of 3.0–10.0 mM. After

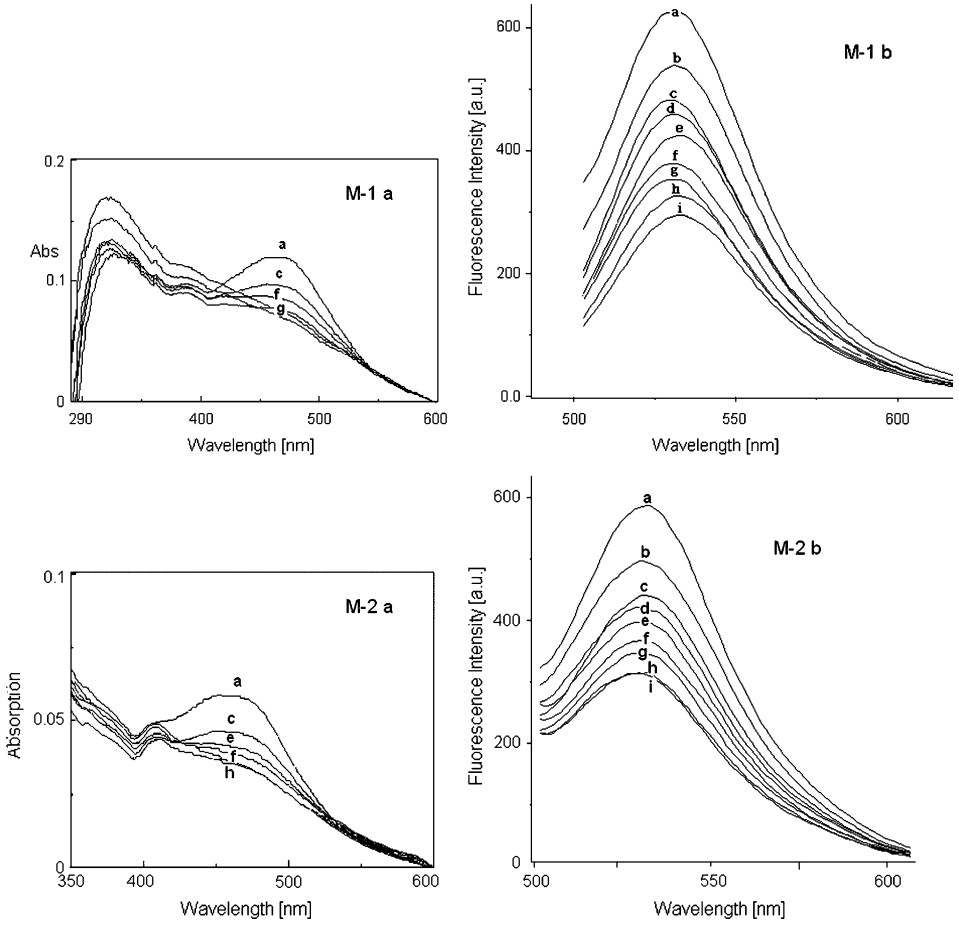

Fig. 3. Absorption (a) and emission (b) spectra of M-1 and M-2 after exposure to (a) 0.00, (b) 1.00, (c) 2.00, (d) 4.00, (e) 6.00, (f) 8.00, (g) 10.0, (h) 12.5, (i) 15 mM glucose concentrations in BES buffer.

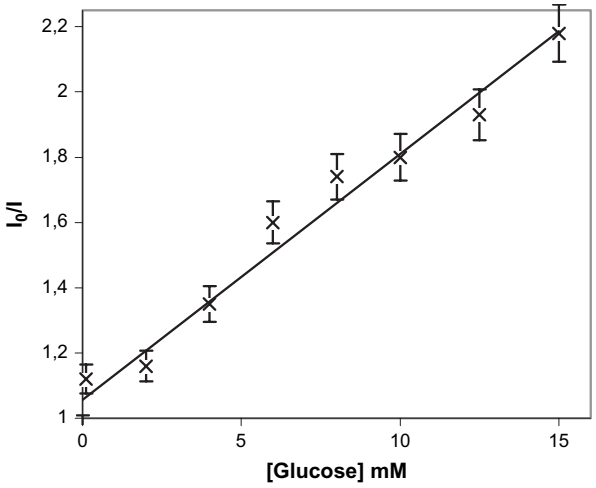

Fig. 4. Plot of normalized emission intensity (I_0/I) vs. glucose concentration for monolayer configuration, M-1.

the first cycle, there was a significant drift (about 10%) in sensor response of M-1, which can be concluded as the conditioning step. Once encapsulated and conditioned, leaching of the DPO out of the sol–gel matrix is very low, which is an advantage of the matrix. The following second and third cycles did not result in any further large drifts (Fig. 5). The reproducibility of the optical responses was assessed by repeatedly introducing a sample of [glucose] = 3.0 and 10.0 mM under batch conditions. At each time the reagent phase was regenerated by a $H_2PO_4^-/HPO_4^{2-}$ buffer system (titrisol) with a pH of 7 ± 0.02. Between 2nd and 5th cycles, the level of reproducibility achieved corresponded to an RSD of 1.8%. The regeneration time under batch conditions was about 14 and 35 min for M-1 and M-2, respectively.

Dynamic working range of the sensor covers the glucose levels of normal blood which is about 5 mM, but in pathological cases it can increase to 45–50 mM

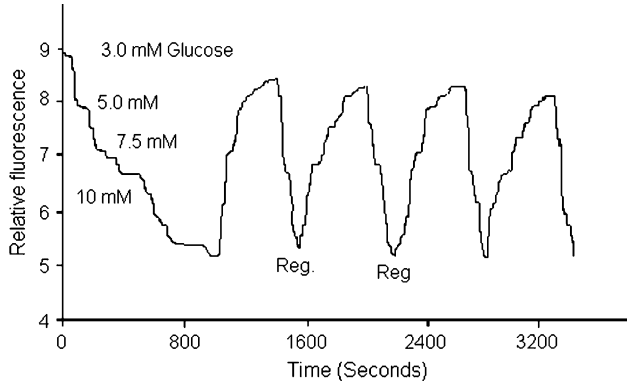

Fig. 5. Response curve of M-1 after exposure to certain glucose concentrations and by repeatedly introducing samples of [glucose] = 3.0 and 10.0 mM.

[17,18]. In the presence of 135 mM NaCl in glucose working solutions and real diluted serum samples, relative signal change loses extend to 17–20% for both the compositions.

3.4. pH effects on signal intensity

In order to avoid the other interfering pH dependent signal fluctuations, we recorded all of the spectra in BES buffered solutions. An important consideration in the preparation of enzyme containing sol–gel compositions is the pH of the reaction media. The initial hydrolysis of the sol–gel process requires catalysis at either acidic or basic pH, and, direct addition of the dye and enzyme into the acid catalysed sol–gel composition may result in a less sensitive sensor glass, because the pH sensitivity of glucose oxidase, and, the emission signal of the dye decreases in acidic solutions. Increasing the pH of the sol–gel composition just prior to the addition of the enzyme and dye by using a sufficient quantity of concentrated phosphate buffer (250 μL, 0.14 M, pH = 6) enhances the signal intensity.

The harmful effects of the acidogenic species such as gaseous CO_2, SO_2 and NO_xs in laboratory atmosphere or environment must also be considered. To avoid such effects we kept the sensor slides in a desiccator and in a refrigerator.

In order to provide crack-free monoliths and good homogenity and prevent the densification of the pore structure during aging sorbitol was added to the sol–gel compositions. Before measurements sensor slides were conditioned in BES buffer and sorbitol washed out of the matrix as mentioned in literature [7].

3.5. Sensor stability

Sensor stability was tested on 3 consecutive months at different glucose concentrations in BES buffer and was found to be satisfactory. Fig. 6 shows the fluorescence intensity ratio vs. glucose concentration for the same sensor for 3 consecutive months of

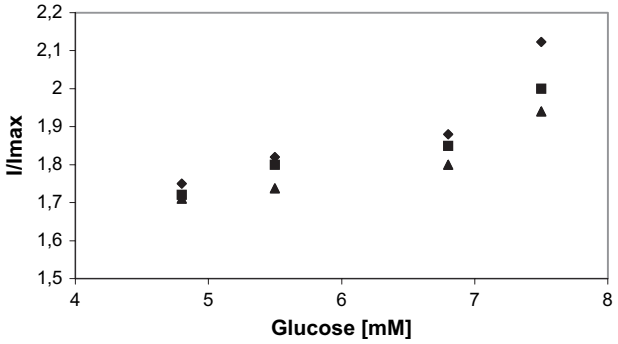

Fig. 6. Relative fluorescence intensity (I/I_{max}) vs. glucose concentration (mM) for the sensor membrane (M-1) for 3 consecutive months.

measurements. The data displayed an average correlation coefficient of 0.992.

4. Conclusion

We have demonstrated that fluorescent DPO dye may be an alternative indicator for enzymatic glucose sensing. Sensor slides are fully reversible in glucose measurements within determined working range. The relative signal change in fluorescent-based measurements was satisfactory and approximately 60%. Due to the promising spectral characteristics DPO dye, can be used in sensor designs containing inexpensive LEDs, optical fibers, and other solid-state components.

Acknowledgement

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