Offline Glucose Biomonitoring in Yeast Culture by Polyamidoamine/ Cysteamine-Modified Gold Electrodes

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This article deals with the use of pyranose oxidase (PyOx) and glucose oxidase (GOx) enzymes in amperometric biosensor design and their application in monitoring fermentation processes with the combination of flow injection analysis (FIA). The amperometric studies were carried out at −0.7 V by following the oxygen consumption due to the enzymatic reactions for both batch and FIA modes. Optimization studies (enzyme amounts and pH) and analytical parameters such as linearity, repeatability, effect of interference, storage, and operational stabilities have been studied. Under optimized conditions, for the PyOx-based biosensor, linear graph was obtained from 0.025 to 0.5 mM glucose in phosphate buffer (50 mM) at pH 7.0 with the equation of $y = 3.358x + 0.028$ *and* $R^2 = 0.998$. *Linearity was found to be 0.01–1.0 mM in citrate buffer (50 mM and pH 4.0) with the equation of* $y = 1.539x + 0.181$ *and* $R^2 = 0.992$ *for the GOx biosensor. Finally, these biosensor configurations were further evaluated in a conventional flow injection system. Results from batch experiments provide a guide to design sensitive, stable, and interference-free biosensors for FIA mode. Biosensor stability, dynamic range, and repeatability were also studied in FIA conditions, and the applicability for the determination of glucose in fermentation medium could be successfully demonstrated. The FIA-combined glucose biosensor was used for the offline monitoring of yeast fermentation. The obtained results correlated well with HPLC measurements.* © 2011 American Institute of Chemical Engineers *Biotechnol. Prog.*, 27: 530–538, 2011
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Introduction

Glucose analysis is very important in numerous areas, especially in clinical applications[1–3] and biotechnological processes.[4,5] As glucose is the main carbon source and also growth-limiting substrate for most of the microorganisms during fermentation or biotransformation procedures, an excess of this substrate may lead to overproduction of main final product, which may cause feed-back inhibitions. Furthermore, this may cause production of undesirable byproducts, and these factors affect fermentation yield and product quality directly. Hence, optimizing product quality[6] and minimizing process cost[7] are the main goals in beverage and food industries. Consequently, according to the importance of glucose in the bioprocesses, effective, reliable, fast, and real-time monitoring systems should be required for controlling the system.

Among various glucose detection techniques, such as polarimetry,[8] spectrometry,[9] amperometry,[10] capillary electrophoresis,[11] and HPLC,[12] biosensors have been widely used due to their crucial advantages related to response time, high specificity to perform measurements in complex media, sensitivity, size, accuracy, cost, and simplicity.[13,14] Photometric-, colorimetric-, optic-, and amperometric-based biosensors have been reported for glucose assay.[15,16] However, enzyme-based amperometric biosensors are considerably convenient for bioprocess control in some aspects. First, viable microorganisms are used for fermentation, and this makes it difficult to control system according to the necessity of making measurements in a complex matrix. Enzyme-based biosensors present high selectivity even in complex matrixes. Also, they can be incorporated in flow injection systems easily and, thus, can be effectively adapted for online and atline monitoring.[17,18]

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Flow injection analysis (FIA) is a considerably proven device for online and offline monitoring of bioprocesses.[19] The application of an automatic FIA system is suitable for process control, because it has various advantages as, for example, no feedback to control, reduced waste, no necessity of expert operator, high speed, and precision. Also, FIA is a multiorganized tool, which consists of many components. Basically, constituents are high quality multichannel peristaltic pump, an injection valve, selector valves, an auto-sampler, and a detector. Enzyme electrodes are the detecting units in FIA systems, and they can combine the specificity of enzyme and sensitivity of amperometric signal transduction in a complex medium.[20,21] Another proficiency of a FIA device is its usage for performing multiple parallel measurements.[22]

Especially for bioprocess monitoring, there are two state of the art biosensor-based methods for glucose detection. One of those is using a sensor system outside the bioreactor, whereas the second one is operated in situ.[23] According to the first approach, the biosensor is not connected directly to the reactor, thus, sensor is used as an external sensing element. Contrary to the first strategy, biosensor is placed into the reactor directly and is immersed in the process medium with in situ operations. There are several advantages and disadvantages for both types. As it is known, bioreactors are closed systems, and the risk of contamination is the basic problem for most processes. Thereby, biosensor must be sterilized for in situ operations. However, it is difficult to take and transport samples among sterile conditions, in situ sensors are the advantageous.[23,24] Considering the types of measuring systems, it is possible to use biosensors for performing offline analysis by FIA.

Basically, there are two types of enzymes that can be used as biorecognition element with FIA-based enzyme biosensors. Glucose oxidase (GOx, glucose 1-oxidase, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is a suitable enzyme for amperometric glucose biosensors. It contains two identical subunits, which are flavine adenine dinucleotide (FAD) cofactor bound polypeptide chains. According to the reaction scheme, the enzyme uses β-D-glucose as substrate and converts it to D-glucono-1,5 lactone and hydrogen peroxide by oxidizing glucose at carbon-1.[25]

$$\beta\text{-D-Glucose} + O_2 \rightarrow \text{D-glucono-1, 5lactone} + H_2O_2$$

One disadvantage of GOx is about its anomeric selectivity. Thus, only the β-form of glucose can be used as a substrate.[26] Some sugar derivates are oxidized by GOx, but its affinity is relatively high for D-glucose ($K_m \sim 1$ mM).[26,27] As the opposite of this, pyranose oxidase (PyOx) can oxidize both α and β forms of glucose.

PyOx (glucose 2-oxidase, pyranose: oxygen 2-oxidoreductase, EC 1.1.3.10) is also a homotetrameric flavoprotein, which also has FAD cofactor. Different to GOx, this enzyme catalysis the glucose at carbon-2 with high affinity and generates 2-keto sugars and H_2O_2 as shown below[28]:

$$\text{D-Glucose} + O_2 \rightarrow \text{2-Dehydro-D-glucose} + H_2O_2$$

The performance of the biosensor is strongly depended on the enzyme stabilization via immobilization. In addition, the stability of the multimeric enzymes is depended on dissociation of the subunits. Multipoint immobilization of multimeric enzymes may prevent subunit dissociation by intersubunit crosslinking and also reduces unwanted conformational changes by intrasubunit crosslinking.[29] Stabilization of the quaternary structure of multimeric enzymes such as GOx and PyOx may require the use of a further crosslinking step to prevent subunit dissociation.[30,31] The glutaraldehyde technique is very versatile to achieve multipoint attachments.[32] In terms of stabilization, the treatment of enzymes that previously adsorbed on dendrimers bearing primary amino groups with glutaraldehyde, in many cases offers very good results, because this technique permits the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the dendrimer.[33] The primary amino groups of both enzyme and the dendrimer possess on its surface are activated with the molecule of glutaraldehyde.[34] Hence, an intense crosslinking between dendrimer and enzyme seems to be achieved, and this multiple covalent links between the enzyme and dendrimer stabilize the quaternary structure of the protein, and also rigidity of the subunit structures is increased.[35] The intense amino groups on polyamidoamine (PAMAM) surface make the subunit structures of PyOx and GOx to involve in the immobilization. This leads to high enzyme stabilization. Recently, new perspectives for the development of novel enzyme immobilization strategies for biosensor applications that use dendrimer are reported in the literature.[36–41]

This article has been organized in two sections; initially various parameters effecting biosensor efficiency were optimized and characterized in batch system for both GOx and PyOx such as optimum pH, enzyme loading, linear range, operational stability, and shelf life. In the next section, offline FIA experiments were investigated. Finally, the use of the proposed systems for the glucose monitoring during yeast cultivation was carried out.

Materials and Method

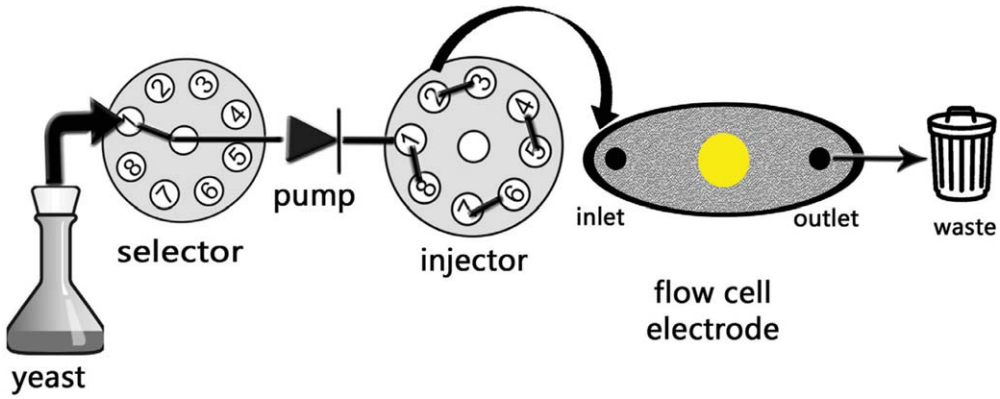
Reagents

Pyranose oxidase (EC 1.1.3.10, 9.4 unit/mg) and glucose oxidase (EC 1.1.3.4, 50 units/mg) were acquired from Sigma Aldrich (Dorset, UK). Cysteamine hydrochloride and sodium borohydride were acquired from Fluka (Steinheim, Germany), PAMAM (25% C_{12} Dendrimer Generation 4), glutaraldehyde solution (% 25, v/v), ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)], and D-glucose were obtained from Sigma Aldrich (Dorset, UK). All of the other compounds were analytical-reagent grade. Distilled water (Milli-Q) was used for preparing the solutions.

Apparatus

All the amperometric data were monitored by using a PalmSens Electrochemical Measurement Unit (Palm Instruments, Houten, Netherlands) with connection to a computer. In batch operations, three electrodes configuration, consisting of gold working electrode (BASI), Ag/AgCl reference electrode (with 3 M KCl saturated with AgCl as the internal solution, Metrohm, Switzerland) and platinum counter electrode (BASI) were used.

Flow injection mode of analysis was performed using a single line flow injection manifold with an electrochemical flow through cell of the cross-flow type with gold working, Ag/AgCl reference, and Pt auxiliary electrodes (CHI130, Austin, www.chinstruments.com). A peristaltic pump (FIAtron, Oconomovoc, WI) equipped with silicon tubing (0.89 mm inner diameter) pumped the working buffer solution as the carrier into the flow line with a flow rate of 1.3 mL min^{-1}.



Scheme 1. The schematic representation of FIA-biosensor measurement system.

The flow line was made of Teflon tubing (0.5 mm inner diameter). Sample solution (100 μL) containing substrate was injected into the carrier stream via an eight-port injection valve (FIAtron, Oconomovoc, WI). The FIA system was connected to a PalmSens potentiostat for the electrochemical measurements. The schematic representation of FIA-biosensor measurement system was shown in Scheme 1. The FIA system was assisted by software program written in C#, which was developed at the Institute for Technical Chemistry, Leibniz University (Hannover, Germany).

Additionally, HPLC with a refractive index detector controlled by a HP-Chemstation from Agilent (Karlsruhe, Germany) was used as the reference method for independent analysis of the glucose and ethanol content. HPLC column [GL Sciences, Inertsil NH_2 5.0 μm (4.6 I.D $\times$ 250 mm), Japan] was used for the chromatographic separation at 30°C. Injection volume was 20 mL. The mobile phase was H_2SO_4 (5 mM).[42] The flow rate was 0.6 mL/min. Initially, a standard calibration curve for glucose and ethanol were plotted (2.5–50 mM for glucose with the equation of $y = 303971.24x$; $R^2 = 0.999$ and 2.5–50 mM for ethanol with the equation of $y = 7097.29x$; $R^2 = 0.994$). After dilution with the mobile phase and a centrifugation step, the samples were applied to the column, and then glucose and ethanol concentrations were calculated using the calibration plots.

Electrochemical measurements

Both PyOx and GOx catalyze the oxidation of glucose in the presence of molecular oxygen. The enzymatic reaction yields the reduction of O_2 to hydrogen peroxide (H_2O_2). According to depletion of O_2 in the bioactive layer through the reaction, decreases in current levels were monitored. The current values were correlated indirectly to the glucose concentration consumed during enzymatic reaction. Alterations of the currents between two steady states (before and after addition of substrate solutions) were monitored amperometrically and recorded as, ΔI [μA] at -0.7 V. The obtained current signals were plotted vs. standard glucose concentrations. This calibration curve was also used to calculate the glucose amount in samples. All the measurements were carried out at 25°C under constant magnetic stirring rate, and it took less than 100 s to obtain a stable signal for each measurement in batch mode.

Biosensor preparation

In the construction of the glucose biosensors either GOx or PyOx were used as biological component. The immobilization procedure was explained in our previous study.[43] As mentioned, the enzyme was immobilized onto the gold electrode surface by the means of PAMAM dendrimers through multipoint covalent linkages in the presence of glutaraldehyde crosslinking agent. The prepared biosensors were immersed into their working buffer solutions and stored at 4°C when not in use.

To estimate immobilization yields, PyOx and GOx activities were determined spectrophotometrically at 420 nm and 30°C by measuring the formation of H_2O_2 for 3 min with a peroxidase-coupled assay using ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] ($\varepsilon_{420} = 43{,}200\ M^{-1}\ cm^{-1}$). The standard assay mixture (total volume: 3 mL) contained 1 mL of ABTS (0.003 M) in 50 mM phosphate buffer (pH 6.0), 300 μL of horseradish peroxidase (6 U), 1 mL of D-glucose (0.3 M), and 100 μL of the PyOx sample.

One unit of PyOx activity was defined as the amount of enzyme necessary for oxidation of 1 μmol of ABTS per min under the conditions described above.[44]

Yeast cultivation

Glucose biosensors were tested in yeast culture medium, and the medium was directly injected to the reaction medium. Cultivation experiments were carried out according to a procedure described in our previous study.[43] The consumption of glucose by yeast during fermentation was monitored amperometrically for 8 h by FIA system. Obtained results were also compared with HPLC as reference method.

Results and Discussion

In the case of many cycles of high yield processes are desired, catalytic properties of enzymes have to be usually improved before the treatment. Generally, soluble enzymes have to be immobilized to be reused for a long period in biotechnological processes, in addition to that some other critical enzyme properties have to be improved such as stability, activity, inhibition by reaction products, and selectivity toward non-natural substrates. In this way, immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols. For example, multipoint and multisubunit covalent immobilization improve the stability of monomeric or multimeric enzymes.[45] In this study, using glutaraldehyde as crosslinking agent leads to multipoint attachment of the multimeric enzymes to dendrimer-modified electrode surfaces.

To estimate if there were any enzymatic activities left in buffer solutions, where the biosensors were washed and kept after preparation, ABTS method was applied. Obtained results were compared with corresponding free enzyme activities, which equal to that of enzyme used for the immobilization process. The percentage of enzyme activities that leaked from the electrode surface were calculated for each enzyme amounts used in biosensor optimization studies. According to the results, there were no significant activities in buffer solutions. The maximum enzyme activity loss was found 0.4%. As a result, it can be claimed that the applied multipoint covalent attachment strategy is effective to provide a stable biomatrix that avoided enzyme leakages.

Optimization studies

In batch operations for the examination of the optimum pH values of the glucose biosensors, different buffer solutions were tested. For PyOx-based biosensor, 0.5 mM glucose standard solution was applied between pH 5.5–7.5 sodium phosphate buffer solutions (50 mM). As shown in Figure 1a, the highest biosensor response was obtained in pH 6.0. In the case of GOx-based biosensor, the pH dependence was investigated over the pH range 3.5–6.5 with sodium citrate and sodium phosphate buffers (50 mM) in the presence of 0.5 mM glucose. The biosensor showed the best result at pH 4.0 as given in Figure 1b. For the further experiments, sodium phosphate (pH 6.0) and sodium citrate (pH 4.0) for PyOx and GOx biosensors were used as the working buffer solutions, respectively. It was observed that for the enzyme electrodes, the pH value of maximum activity shifted toward the acidic region when compared with that of the free enzymes. The optimal pH values of free GOx and PyOx were known as 5.5 and 7.0, respectively. When these enzymes were immobilized by means of PAMAM dendrimer, their optimum pH shifted toward acidic sides as 4.0 and 6.0. This can be explained by partitioning of protons. The change in pH/activity profile is mostly due to the PAMAM dendrimers. The positive charge of amine groups on the surface of the dendrimers repels the protons toward the surface of electrode. It causes relative higher proton concentrations around the surface. Thus, optimum pH values of immobilized enzymes shifts toward acidic regions.[46]

The amperometric response was examined as a function of the enzyme amounts. For the PyOx-based biosensor, three different electrodes were prepared with 0.5 mg (4.7 unit), 1.0 mg (9.4 unit), and 2.5 mg (23.5 units) of enzyme. Also for the GOx-based biosensor, 1 mg (50 unit), 2 mg (100 unit), and 4 mg (200 units) of enzyme amounts [in 10 μl, 50 mM phosphate buffer (pH 6.0)] were immobilized on PAMAM-modified gold electrodes. The amounts of other components were kept constant. The highest signals were obtained with the biosensors with 1 mg (9.4 units) PyOx (Figure 2a) and 2 mg (100 units) GOx (Figure 2b) in immobilization matrix while no increase for the higher loadings were found. By increasing the enzyme concentrations to achieve intense multipoint covalent attachment becomes more possible (which is a strong enzyme-dendrimer interaction). There will be more primary amine groups to bind via crosslink to dendrimer molecules. More crosslinkages will lead to more sufficient enzyme immobilization, but at the same time, these intense crosslinkages can induce conformational changes. Finally, the possible conformational changes and diffusional problems may lead to loss of enzyme activity.[43]

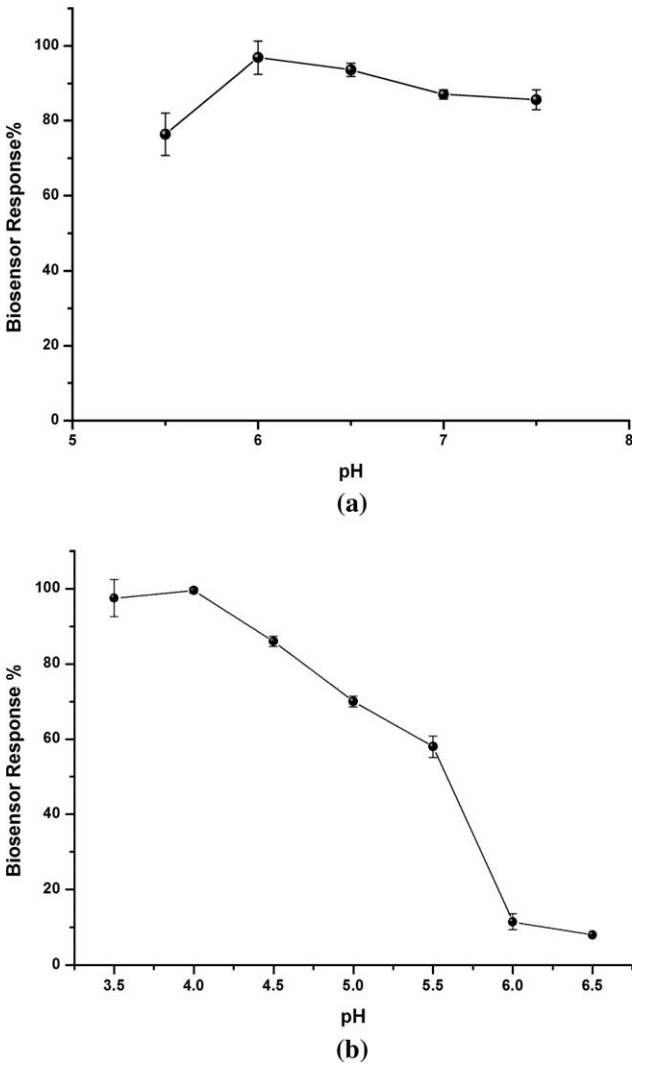


Figure 1. Effects of pH for PyOx- and GOx-based biosensor.

(a) Effect of pH for PyOx-based biosensor (50 mM, Sodium phosphate buffers at pH 5.5; 6.0; 6.5; 7.0; 7.5;−0.7 V, [Glc]; 0.5 mM, error bars show S.D). (b) Effect of pH for GOx-based biosensor (50 mM, sodium citrate buffers at pH 3.5; 4.0; 4.5; 5.0 and sodium phosphate buffers at 5.5; 6.0; 6.5; −0.7 V, [Glc]; 0.5 mM, error bars show S.D).

One of the most important advantages of enzyme-based biosensors on chemical measurement systems is their specificity to certain substrates. GOx and PyOx are responsible for glucose oxidation, and PyOx can also oxidize a number of common monosaccharides on carbon-2 in the presence of molecular oxygen to yield the corresponding 2-keto sugars and hydrogen peroxide. To investigate the substrate specificity of the PyOx-based biosensor, xylose, glucose, galactose, mannose, sucrose, lactose, and maltose solutions (0.25 mM) were introduced to working buffer solution, respectively. Relative biosensor responses were determined and are shown in Figure 3. As a result, xylose and galactose were detectable besides glucose by the PyOx-based biosensor. In the application of the biosensor in fermentation medium, this does not possess a problem, because as carbon sources for yeast cells only glucose is used in Schatzmann fermentation medium. On the other hand, wider substrate specificity could be also advantageous especially for the use of biosensing systems as an electrochemical bio-detector after chromatographic separations.[47]

Batch experiments were about optimization and analytical characterization of PyOx and GOx biosensors. In the next

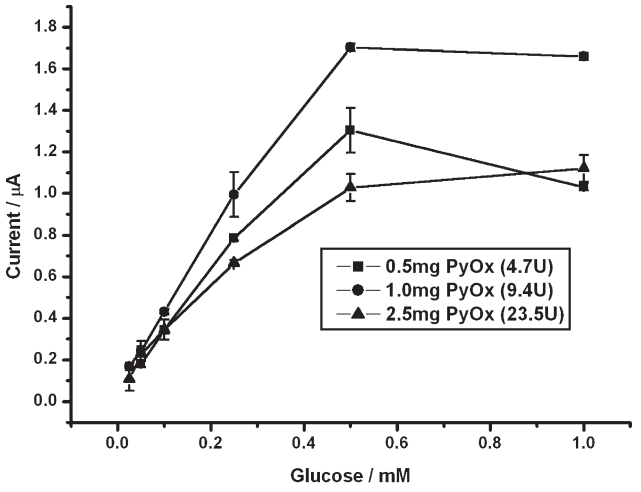


Figure 2. Effects of enzyme amount on the PyOx- and GOx-based biosensor.

(a) Effect of enzyme amount on the PyOx-based biosensor response (in sodium phosphate buffer, 50 mM, pH 6.0; −0.7 V, error bars show S.D). (b) Effect of enzyme amount on the GOx-based biosensor response (in sodium citrate buffer, 50 mM, pH 4.0; −0.7 V, error bars show S.D).

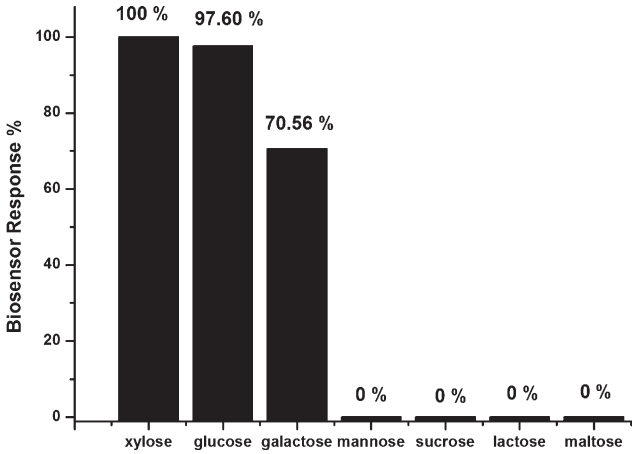


Figure 3. Comparison of the PyOx biosensor responses for the various sugars (in sodium phosphate buffer 50 mM, pH 6.0; −0.7 V; Substrate conc: 0.25 mM).

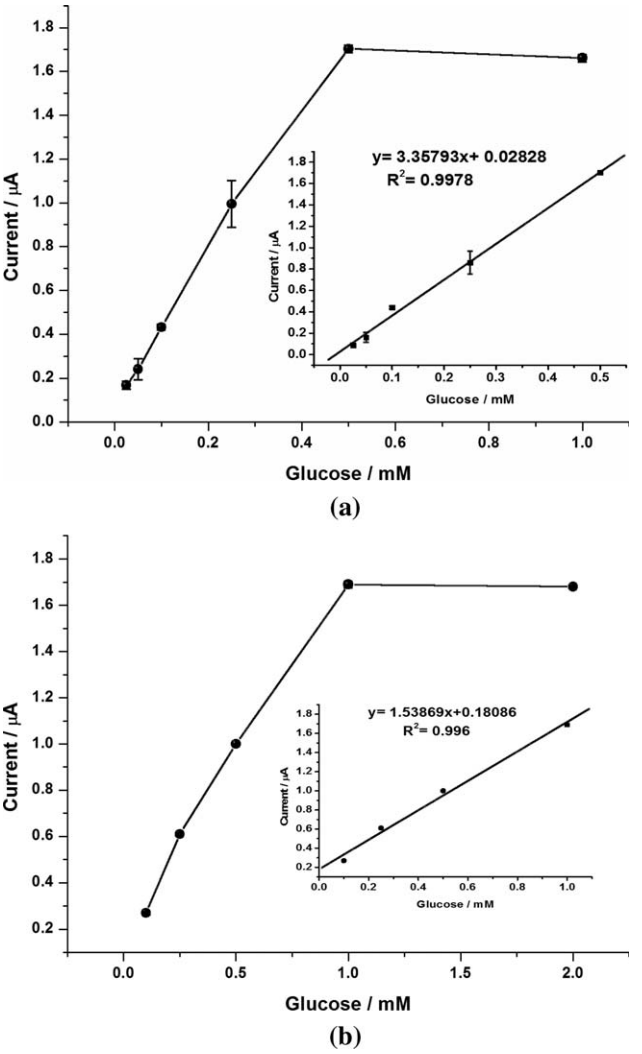


Figure 4. Calibration curves for PyOx- and GOx-based biosensor.

(a) Calibration curve for PyOx-based biosensor (in 50 mM sodium phosphate buffer, pH 6.0; −0.7 V, error bars show S.D). Inset: calibration plot for glucose. (b) Calibration curve for GOx-based biosensor (in 50 mM sodium citrate buffer, pH 4.0; −0.7 V, error bars show SD). Inset: calibration plot for glucose.

part of this work, the control of fermentation processes with the setup FIA system[43] was tested, and it was obvious that selecting the most appropriate and advantageous enzyme-based FIA combined biosensor from every point is a pivotal point of all.

Analytical approaches

In batch mode for the PyOx-based biosensor, a strict linear calibration curve was obtained from 0.025–0.5 mM glucose in 50 mM phosphate buffer at 7.0 with the equation of $y = 3.358x + 0.028$ and $R^2 = 0.998$ (inset of Figure 4a). On the other hand, the biosensor signals corresponding to 0.1 mM glucose standard solutions were measured for 10 times to achieve repeatability of the biosensor response. Relative standard deviation (RSD%) were calculated as 4.9%. For GOx-based biosensor linear range was found to be 0.01–1.0 mM for glucose in sodium citrate buffer (pH 4.0) with the equation of $y = 1.539x + 0.181$ and $R^2 = 0.992$ (inset of Figure 4b). RSD corresponding to 0.5 mM glucose solution for 10 repeated measurements was calculated as 4.38%.

Moreover, limit of detection (LOD) values were calculated as 7.45 μM for PyOx-based biosensor and 11.84 μM for GOx-based biosensor according to $S/N = 3$. All the signals were recorded under the same experimental conditions. According to the data, it can be claimed that this PAMAM-modified enzyme-based glucose biosensors presented a good repeatability in our optimized experimental conditions.

Moreover, K_M and I_{MAX} values were estimated for the both glucose biosensors by using the response curves (substrate concentrations vs. currents). For PyOx-based biosensor, those were calculated as 1.17 mM and 5.70 μA, respectively. For GOx-based system, K_M and I_{MAX} values were calculated as 1.67 mM and 4.49 μA, respectively. According to literature K_M, values of GOx from *Aspergillus niger* sp. changes between a wide range of 1.59–130 mM for beta-D-glucose.[48–52] Also the free form of pyranose oxidase from *Cariolus hirsutus* and *Cariolus versicolor* sp. has K_M values 0.83 and 1.7 for D-glucose, respectively.[53] It is well known that the source of the enzyme and the method used are very important parameters that affect the kinetic properties of the enzymes. It is clear that there are no significant changes on the affinity of the enzymes to the substrates. We can claim that immobilization via dendrimers and glutaraldehyde chemistry as crosslinking agent leads enzymes to stabilize their native forms. It is very important to protect the enzymes from any negative kinetic changes during immobilization process. Our results show that applied immobilization strategy is compatible with the enzymes nature.

During the characterization studies of the FIA system, the same experimental conditions that were obtained in batch experiments such as optimal pH and enzyme concentrations were used for both glucose biosensors. Thus, the optimal pH for GOx- and PyOx-based biosensors were 4.0 (50 mM sodium acetate buffer) and 6.0 (50 mM sodium phosphate buffer), respectively, for further FIA studies. It is logic that optimal pH values are the same for both batch and FIA systems, because both immobilization procedure and microenvironment of enzymes are identical. Also, enzyme amounts were used 2 mg (100 U) and 1 mg (9.4 U) for GOx and PyOx, respectively.

For the analytical characteristics, Figures 5a,b (calibration plots were given in inset) show the linear graph in which biosensor response as current (y; μA) was plotted vs. glucose concentration (x; mM substrate). Initially, linearity between current and glucose concentration started from 0.05 mM and continued until 2.5 mM substrate for proposed GOx-FIA system. Then, a steady state was reached beyond 2.5 mM glucose. The linearity was defined by the equation of $y = 0.075x + 0.007$, with a correlation coefficient $R^2 = 0.995$. For the PyOx-based FIA system, linearity was obtained in the range of 0.025–1.0 mM glucose given by the equation of $y = 1.346 + 0.0004$, with a correlation coefficient $R^2 = 0.999$. At higher concentrations than 1 mM, linearity was deviated and reached a steady state. Moreover LOD values were calculated as 1.27 μM for PyOx-based biosensor and 38.97 μM for GOx-based biosensor according to $S/N = 3$.

Repeatability was also examined. RSD were calculated as 1.6% ($n = 10$) and 2.8% ($n = 10$) for GOx and PyOx biosensors, respectively. All repeated analyses were evaluated by using 0.5 mM glucose solutions for both biosensors. All the characterization results are also summarized in Table 1.

Furthermore, interference study was performed in batch system. For the examination of the potential interferents, the amperometric response of GOx and PyOx biosensors were registered toward 0.5 mM glucose solutions in the presence of various interfering substances such as ascorbic acid, 3-acetamidophenol, and Schatzmann medium, which is widely used as a standard medium in fermentation processes of yeasts. The differences in current levels were compared with that of standard glucose solutions and were calculated as a relative response value. The effect of interferents to biosensor signals was also evaluated by using the working potentials of +0.7 V (in which produced peroxide through the enzymatic activities are measured) and −0.7 V. The results are displayed in Table 2. It is known that at higher potentials, most common metabolites such as uric acid and ascorbic acid get oxidized and interfere with the electrochemical signal. It is therefore essential to apply the lowest possible electrode potential. According to the results given in Table 2, use of of −0.7V as the working potential is favorable because here none of the compounds tested could interfere with the biosensor signal. In addition, higher amounts of

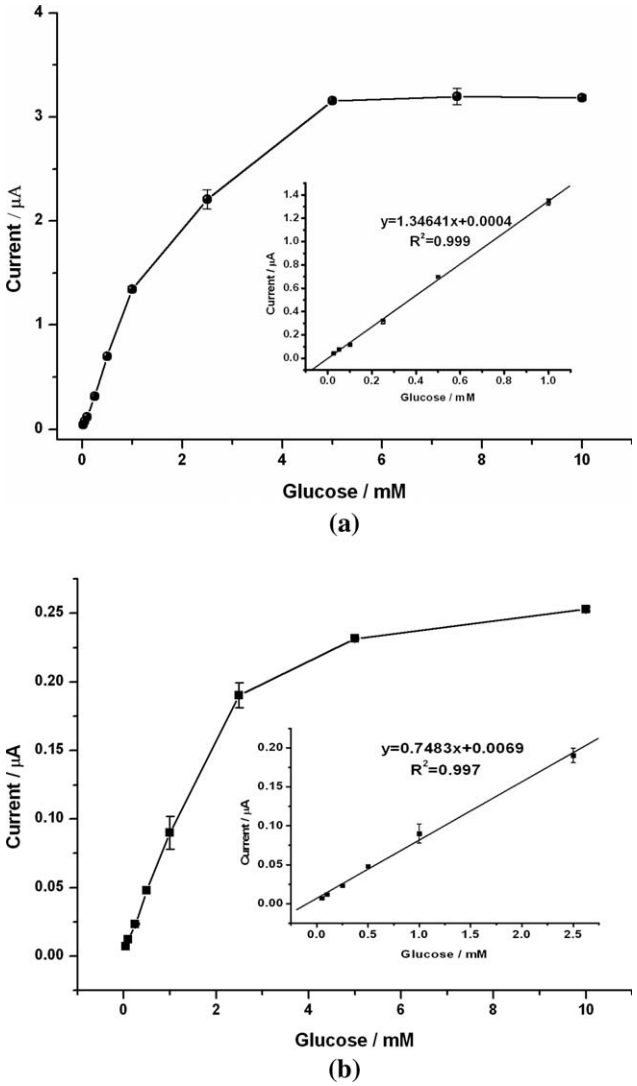


Figure 5. Calibration curves for PyOx- and GOx-based FIA-biosensor.

(a) Calibration curve for PyOx-based FIA-biosensor (in 50 mM sodium phosphate buffer, pH 6.0; −0.7 V, error bars show S.D). Inset: calibration plot for glucose. (b) Calibration curve for GOx-based FIA-biosensor (in 50 mM sodium citrate buffer, pH 4.0; −0.7 V, error bars show S.D). Inset: calibration plot for glucose.

Table 1. Comparison of the Characteristic Parameters of Proposed Glucose Biosensors

	Linear range (mM)	Equation* (R^2)	LOD†	RSD	Optimum pH	Enzyme amount	Operational stability	Storage stability
Batch system								
PyOx biosensor	0.025–0.5	$y = 3.358x + 0.028$ (0.998)	7.45 μM	5.07‡	6.0 (Phosphate buffer)	1 mg (9.4 U)	No activity lost during 8 h	27.71% of initial activity was lost during 1 month
GOX biosensor	0.01–1.0	$y = 1.539x + 0.181$ (0.992)	11.84 μM	4.4§	4.0 (Citrate buffer)	2 mg (100 U)	No activity lost during 8 h	No activity lost during 1 month
FIA mode								
PyOx biosensor	0.025–1.0	$y = 1.346x + 0.0004$ (0.999)	1.27 μM	2.8§	n.d	n.d	9% activity loss during 3.5 h	n.d
GOX biosensor	0.05–2.5	$y = 0.748x + 0.0069$ (0.997)	38.97 μM	1.6§	n.d	n.d	No activity loss during 3.5 h	n.d

* In all equations, y and x values indicate response signals as μA and concentration as mM, respectively. † LOD was calculated according to three times of S/N. ‡ For 0.1 mM glucose. § For 0.5 mM glucose. n.d not detected.

Table 2. Effect of Potential Interferents on Glucose (0.5 mM) Responses

	Relative response (%)*			
	GOX biosensor		PyOx biosensor	
Substrate	at −0.7 V†	at +0.7 V†	at −0.7 V†	at +0.7 V†
Glc (0.5 mM)	100	100	100	100
Glc (0.5 mM) + AA (0.1 mM)	84.8	129.0	96.1	137.7
Glc (0.5 mM) + AP (0.1 mM)	99.8	97.5	105.1	130.2
Glc (0.5 mM) + SM (v/v, % 0.1 in reaction medium)	92.9	100.8	99.7	107.8

AA, ascorbic acid; AP, 3-acetamidophenol; SM, Schatzman medium.
* All measurements were repeated three times. † Vs. Ag/AgCl reference electrode.

Schatzmann medium cause a large decrease in current levels in the presence of glucose when the potential 0.7 V is applied. Thus, in all experiments, −0.7 V vs. Ag/AgCl electrode potential was used to obtain interference free signals.

Stability

Operational and storage stabilities are important parameters for immobilized enzymes. Immobilization procedure affects enzyme activity mostly in a negative way, because enzymes are removed from their native environment. However, while controlling biotechnological processes, it is very crucial to keep enzyme activity constant.

Operational stability and shelf life were investigated in the batch mode. Proposed glucose biosensors were operated during 8 h. Glucose standard solutions, 0.1 and 0.5 mM, were used to test the stabilities of PyOx and GOx based biosensors, respectively. Consequently, we did not observe any loss of activity during the measurements for both biosensors (Table 1). Although determining their shelf life, the responses of enzyme electrodes were measured each 3 days for duration of 1 month and when they were not in use stored at 4°C in contact with their working buffer solutions. Finally, PyOx-based biosensor kept 72% of its initial activity after this stage but GOx biosensor did not show any loss of activity during storage.

Determination of the interested compound by the means of FIA-based biosensors without any loss of activity during operation takes much attention when it is considered to perform the system in off-/online mode. During the measurements with FIA, repeated injections of 0.5 mM glucose over 3.5 h period of time were carried out, and repeatable 34 amperometric responses were obtained. After 3.5 h, the relative enzyme activities were calculated as 100 and 91% for GOx- and PyOx-based FIA biosensor, respectively. Maximum activity was assumed as 100%, and other results were calculated according to the maximum point. The experiments clearly indicated that operational stability of both FIA–biosensor systems is excellent.

Yeast cultivation

Finally, optimized biosensors were applied for offline monitoring of glucose during fermentation. For this purpose, *Saccharomyces cerevisiae* H620 was cultivated in Schatzmann medium according to literature[43] and consumption of glucose was monitored by FIA-based glucose sensors during the fermentation. Two hundred- and three hundred-fold dilutions of the samples were required to adjust the sample concentration to the linear range of the GOx-and PyOx-based FIA biosensors, respectively. Additionally, glucose assay was performed by HPLC as a reference method. All data from biosensors and HPLC are depicted in Figure 6. As it is seen, the obtained results were well correlated with the reference method. Also, an increase of ethanol concentration, which was obtained by HPLC assay, was added to the graph to show that ethanol production is related to glucose consumption. As a result, the developed FIA-based glucose biosensors are applicable to monitor processes in offline mode.

Conclusions

Development of the effective glucose biosensing systems in the area of process monitoring is very crucial. In this article, we aimed to find out the most appropriate enzyme-based biosensor among our developed systems for glucose monitoring. To achieve this, we performed two different enzyme-based biosensors and their flow injection integrated applications. Briefly, both biosensors, which were optimized in batch system and characterized in batch and FIA modes, showed very good performances in fermentation medium. For the future perspective, our goal is to combine multi-analysis system with in situ microscopy of monitoring the

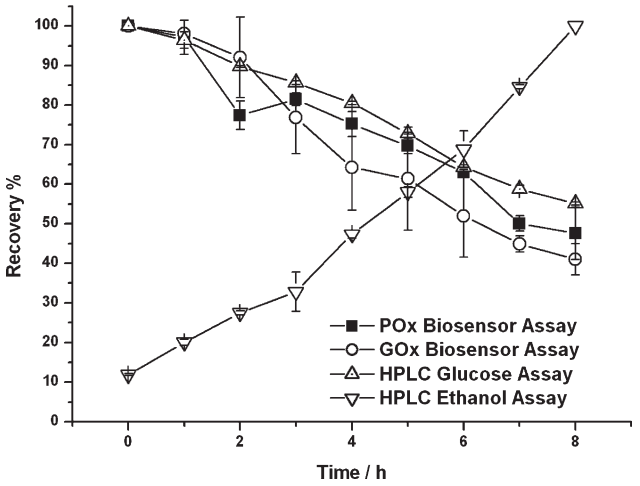


Figure 6. Monitoring of time depending glucose consumption and ethanol production in yeast culture by HPLC (refractive index detector) and glucose biosensors (at −0.7 V), error bars show SD.

surface morphologies of yeast cells to succeed more controllable operations. In our previous study, we improved alcohol-based biosensor,[43] and for the following experiments, we will couple the most suitable glucose biosensor with alcohol based one to detect consumption of glucose that proportional to alcohol production simultaneously. For that reason, we have chosen PyOx-based biosensing system because it is more convenient due to its optimum working buffer solution pH 6.0, lower LOD value. It is already known that optimum pH is one of the most important parameter for enzyme-based biosensors so that PyOx has chosen as a biocomponent, because it has nearly the same working buffer with AOx-based biosensor.

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