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Sol–gel encapsulated glucose oxidase arrays based on a pH sensitive fluorescent dye

Nimet Yildirim [a], Dilek Odaci [a], Gulsiye Ozturk [b], Serap Alp [b], Yavuz Ergun [b], Kay Dornbusch [c], Karl-Heinz Feller [c], Suna Timur [a,*]

[a] Ege University, Faculty of Science, Department of Biochemistry, 35100 Bornova, Izmir, Turkey
[b] University of Dokuz Eylul, Faculty of Arts and Sciences, Department of Chemistry, 35160 Tinaztepe, Izmir, Turkey
[c] University of Applied Sciences Jena, Department of Medical Engineering and Biotechnology, Carl-Zeiss-Promenade 2, D-07745 Jena, Germany

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ABSTRACT

Optical glucose oxidase (GOx) arrays based on pH sensitive fluorescent dye (2-(4-tolyl)-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone) (CPO) has been constructed. The arrays were prepared by spotting of CPO and GOx together with tetraethoxysilane (TEOS)/Chitosan (CHIT) mixture via a microarrayer. After optimization studies, analytical characterization of enzyme arrays were carried out. The fluorescence intensity of the system was linearly correlated to glucose concentration in the range of 1.0–30.0 mM (in potassium phosphate buffer; 2.5 mM at pH 7.0). Furthermore, the developed arrays were used to analyze glucose in some beverages and HPLC was used as a reference method for independent glucose analysis.

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1. Introduction

The development and application of the arrays of immobilized biological compounds (biological microchips) have become a significant trend in modern biology, biotechnology, and medicine. The main advantage of biological microchips over conventional analytical devices is the possibility of massive parallel analysis. Biological microchips are smaller than conventional testing systems and highly economical in the use of specimens and reagents [1].

The concept of the integrated micro-analytical system is based on the necessity to develop a complex modular system and to adapt them to all kinds of different applications. Furthermore, the system should be suitable for gaseous and liquid samples which have implications to the pressure tightness of the micro fluidic components. In order to enable rather different applications and measuring regimes, the development of a problem-tailored data acquisition and data analysis software is essential [2]. Sol–gel processes offer a relatively mild route for the immobilization of biological molecules which are entrapped in the growing covalent gel network rather than being chemically attached to an inorganic material. Many bio molecules, such as enzymes, have already been entrapped in bulk sol–gel matrices for the preparation of biocatalysts and biosensors. Moreover, the transparency of sol–gel matrices enables the optical detection of colour-producing reactions. With regard to stability, bio molecules entrapped in sol–gels typically exhibit improved resistance to thermal and chemical denaturation, and increased storage and operational stability [3]. On the other hand, the preparation conditions of sol–gel matrix have a remarkable effect on the bio catalytic activity of the immobilized enzyme. The local environments of the entrapped enzymes and the ability of analytes to diffuse to the enzyme are the two key issues of developing sol–gel based biosensors. Several studies have been reported regarding the properties of the porous sol–gel matrix, such as pore size distribution, surface area, pore geometry, morphology, and polarity [3–5].

Moreover, glucose sensing takes an important place in controlling various food and biotechnological processes as well as in diagnosis and therapy of diabetes. The crucial demand for continuous, accurate, and relatively non-invasive glucose sensing methods has motivated the design of various sensing materials as well as methodologies. Glucose oxidase (GOx) is by far one of the

* Corresponding author. Tel./fax: +90 232 3438624.
E-mail address: sunatimur@yahoo.com (S. Timur).

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most intensively studied enzyme and capable of catalyzing the oxidation of D-glucose by molecular oxygen to D-gluconic acid [6]. Furthermore, fluorescence sensing of chemical and biochemical analytes is the dominant analytical approach in medical testing, biotechnology, and drug discovery. This method has become one of the most sensitive and is often used for different bio analytical purposes applying fluorescence dyes and quantum dots as labels [7]. Additionally, it is obvious that analytical methods based on the amalgamation of an enzymatic reaction with molecular fluorescence are one of the most interesting and promising analytical alternatives [8–12]. Previously, GOx was immobilized on the polyvinyl alcohol–pyrene (PVA–Py) matrix prepared by "Click" chemistry approach and used as a water-soluble probe molecule for the fluorescence glucose sensing by our group. In that work glucose detection was carried out by following the increase in fluorescence intensity due to the diminished quenching of the photo excited Py molecules based on oxygen consumption through the enzymatic reaction [7]. On the other hand, fluorescent pH probes are also interesting due to significant advantages over other techniques, such as non-destructive character, high sensitivity and specificity [13,14]. Measurement of pH by fluorescence-based techniques is well established for both imaging and sensing applications. Therefore, fluorescence offers significant advantages over other methods for physiological pH measurements and the wide range of indicator dyes available [15]. Fluorescent pH indicators within the physiological range are an attractive target in molecular design and synthesis.

Here we have presented the planar sol–gel based fluorescent GOx arrays. A pH sensitive fluorescent dye (2-(4-tolyl)-4-[4-(1,4,7,10-tetraoxa-13-azacyclopentadecyl)benzylidene]-5-oxazolone), (CPO) was mixed in tetraethoxysilane (TEOS)/Chitosan (CHIT) together with the enzyme and spotted onto the glass slides via a microarrayer. The response signal of the arrays was measured by following of the pH induced changes in fluorescence intensity of the dye due to the enzymatic reaction. As well as optimization studies, analytical characterization sample application were carried out.

2. Experimental

2.1. Chemicals and materials

Glucose oxidase (GOx; EC 1.1.3.4, from *Aspergillus niger* – Type II-S, 50 000 U/mg), chitosan (CHIT, from crab shells, minimum 85% deacetylated), tetraorthosilicate (TEOS) were purchased from Sigma (Germany). Tetrahydrofuran (THF) was purchased from Merck, KH_2PO_4 was purchased from Riedel-de Haen® (Germany), D-glucose and glutaraldehyde were purchased from VK Labor und Feinchemikalien (Germany) and Alfa Aesar (Germany), respectively. The CPO derivative was synthesized and purified using the general procedure according to our previous study [16].

Different beverages, such as Trendy Cola, apple juice, lemon juice (brand I), lemon juice (brand II), and mixed fruit juice were used to analyze glucose content and purchased in a local market. Initially, the samples were degassed and used as stock substrate solution with dilution by working buffer.

Sol–gel/chitosan hybrid solution was prepared by mixing different ratios of TEOS, 60 μl of ethanol, 150 μL of H_2O, 600 μL of 0.5 mg/mL CHIT solutions (final concentration 0.3%). This mixture was stirred for 1 h until a clear sol–gel composite was formed and its pH was adjusted to 4.0–6.0 by using 0.1 M NaOH solutions [17].

2.2. Instrumentation

Spotting of TEOS/CHIT/CPO/GOx on the surface of glass slides was carried out with a BioRobotics MicroGrid, a High Throughput Automated Microarrayer (England). The optical measurements were taken with a fluorescence reader from Sensovation AG, Stockach (Germany). Fluorescence spectral data were recorded on Perkin Elmer, Luminescence Spectrometer, LS 50B (Germany). Spectrophotometric measurements were performed with a Perkin Elmer UV/VIS Spectrometer-Lambda 2 (Germany). JASCO HPLC (MD-1510 PDA Detector), (Germany) was used as a reference method for the analysis of glucose content in real samples.

2.3. Set-up of the fluorescence measuring system

The main part of the micro-analytical system is the measuring cell. Apart from fluorescence detection in transmittive and reflective mode, the measuring console was suitable for measurements under different measuring regimes (as fluorescence alone, absorption combined with fluorescence). The measuring cell is located in front of the fluorescence camera which is gathering as much fluorescence light as possible. The excitation light, passing the filters for wavelength selection, is directed onto the front of the measuring cell via mirrors. Additional filters in front of the camera enable a reduction of the stray light and a pre-selection of the detection wavelength region [18]. The excitation light can be also directed to the backside of the cell for adsorption measurements. Such a modular set-up made the measuring console very flexible to various measuring regimes. The emitted radiation is taken by a deep cooled CCD camera. The measuring arrangement, a fluorescence reader (Sensovation AG, Stockach), consists of a lamp, a shutter, filters for excitation and emission (Fig. 1) and a 3-stage Peltier cooled CCD image sensor with integrated micro-lenses and a high quantum efficiency (up to 90%) for detecting the fluorescence signal.

2.4. Preparation of TEOS/CHIT/CPO/GOx on the surface of microscope glass

For the preparation of TEOS/CHIT/CPO/GOx; GOx (1 mg), TEOS/CHIT (100 μL) and CPO (75 μL, 1 mg/mL) were mixed and spotted to the cleaned glass surface and allowed to dry at room temperature for 5 min. After that, spots were treated with glutaraldehyde solution (2.5% in potassium phosphate buffer, 50 mM, pH 7.5) for 2 min and washed with distilled water and phosphate buffer solution, respectively.

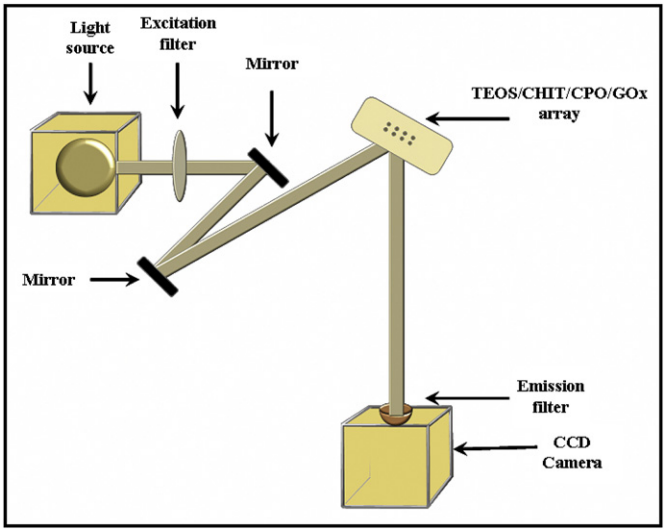

Fig. 1. Schematic view of the fluorescence measuring set-up.

2.5. Measurements

The TEOS/CHIT/CPO/GOx array spotted on glass surface was placed in front of fluorescence camera. Fluorescence intensity of each array was measured at 480 nm extinction and 523 nm emission wavelengths (I_0). Then, a known amount of glucose in buffer solution was dropped onto the surface and allowed to stand 1 min at the room temperature (25 °C). When the enzyme arrays were treated with the glucose substrate, gluconic acid was produced due to the bio catalytic reaction that caused pH changes at the microenvironment of the matrix. This in turn alters the fluorescence signal of the dye which is subsequently correlated to the substrate concentration, (I_1). Fig. 2 shows schematic representation of the reaction principle of TEOS/CHIT/CPO/GOx. Finally, the surface was washed for 5 min by gently treatment with working buffer solution for the regeneration prior the next measurement.

For the spectroscopic studies of GOx/CPO in solution, enzyme (4000 U or 0.08 mg/mL) and CPO (30 μL, 1 mg/mL in THF) were mixed in potassium phosphate buffer solution (3 mL, 2.5 mM, pH 7.0) in the quevette and then absorption and fluorescence emission spectra were recorded. Fluorescence intensity of both TEOS/CHIT/CPO/GOx array and GOx/CPO in solution was defined as ΔI which was calculated as follow: $\Delta I = I_0 - I_1$. The relative fluorescence intensity was also calculated as $\Delta I \times 100/I_1$

2.6. Sample application

Proposed arrays were used to apply glucose analysis in real samples. No sample pre-treatment was required for the analysis. Samples were added to reaction medium instead of substrate and measurements were performed. Additionally, HPLC with a refractive index detector was used as a reference method for independent analysis of the glucose content. 125/4 NUCLEOSIL 100-5 C-18 HD HPLC column (ReproGel-Ca 9 μm (250 × 8 mm), Germany) was used for the chromatographic separation of monosaccharide at 80 °C. Injection volume was 10 μL. The mobile phase was water. The flow rate was 0.5 mL/min. Initially, standard curve for glucose was plotted (0.1–10.0 mM), then samples were applied to the column and glucose levels were calculated using calibration plot.

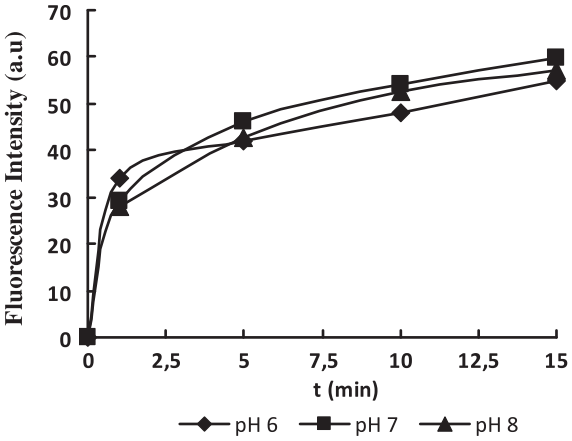

Fig. 3. Optimization of response time (Glucose; 5 mM, CPO; 0.005 mg/mL, GOx; 0.08 mg/mL in potassium phosphate buffers (2.5 mM) between pH 6.0–8.0, 2.5 mM).

3. Results and discussion

3.1. Fluorometric response of GOx/CPO in solution

Absorption and fluorescence emission based photo physical parameters of CPO were investigated and reported in our previous study [19]. The response time of GOx/CPO system (GOx; 0.08 mg/mL, CPO; 0.005 mg/mL) was investigated in 2.5 mM potassium phosphate buffer at different pHs between 6.0 and 8.0 in the presence of 5 mM D-glucose substrate. The relationship between the fluorescence intensity and the response time is shown in Fig. 3. It is clearly observed that the fluorescence intensity achieved its maximum at 5 min. Therefore, we used this response time in our further experimental steps.

3.1.1. Effect of ionic strength

The effect of ionic strength on the fluorometric response was carried out. For this purpose, phosphate buffer solutions (pH 7.0) with different ionic strength (1, 2.5, 5, 10, 25 and 50 mM) were prepared and fluorescence intensity of the enzyme/arrays in the presence of

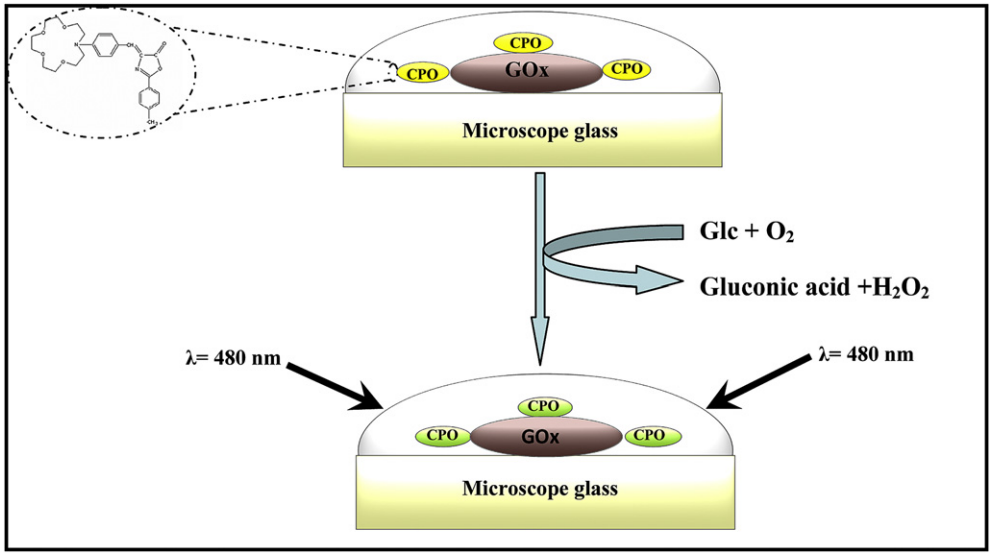

Fig. 2. Schematic representation of TEOS/CHIT/CPO/GOx reaction principle.

the substrate in these buffer solutions was registered in 5 min of response time. The highest response was obtained with 2.5 mM buffer and it was chosen as the most appropriate measurement medium. The corresponding Ionic strength – fluorescence intensities profile was shown in Fig. 4.

3.1.2. Linear range for Glc using GOx/CPO in solution

After optimization of response time and ionic strength, a calibration curve was plotted for the fluorescence intensities depending on the glucose amounts. A linear calibration plot using GOx/CPO was defined in the range of 0.5–8.0 mM glucose by the equation of $y = 12.501x + 5.5971$ ($R^2 = 0.991$), in which x and y show glucose concentration in mM and fluorescence intensity (ΔI, a.u), respectively.

3.2. Fluorometric response of TEOS/CHIT/CPO/GOx array system

3.2.1. Effect of CPO amount

In order to find optimum dye concentration in the array matrix, TEOS/CHIT (100 μL) and known amount of CPO (75 μL) were mixed and spotted onto the microscope glass surface. Fluorescence intensity of TEOS/CHIT/CPO/GOx spots was measured at 480 nm extinction and 523 nm emission wavelengths. Different concentrations of CPO within a range of 0.5–1.5 mg/mL were tested and the maximum response was found as 1 mg/mL, (Fig. 5).

3.2.2. Influence of enzyme loading

The effect of enzyme loading on the fluorescence intensity of the array was tested by using various amounts of enzyme within the range of 1.25–20 mg/mL (which equals to 62,500–100,000 U). It is observed that after 10 mg/mL of GOx loading, the signals were kept constant and no further increments were observed, hence the optimum enzyme concentration was chosen as 10 mg/mL, (Fig. 6).

3.2.3. Analytical characteristics of the array system

The fluorescence intensity of the arrays versus substrate concentration was investigated and the linearity was found to be in the range of 1.0–30.0 mM glucose and defined with the equation of $y = 0.322x + 855$, ($R^2 = 989$). Furthermore, the limit of detection (LOD) was calculated as 0.064 mM by using signal to noise (S/N) ratio of 3. In the equation x and y show glucose concentration in mM and fluorescence intensity (ΔI), respectively.

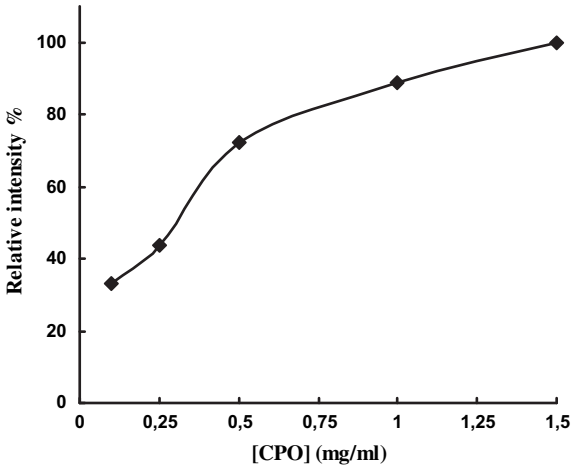

Fig. 5. The effect of CPO amount on the fluorescence intensity of TEOS/CHIT/CPO/GOx arrays (Glucose conc.; 5.0 mM in 2.5 mM (pH 7.0) phosphate buffer solution).

The repeatability of the TEOS/CHIT/CPO/GOx arrays was tested by four successive measurements for 10 mM of glucose and the standard deviation (SD) and coefficient of variation (cv, %) were calculated as ±0.29 mM and 5.01%, respectively.

To examine the operational stability TEOS/CHIT/CPO/GOx arrays were allowed to treat with working buffer and measurements were carried out within every half an hour. Data showed that developed arrays retained 70% of its initial fluorescence intensity after 5 h. These findings indicate that the stability of the arrays were satisfactory.

3.3. Sample application

Finally, the TEOS/CHIT/CPO/GOx system was tested to analyze glucose in some beverages. The samples were used as stock substrate solutions with different dilution amounts with buffer and dropped to the array surface instead of glucose substrate. Then the fluorescence intensity of the TEOS/CHIT/CPO/GOx array was measured. Additionally, HPLC was used as a reference method for independent analysis of the glucose content in the same samples. Glucose standard curve obtained from the HPLC was defined by the equation of $y = 119893.905x$ ($R^2 = 0.998$), in which x and y show glucose concentration in mM and peak area in the range of

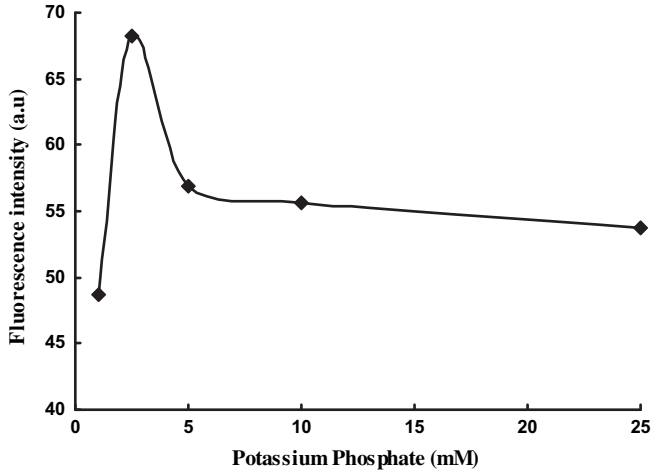

Fig. 4. The effect of ionic strength on the fluorescence intensity of GOx/CPO in solution (in potassium phosphate buffer, pH 7.0, reaction mixture composed of GOx: 0.08 mg/mL and CPO: 0.005 mg/mL, (Glucose; 5.0 mM)).

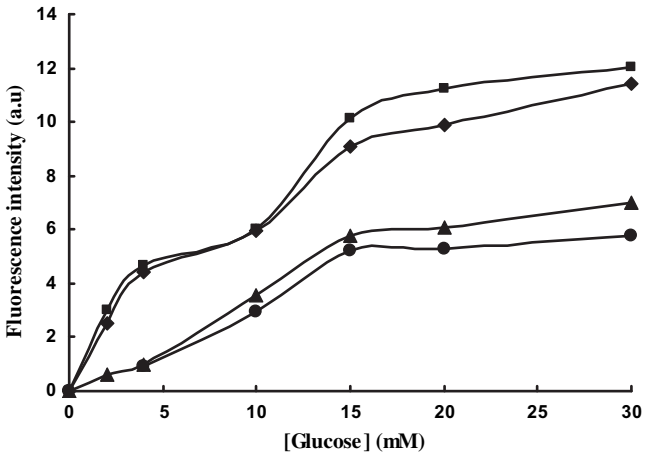

Fig. 6. The effect of enzyme loading (CPO; 1 mg/mL; Glucose; 5 mM in 2.5 mM (pH 7.0) potassium phosphate buffer solution, GOx; ● 2.5 mg/mL, ▲ 5 mg/mL, ◆ 10 mg/mL, ■ 20 mg/mL).

Table 1
Results for glucose analysis in real samples by TEOS/CHIT/CPO/GOx array and HPLC.

Sample	Glucose (g l^{-1})		
	TEOS/CHIT/CPO/GOx array	HPLC	Recovery (%)
Cola	49.045	50.15	97.80
Apple juice	16.14	15.13	106.62
Lemon juice (brand I)	3.65	3.86	94.54
Lemon juice (brand II)	12.75	12.85	99.29
Mixed fruit juice	33.04	33.52	98.58

0.1–10 mM glucose. Obtained data from both HPLC and array systems are presented Table 1 and according to data it can be said that the use of array system resulted in very similar results in comparison to the HPLC. Hence, the proposed system could be a good alternative to obtain practical glucose sensing system.

4. Conclusion

GOx-containing sol–gel arrays were prepared by spotting micro matrices on microscope slides. The advantage of this kind of entrapment in sol–gel is that the enzyme and the dye are more stable in solid phase in comparison to solution phase. Another aspect of the TEOS/CHIT/CPO/GOx array is sensitivity when compared with the results obtained in GOx/CPO. Finally, the developed TEOS/CHIT/CPO/GOx arrays were applied for the glucose analysis in real samples. The results achieved with the TEOS/CHIT/CPO/GOx array are in good agreement with the results obtained with HPLC, which makes the method developed suitable for commercial applications.

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