Alcohol Biosensing by Polyamidoamine (PAMAM)/Cysteamine/Alcohol Oxidase-Modified Gold Electrode

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A highly stable and sensitive amperometric alcohol biosensor was developed by immobilizing alcohol oxidase (AOX) through Polyamidoamine (PAMAM) dendrimers on a cysteamine-modified gold electrode surface. Ethanol determination is based on the consumption of dissolved oxygen content due to the enzymatic reaction. The decrease in oxygen level was monitored at −0.7 V vs. Ag/AgCl and correlated with ethanol concentration. Optimization of variables affecting the system was performed. The optimized ethanol biosensor showed a wide linearity from 0.025 to 1.0 mM with 100 s response time and detection limit of (LOD) 0.016 mM. In the characterization studies, besides linearity some parameters such as operational and storage stability, reproducibility, repeatability, and substrate specificity were studied in detail. Stability studies showed a good preservation of the bioanalytical properties of the sensor, 67% of its initial sensitivity was kept after 1 month storage at 4°C. The analytical characteristics of the system were also evaluated for alcohol determination in flow injection analysis (FIA) mode. Finally, proposed biosensor was applied for ethanol analysis in various alcoholic beverage as well as offline monitoring of alcohol production through the yeast cultivation. © 2010 American Institute of Chemical Engineers *Biotechnol. Prog.,* 26: 896–906, 2010
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Introduction

Recently, there is a need of rapid and reliable determination of ethanol in a large variety of areas such as industry, clinic, forensic science, agricultural analysis, and environmental analysis. Thus, monitoring of ethanol in these samples has received much attention due to its economic and commercial importance. The quantitative measurement of alcohol is very important to control the fermentation processes, product quality in some industrial fields, including the pulp, food, and beverages industries.[1] Numerous analytical methodologies and various approaches have been developed for such analysis, including redox titrations,[2] colorimetric techniques,[2] refractometry,[3] liquid chromatography,[4] gas chromatography,[5] and spectrophotometry.[6] However, these methods are relatively expensive, time consuming, complex to perform, require laborious sample pretreatment, good skilled operator, and expensive instrumentation.[7,8] Industrials such as food and beverages need rapid and cheap methods to determine compounds of interest.[9] So that, biosensors have been developed to alleviate the analysis in routine of industrial products. Comparing analytical methods with biosensors, biosensors have some advantages as high selectivity and specificity, relative low cost of construction and storage, potential for miniaturization; they facilitate automation and are simple and fast to operate. Furthermore, they enable the construction of portable equipment for fast analysis and monitoring in platforms of raw material reception, quality control laboratories, or any stage during the biotechnological processing.[10] Enzyme-based biosensors use selectivity, sensitivity, and specificity characteristics of enzymes; for this purpose, enzyme-based biosensors are developing devices in the detection of small quantity of ethanol in industrial and fermentation broth.

The alcohol biosensors have been developed using either alcohol dehydrogenase (ADH) or AOX, by immobilizing these enzymes onto suitable transducers. ADH requires the presence of NAD^+ as cofactor which increases the overall cost of manufacture. Also, the cofactor needs to be close to the electrode surface where the enzyme is deposited. Because of such problems, sensitivity decreases.[11] For AOX biosensors, no other cosubstrate than O_2 is needed.[12] Different types of alcohol biosensors reported previously have been summarized in Table 1.

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In literature, there are lots of enzyme-immobilization platforms for constructing alcohol biosensors. As such, there is an increasing need for suitable immobilizing methods because of the limited storage stability of AOX. Nanjo and Guilbault were the first who immobilized AOX for ethanol measurement in 1975.[23] So far, AOX has been immobilized on various substrates including nylon cloth,[23] pig intestine,[24] nylon net,[25] polypropylene membrane,[26] acetylcellulose filter,[27] polyacrylamide membrane,[28] Nafion membrane,[29] carbon paste by covalent coupling,[30] nitrocellulose or polycarbonate membranes,[11] poly(carbamoyl) sulfonate using screen-printing technology,[12] polyvinyl ferrocenium matrix through electrostatic process,[31] graphite Teflon by physical inclusion,[32] electropolymerised film of 2,6-dihydroxynaphthalene and 2-(4-aminophenyl)-ethylamine,[33] Teflon membrane[34] nonconducting polypyrrole film by cross-linking,[35] hydrophobic semisolid matrices,[36] polypyrrole and CP-copolypyrrole,[37] and poly(neutral red) film.[38] One possible and effective way of improving the stability and activity of an immobilized enzyme is to incorporate the enzyme into some biomaterials that are more biocompatible such as dendrimers. Using highly branched dendritic macromolecules as a structural component for the organic films is of great interest.[37,39] They possess a unique surface of multiple chain ends, these ends have surface groups that can be controlled according to the objective aim as a function of synthetic generations.[40] Fourth-generation (G4) poly(amidoamine) (PAMAM) dendrimers have 64 surface amino groups. The amine groups that PAMAM contains lead to a high surface for enzyme to bind. In addition to this, synthetic modifications for the molecularly ordered nanostructures can be achieved by the high concentration of functional end groups of dendrimers.[41] From the aspect of biosensing application, dendrimers regarding multilayered configurations have several advantages. First, the dendrimers provide multiple conjugation sites, and a densely functionalized and structurally stable architecture can easily be obtained. Second, because of the interior void structure of dendrimers,[42] the resulting multilayer film will provide minimal diffusion restriction for analytes and electron-transferring substances (mediators)[42,43] ensuring desired enzymatic and electrocatalytic reactions over the whole range of multilayers. Third, the remaining reactive groups after film formation are accessible for further modifications with artificial redox mediators for the reagentless biosensing or pendant groups for specific purposes.[44] PAMAM dendrimers have been recognized as promising building blocks of molecularly organized nanostructures. Multistep immobilization of proteins using dendrimers increases the proteins attachment capacity of the surfaces.[45] According to the literature, it was reported that PAMAM dendrimer Generation 4 enhances surface loading capacity and efficiency for selective and sensitive detection of interested molecule.[46] Furthermore, high density of active groups on the surface of such dendrimers tends to show remarkably higher chemical activity to their activity when present in other molecules.[47] Additionally, the polyamidoamine structures mimic three-dimensional structure of biomacromolecules and their good biocompatibility.[48,49] PAMAM, thus has been utilized as the bioconjugating reagent for construction of films with biomolecules.

From the point of enzyme-based biosensors, the performance of the biosensor is strongly depended on the enzyme stabilization and also 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.[50] Stabilization of the quaternary structure of multimeric enzymes such as AOX may require the use of a further cross-linking step to prevent subunit dissociation.[51,52] The glutaraldehyde technique is very versatile to achieve multipoint attachments. In terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed in dendrimer bearing primary amino groups offers in many cases very good results, because they permit the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the dendrimer.[53] The primary amino groups of both enzyme and the dendrimer posses on its surface are activated with the molecule of glutaraldehyde. Hence, an intense crosslinking between dendrimer and enzyme seems to be achieved and this multiple covalent links between the enzyme and dendrimer stabilizes the quaternary structure of the protein and also rigidity of the subunit structures is increased.[54] The intense amino groups on PAMAM surface make the subunit structures of AOX 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.[55–60]

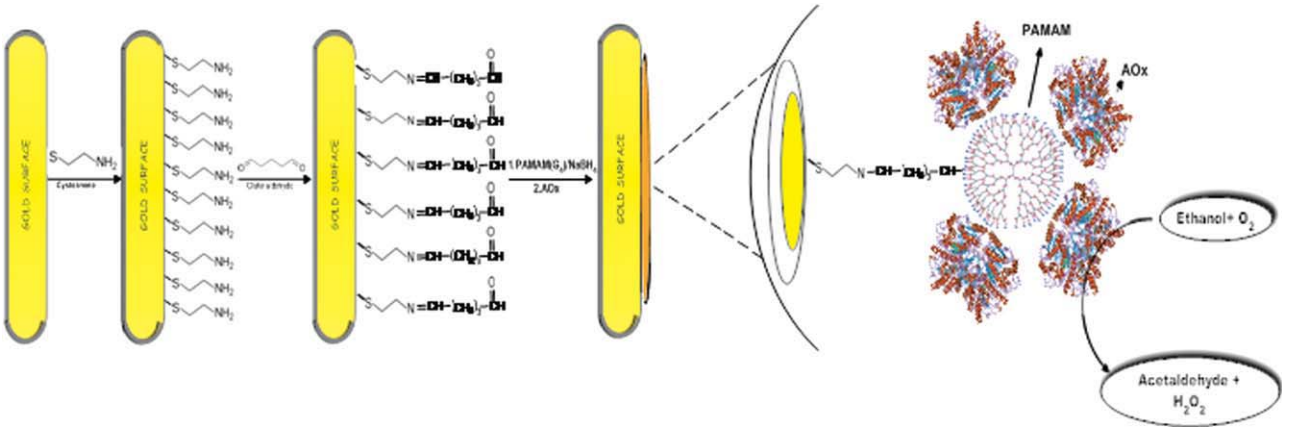
Covalent immobilization of the biocomponents onto metallic surfaces such as gold, silver, and platinum through thin film self-assembled monolayers (SAMs) has been considerably grown. Hence, SAMs involve an attentively designed bond between a functional group of the biomaterial and the appropriate group of SAM.[61] Self-assembled monolayers (SAMs), especially those formed on gold surface (Au-SAMs), have attracted considerable attention during the last years as powerful tools to prepare structurally well defined and stable chemical interfaces and thin films with controllable thicknesses and desirable function.[40] Because of these unique specialties that SAMs have, they are ideally suitable to work about both fundamental and practical issues, such as biocatalysis, electro-optic devices, corrosion, lubrication, adhesion, electron/energy transfer, molecular recognition, and sensors devices as model systems.[62–64] Appropriate molecules containing sulfur functional groups, such as thiol, disulfide, and sulfide adsorb on Au surface via Au-thiolate bond and results in formation of Au-SAMs. Immobilization of biomolecules onto gold-thiol SAMs have potential applications in construction of microdevices and nanodevices[65,66] for in vivo and ex vivo measurements of metabolites such as glucose,[67–69] cholesterol,[70] proteins,[71] antioxidants,[72] vitamins,[73] neurotransmitters,[74,75] antibodies and antigens,[76] DNA,[77,78] and amino acids.[73,79] Nowadays, SAM's of amine-terminated generation 4 (G4-NH_2) PAMAM dendrimers are used as an efficient immobilization matrix on gold substrates in electrochemical sensing devices.[80]

This work describes the construction of an alcohol biosensor by using SAMs on Au electrode surface that consist of alcohol oxidase as biocomponent, PAMAM dendrimer and glutaraldehyde as crosslinking agent. Also optimization, characterization, and applications of PAMAM/AOX biosensor are achieved. The optimized biosensor was used for ethanol analysis in beverages and for offline control of ethanol fermentation processes.

Table 1. Comparison of Performances of Various AOX-Based Biosensors

Biosensor Configuration	Stability	Linearity for Ethanol	Limit of Detection	Working Potential	Real Samples	Reference
GE/HRP/Os-Ap59/AOX/Cp	After 14 days, 50% of activity lost. After 1,000 measurements, 40% of activity lost.	Up to 2 mM	n.r.	−50 mV (vs Ag/AgCl)	Red and white wines	13
CFE/PNR/AOX	After 3 weeks, 57.6% of activity lost.	Up to 0.7 ± 0.1 mM	29.7 ± 1.5 μM.	−300 mV (vs SCE)	Red and white wines	14
CHIT/AOX-eggshell membrane (Batch)	After 3 months, 13.4% of activity lost. No loss after 20 measurements carried out in 8 h;	0.06–0.8 mM	30 μM	n.r.	Beers, liquors	15
PPy/AOX and CP-co-PPy/AOX	CP-co-PPy matrice: after 20 days 75% of activity lost. PPy matrice after 20 days 80–85% of activity lost.	n.r.	n.r.	n.r.	n.r.	16
CoPC-SPCE, coated with AOX	n.r.	Up to 2 mM	n.r.	−400 mV (vs. Ag/AgCl.)	Beer	11
RVC-epoxy resin-AOX-HRP-ferrocene	n.r.	4 μM–2.5 mM	1.5 μM	0.0 V	Beer	17
Pt/AOX-poly(carbamyl) sulfonate hydrogel (Batch)	After 18 days 30–35% of activity lost. After 12 h 68% of activity lost	0.02–3.75 mM	n.r.	+600 mV (vs Ag/AgCl)	Red and white wines	12
CP/AOX-HRP-osmium (FIA)	10% of sensitivity lost after 270 injections carried out in 9 h	0.25–2 mM	n.r.	n.r	n.r	18
Pt/polyvinylferrocenium matrix-AOX (Batch)	After 36 days 98% of of activity lost (132 measurements)	Up to 3 mM	n.r.	+700 mV (vs SCE)	n.r.	19
Au/PPY_{ox}/AOX-glutaraldehyde-BSA(FIA)	no loss after 90 injections carried out in 3 h; 5% of sensitivity lost 6 h of on-line continuous monitoring	0.01–0.75 mM	2.3 μM	+700 mV (vs Ag/AgCl)	Red wine	20
Pt SPE/AOX- Resydrol Polymer	After 5 days 4% of activity lost	n.r.	3.5×10^{-2} (% v/v) of ethanol	From 0 mV to +600 mV	Red and white wine Process of wine fermentation	21
CP/(graphite-Teflon)/AOX-HRP-ferrocene (FIA)	no loss after 15 days (three measurements a day)	0.2 μM–2.0 mM	0.18 μM	0.00 V	Beer, white wine, red wine, liquor	22

GE, graphite electrode; CFE, carbon film electrode; CHIT, chitosan; CP, carbon paste; SPE, screen printed electrode; AuE, gold electrode; RVC, reticulated vitreous carbon; SPCE, screen-printed carbon electrode; HRP, horseradish peroxidase; CP, (3-methylthienyl methacrylate and pvinylbenzyloxy poly(ethyleneoxide)); CoPC, cobalt phthalocyanine; Ap, anodic electrodeposition paints; Cp, cathodic electrodeposition paints; PNR, poly neutral red; PPy, polypyrrole; n.r., not reported.



Scheme 1. Surface modification and immobilization procedure.

Materials and Methods

Chemicals and reagents

Alcohol oxidase (AOX; Alcohol: O_2 oxidoreductase, EC 1.1.3.13. from *Pichia pastoris* species) with a specific activity of 22 U/mg of protein was purchased from Sigma Chem. Co. (St. Louis, MO), cysteamine hydrochloride, and sodium borohydride were acquired from Fluka (Steinheim, Germany), Poly (amidoamine) (PAMAM- 25% C_{12} Dendrimer Generation 4) and glutaraldehyde solution (% 25, v/v) were obtained from Sigma Aldrich (Dorset, UK). Alcohol (% 96, v/v) was obtained from Riedel-de Haen (Germany). The other chemicals were of analytical grade. Distilled and deionized water (GFL, Germany) was used through this work. Commercial alcohol beverages were purchased from local market. Sodium phosphate buffer (50 mM, pH 7.0) was used as a working buffer solution.

Apparatus

Measurements were carried out by PalmSens Electrochemical Measurement Unit (Palm Instruments, Houten, Netherlands). Three electrodes configuration was employed, consisting of gold working electrode (BASI), Ag/AgCl reference electrode (with 3M KCl saturated with AgCl as the internal solution, Metrohm, Switzerland), and platinum counter electrode (Metrohm, Switzerland).

Flow injection mode of analysis was performed using a single-line flow injection manifold with an electrochemical flow through cell of the wall-jet 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 i.d) propelled the working buffer solution as the carrier into the flow line with a flow rate of 1 mL min^{-1}. The flow line was made of Teflon tubing (0.5 mm i.d). Sample solution (50 μL) containing substrate was injected into the carrier stream via a eight-port injection valve (FIAtron, Oconomovoc, WI). FIA system was connected to Palmsens potentiostat for the electrochemical measurements. FIA system was assisted by software program written in C# which developed at Institute of Technical Chemistry, Leibniz University, Hannover.

Additionally, HPLC with a refractive index detector (RID) controlled by a HP-Chemstation from Agilent (Karlsruhe, Germany) was used as the reference method for independent analysis of the 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 μL. The mobile phase was H_2SO_4 (5 mM).[81] The flow rate was 0.6 mL/min. Initially, standard curve for ethanol was plotted (2.5–50 mM for ethanol). After dilution with mobile phase and centrifugation, samples were applied to the column and then ethanol levels were calculated using calibration plot.

Construction of PAMAM/AOX biosensor

The gold surface was initially polished with alumina powder (Gamma, 0.05 μm). Then, etched in 0.5M H_2SO_4 solution by cyclic-potential scanning between 0 and +1.5 V until a reproducible voltammetric response was obtained. Each preparation step of alcohol biosensor was summarized in a Scheme 1. The cleaned gold electrode was first immersed in 100 mM of aqueous cysteamine solution for 30 min. To remove the excess cysteamine, the surface was fully rinsed with distilled water. After that it was placed into glutaraldehyde solution (5%) in 0.05M sodium phosphate buffer (pH 7.0) for 30 min and rinsed with distilled water. So that, crosslinkages were formed in between amine groups of PAMAM and cysteamine on the surface. Then, the electrode was transferred into G4 PAMAM dendrimer (1%) dissolved in 50 mM sodium phosphate buffer (pH 7.0) for 1 h. The Schiff bases formation was reduced by treatment with $NaBH_4$ solution (5 mM) for 30 min.[82] Finally, 5 μL AOX (6.25 U) and 10 μL of glutaraldehyde solution (1% in pH 7.0, 50 mM sodium phosphate buffer) were dropped onto the electrode surface, respectively. Then, the electrode was allowed to dry at ambient conditions. The obtained biosensor was stored in contact with the working buffer at 4°C when not in use. Daily prepared electrodes were used during the experiments.

Surface characterization

The morphology of cysteamine/PAMAM and cysteamine/PAMAM/AOX-modified gold surfaces were imaged by Atomic Force Microscopy (AFM, NanoMagnetics Istruments, UK). Imaging was carried out at ambient temperature in noncontact mode.

Measurements

The response of the ethanol biosensor shows the decrease of dissolved O_2 content upon exposure to ethanol solution.

The decrease of dissolved O_2 concentration could be determined as the analytical signal of the biosensor.[65] Related reaction diagrams are given below,

$$\text{Cathodic reactions : } O_2 + 2H_2O \rightarrow 2H_2O_2 \quad (2)$$

$$2H_2O_2 + 4_e{}^- \rightarrow 4OH^- \quad (3)$$

$$\text{Anodic reaction : } 4^-_{Ag} \rightarrow 4Ag^+ + 4e^- \qquad 4Ag^+ + 4Cl^- \rightarrow 4AgCl \quad (4)$$

$$\text{Total reaction : } 4Ag + 4Cl^- + O_2 + 2H_2O \rightarrow 4AgCl + 4OH^- \quad (5)$$

Proposed system is based on the chronoamperometric monitoring of the current that occurs due to the oxidation of the ethanol by enzymatic reaction of AOX. All measurements were carried out at 25°C under continuous and constant magnetic stirring. The three electrodes were immersed into the electrochemical cell that contains 10 mL working buffer. After each run, the electrodes were washed with distilled water. When the current background was stable, ethanol solutions were added to the electrochemical cell and the steady-state current values recorded as amount of current (ΔI, μA) which were followed by a potentiostat at −0.7 V. The steady-state current was typically achieved in less than 100 s. The obtained current signals were plotted vs. various standard ethanol concentrations. This calibration curve was used to calculate the ethanol amount in real samples. Cyclic voltammetry was carried out in working buffer including $[Fe(CN)_6]^{4-/3-}$ (5 mM).

Sample applications

Proposed alcohol biosensor was applied to both commercial beverages and fermentation medium. Samples were injected into the reaction medium instead of substrate solution. Commercial samples were diluted with working buffer to adjust the ethanol content of the sample to be in the linear range, but fermentation broth was injected to the reaction medium with no pretreatment.

Biological Material for Fermentation Experiments. The distillery strain of *Saccharomyces cerevisiae* H620 was subcultured in agar medium containing 4 g L^{-1} glucose, 4 g L^{-1} yeast extract, and 10 g L^{-1} malt extract. Yeast cells were inoculated in liquid-modified Schatzman medium of the following composition (in g L^{-1}): Glucose 30 g L^{-1}, sodium citrate 1.25 g L^{-1}, 1 mL of vitamin solution (Myo-Inosit(ol); 6.0×10^{-2} g L^{-1}, calcium-D-pantothenate; 3.0×10^{-2} g L^{-1}, thiaminhydrochloride (vit B_1); 0.6×10^{-2} g L^{-1}, pyridoxolhydrochloride (vit B_6); 0.15×10^{-2} g L^{-1}, biotin; 0.3×10^{-4} g L^{-1} and 1 mL of antibiotic solution (Ampicilin; 0.5×10^{-4} g L^{-1}, tetracycline; 0.2×10^{-4} g L^{-1}, chloramphenicol; 0.3×10^{-4} g L^{-1}). After precultivation, 12.5 mL of fermentation broth was transferred into 62.5 mL of fresh liquid Schatzman medium containing 2.5 g glucose dissolved in 25 mL distilled water, 75 μL vitamin, and 75 μL antibiotic solution.[83] Cultures were incubated in an orbital shaker (New Brunswick Scientific) at 30°C and 200 rpm for 16 h. Batch ethanol fermentation process was monitored and samples were collected from fermentation broth during 10 h.

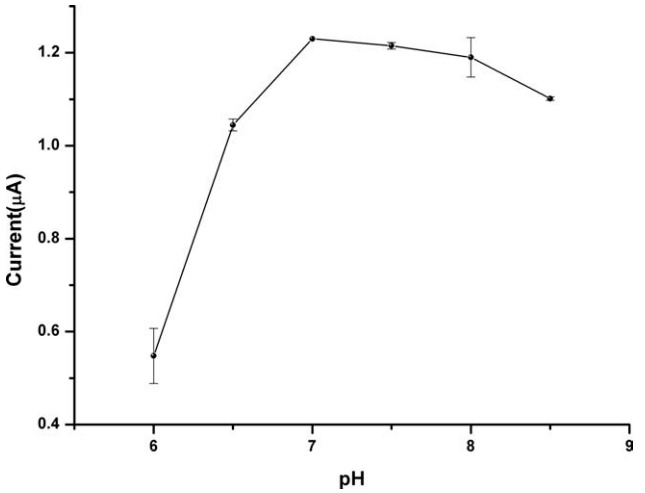


Figure 1. Effect of pH (sodium citrate buffer at pH 6.0; 6.5, sodium phosphate buffer at pH 7.0; 7.5; 8.0 and Tris-HCl buffer at pH 8.5, 25°C, −0.7 V, [EtOH]; 0.5 mM).

The cell density was determined by offline counting with Neubauer chamber.

Commercial Samples. Alcohol content in alcoholic beverages (gin, whisky, tequila, raki, and vodka) was measured by ethanol biosensor and results were compared with those given by the company.

Results and Discussion

From the immobilization point of view, a first rapid protein adsorption by ionic exchange, followed by the covalent reaction occurs when glutaraldehyde is used for the immobilization of AOX. The enzyme is immobilized to the previously activated dendrimer via this mechanism. In this form, the glutaraldehyde molecule bound to the both ε-amino groups of lysines and primary amino groups of the enzyme could covalently react with the glutaraldehyde molecule bound to the primary amino groups of PAMAM dendrimer establishing a multipoint covalent enzyme-dendrimer attachment.

This technique suggests that the possibility of achieving an intense multipoint covalent attachment (that is a strong enzyme-dendrimer interaction) are quite high using a mild modification of amino groups in dendrimer and the protein with glutaraldehyde.[84]

Optimization studies

The effect of pH on the biosensor response was investigated in the range pH 6.0–8.5 in stirred solutions at −0.7 V. The biosensor subjected to 0.5 mM ethanol substrate between pH 6.0–8.5 by using sodium citrate, sodium phosphate, and Tris-HCl buffers. AOX from *P. Pastoris* has a pH optimum in the range of pH 7.0–8.0. The sensor with that enzyme showed maximum response in the range of pH 7.0–7.5 (Figure 1). Other pH values resulted in the loss of the enzyme activity. It is logical that the enzyme sensor is more resistant to alkaline pH values. Because of the local proton formation in acidic values, the enzyme could be denatured.[22] Consequently, the optimum pH was chosen at pH 7.0 for further studies.

Moreover, performance of the biosensor strongly depends on the amount of immobilized enzyme because this would affect biosensor sensitivity directly. To examine this effect,

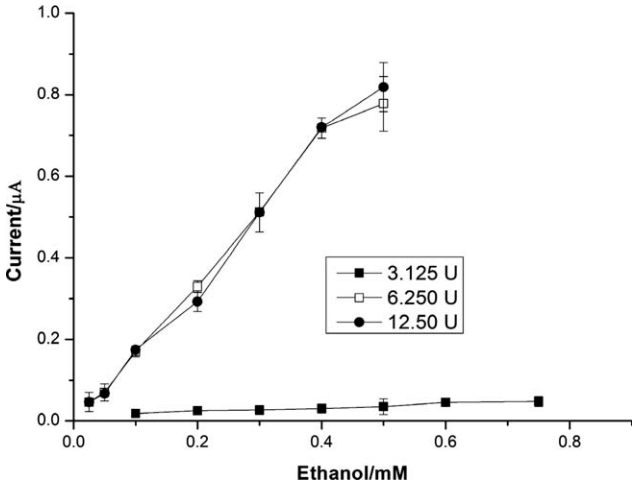


Figure 2. Effect of enzyme amount on the biosensor response (in sodium phosphate buffer, 50 mM, pH 7.0; 25°C, −0.7 V).

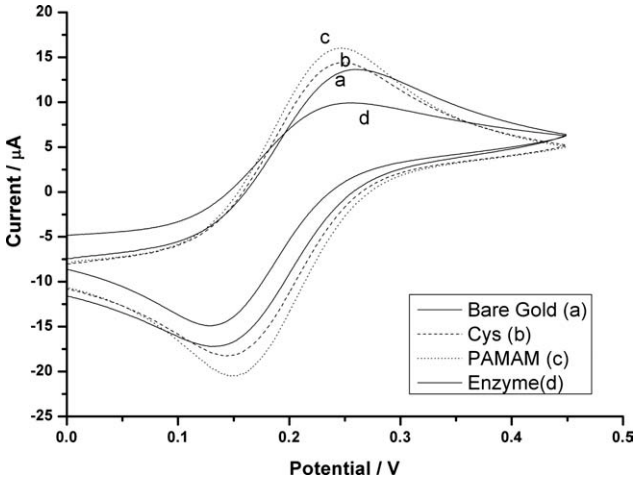


Figure 3. Cyclic voltammograms of bare gold electrode (a), cysteamine (b), cysteamine/PAMAM (c) and cysteamine/PAMAM/AOX (d) in 50 mM sodium phosphate buffer, pH:7.0 with the presence of 5 mM $[Fe(CN)_6]^{4-/3}$ at 100 mV/s

three different biosensors prepared with 3.125 U, 6.25 U, and 12.5 U of AOX drop-dried on the surface. Figure 2 shows that lower enzyme amounts (3.125 U) lead to lower responses due to the insufficient enzyme activity. When enzyme amounts were increased (6.25 U and 12.5 U), higher signal responses were obtained. Moreover, if 12.5 U of enzyme solution were used, biocomponent surface becomes thicker and this causes diffusion problem. This could be the reason to obtain similar signals. It is also possible that the increase in enzyme amount may cause the saturation of the surface because the dendrimer amount is always stable, its capacity of enzyme loading may be overloaded. As a result, optimum enzyme amount (6.25 U) which has nearly the same biosensor sensitivity as 12.5 U enzyme was chosen for further experimental steps.

Characterization

Initially, the stepwise biosensor construction process was followed by cyclic voltammetry using ferricyanide as redox mediator. Figure 3 shows CV responses in working buffer

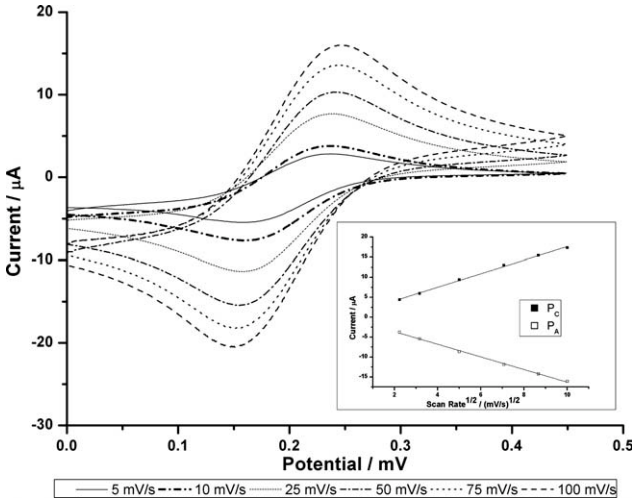


Figure 4. Cyclic voltammograms of cysteamine/PAMAM modified electrode in 50 mM pH 7.0 sodium phosphate buffer with the presence of 5 mM $[Fe(CN)_6]^{4-/3}$ at 5, 10, 25, 50, 75, 100 mV/s. Inset: Plots of anodic and cathodic peak currents vs. scanrate$^{1/2}$

solution containing 5 mM $[Fe(CN)_6]^{4-/3-}$ at a scan rate of 100 mV/s. Well-defined cyclic voltammogram was observed at bare Au surface, self assembly of cysteamine monolayer and PAMAM modified electrodes. Higher peak current values obtained in each step as 13.638 μA ($\Delta E_{Pc} = 0.258$ V; Figure 3a), 14.411 μA ($\Delta E_{Pc} = 0.246$ V; Figure 3b), 16.023 μA ($\Delta E_{Pc} = 0.247$ V; Figure 3c), respectively. This is an expected result and because of the presence of increased number of functional amino groups as a result of step by step modification with cysteamine and PAMAM that attracted the negatively charged $[Fe(CN)_6]^{4-/3-}$ to the electrode surface. However, when biomolecule was added into the surface structure diminished electron transfer properties were observed and this resulted in lower peak current 9.914 μA ($\Delta E_{Pc} = 0.256$ V) due to the possible diffusion layer which can be seen in Figure 3d.

Additionally, Figure 4 shows cyclic voltammograms of cysteamine/PAMAM-modified Au electrode at different scan rates. Both anodic and cathodic peak currents linearly proportional to the square root of scan rate, in the range from 5 to 100 mV/s (Figure 4 (inset), linear regression equations: P_a; $y = 1.688x + 0.762$, $R^2 = 0.998$ and $P_c = -1.587x - 0.443$, $R^2 = 0.998$) which indicates diffusion-controlled electrode process.

Apart from cyclic voltammetry experiments, chronoamperometry was used for analytical characterization of the biosensing system. The enzyme electrode rapidly responses to the ethanol concentration changes and reaches a steady state after less than 100 s. Figure 5 depicts a strictly linear calibration curve obtained from 0.025 to 1.0 mM ethanol in 50 mM potassium phosphate buffer at pH 7.0. Deviation from the linearity for the high ethanol concentration might be due to the AOX saturation by ethanol as well as to oxygen limitation in microenvironment. The results indicate that PAMAM/AOX-modified gold electrode is an advantageous detector for ethanol because a wide linear range is achieved. Moreover, LOD was calculated as 0.016 mM according to $S/N = 3$.

On the other hand, a calibration graph was also plotted by measuring the produced peroxide due to the enzymatic

reaction at +0.7 V at the same working conditions and quite lower response signals were obtained in the same linear ranges of ethanol. In this case, Schatzman medium, which was used as the culture medium for yeast cultivation, caused interferences, which was not observed at −0.7 V so that to avoid this effect in case of bioprocess monitoring experiments, −0.7 V was selected as the working potential and conducted for further experimental steps.

To examine the repeatability of the biosensor responses, signals corresponding to 0.5 mM ethanol standard solutions were measured for eight times. The standard deviation (S.D) and coefficient of variation (c.v) were calculated as ±0.017 mM and 3.3%, respectively. The signals were recorded under the same experimental conditions. According to the data, it can be said that this PAMAM/AOX electrode presented a good repeatability in our experimental conditions.

As for reproducibility, three biosensors were prepared under the same experimental conditions on different days and calibrated for ethanol. For each biosensing system following equations defining the linear graphs were obtained in the range of 0.025 − 1.0 mM ethanol; $y_1 = 1.249x + 0.076$ ($R^2 = 0.978$), $y_2 = 1.435x + 0.024$ ($R^2 = 0.998$), and $y_3 = 1.566x + 0.017$ ($R^2 = 0.995$). Additionally, electrode to electrode reproducibility was calculated with those three independent biosensors by measuring of the current responses to 0.2 and 0.5 mM ethanol at the optimized working conditions, and 0.8% and 4.9% relative standard derivations (RSD) were obtained. Similarity in the slope of equations and RSD values of points calculated indicates good reproducibility for the proposed AOX biosensor.

AOX biosensor was also used in flow injection analysis (FIA) mode and linearity was achieved with $y = 0.270x + 0.118$ ($R^2 = 0.997$), where y is the sensor response in current (μA) and the x is the substrate concentration in mM. When the biosensors were used in the flow injection mode due to the restricted contact time of the substrate with the bioactive layer and the dilution of the sample concentration in the flow system before reaching the electrode surface, lower responses were observed in compare to ones obtained via batch measurements. The repeatability of the system was also evaluated for 0.7 mM ethanol concentration by 10 subsequent injections and SD and CV% values were calculated as 0.68 ± 0.03 and 5.65%, respectively.

Additionally, to monitor the nature of the resulting PAMAM (Figure 6a) and enzyme (Figure 6b) layers obtained onto the gold surface, AFM which provides morphological information was used in noncontact mode. According to surface area coefficient (sa) from the histograms roughness, averages are obtained as 11.715 nm (PAMAM) and 4.810 nm (PAMAM/AOX), respectively. It is clear that after combining biomolecule with dendrimer, more homogeneous structure formed on the surface as it can be seen from roughness averages.

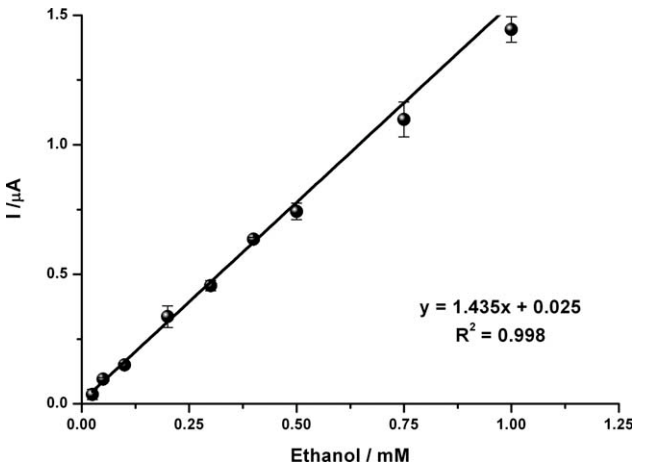


Figure 5. Calibration curve for ethanol (in 50 mM sodium phosphate buffer, pH 7.0; 25°C; −0.7 V).

Substrate specificity

The enzyme electrode measurements were carried out for methanol, ethanol, *n*-butanol, and 2-propanol. A comparison of the relative biosensor responses for different short chain aliphatic alcohols is given in Figure 7. The amperometric response decreased as the number of carbon atoms in the aliphatic chain increased, which is attributed to the stronger steric impediment for the alcohols with longer chains that hinders the access of the substrate to the active center of the enzyme.[85] Moreover, a decrease in the current is also observed when the ramification of the aliphatic chain is higher. An increase on the length of the aliphatic alcohol chain results in a stronger impediment to reach the enzyme active center. The big difference in the response obtained for

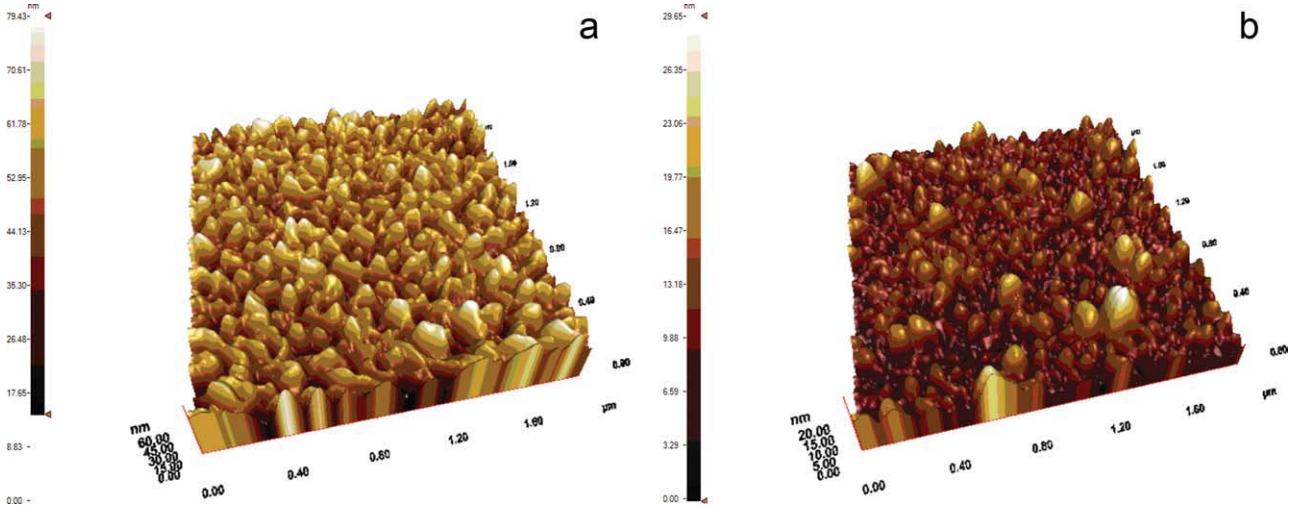


Figure 6. AFM imaging of Cysteamine/PAMAM and Cysteamine/PAMAM/AOX modified gold surface in noncontact mode.

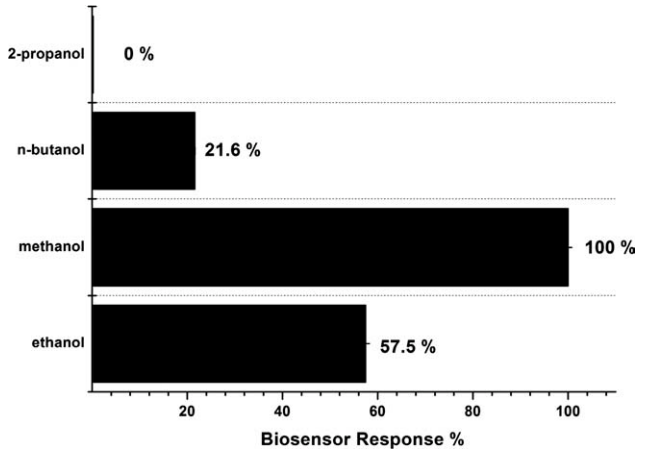


Figure 7. Comparison of the biosensor response for the different aliphatic alcohols (in phosphate buffer 50 mM, pH 7.0; 25°C; −0.7 V, [Alcohol]: 0.5 mM).

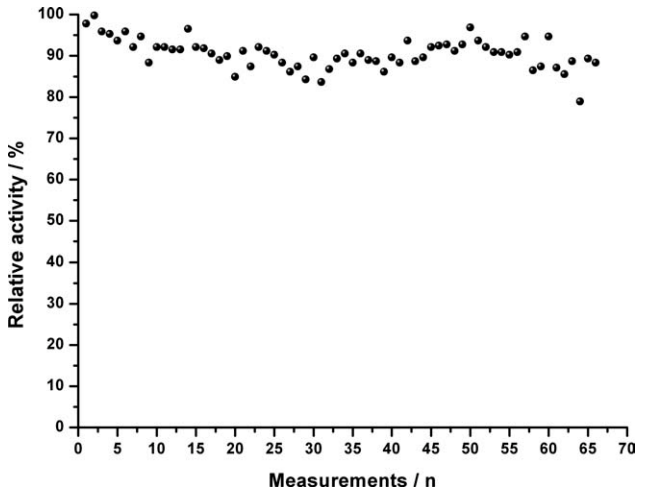


Figure 8. Relative activity (%) vs. consecutively injections of 0.7 mM ethanol in 50 mM sodium phosphate buffer, pH 7.0 during 210 min.

methanol with respect to the rest of alcohols is due to the fact that the product of the methanol oxidation is formaldehyde, which acts also as a substrate of AOX, thus producing an amplification of the analytical signal.[12] It is not given in the figure that the biosensor did not give any signal to 2-propanol because the substrate is a secondary alcohol. As mentioned earlier, the enzyme oxidizes only primary alcohols.

Operational stability and shelf life

Operational stability of the cysteamine/PAMAM-modified ethanol biosensor was investigated for 0.5 mM ethanol at optimal working conditions (25°C and pH 7.0, 50 mM sodium phosphate buffer). After each measurement, biosensor was washed by distilled water and kept in pH 7.0 phosphate buffer until the next measurement. Signal responses were measured each hour during 8 h and 2% activity loss has observed. Similar experiment was carried out in FIA system for 0.7 mM ethanol and biosensor tested with 66 injections for 210 min. Activity loss is calculated as 11.64% after this period. (Figure 8)

The shelf life of any biosensor is a very important parameter for long-term applications. To determine storage stability of the PAMAM/AOX ethanol biosensor, measurements were performed each 2 days for a month by using 0.5 mM ethanol. Biosensor was stored at +4°C in pH 7.0 phosphate buffer when it was not in use. Figure 9 shows that after a month, the response activity decreases by 32% of the original value. In spite of immobilized AOX has limited storage stability, the PAMAM/AOX has the longer storage stability when compared with the literature.[13,14]

Table 2. Results for Alcohol Analysis in Real Samples by Enzyme Electrode

Beverages	Ethanol Amount Labeled with the Manufacturer (% v/v)	PAMAM/AOX (% v/v)*	Recovery (%)
Gin (Brand 1)	40	38.5 ± 0.905	96.2
Gin (Brand 2)	47	48.9 ± 0.17	104
Vodka	40	41.1 ± 2.69	102.7
Whiskey	43	42.4 ± 0.28	98.6
Raki	45	42.4 ± 0.36	94.2
Tequila	38	36.3 ± 1.76	95.5

* Results were given as average ± standard deviation ($n = 3$ or 4).

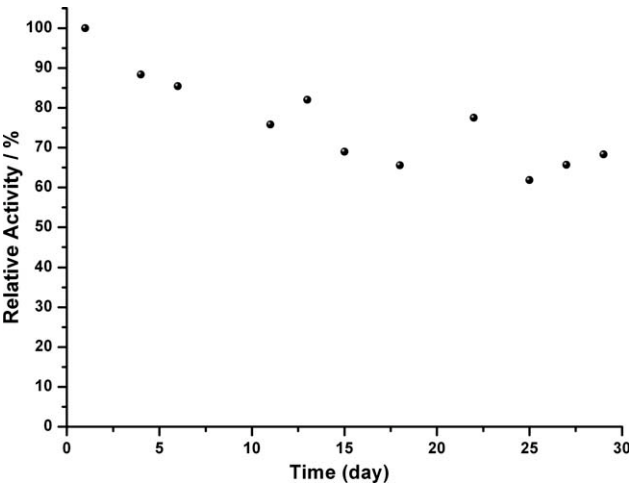


Figure 9. Storage stability of PAMAM/AOX biosensor (in sodium phosphate buffer, 50 mM, pH 7.0; 25°C; −0.7 V).

Sample analysis

The PAMAM/AOX biosensing system allowed its application for the direct ethanol analysis in real samples. For this purpose, some commercial alcoholic beverages and yeast culture media were examined.

Analysis of Alcoholic Beverages. The performance of the biosensor was tested by using vodka, gin, whiskey, tequila, and raki. Each sample was diluted with working buffer solution and used instead of substrate solution. Measurements were repeated twice and obtained values were compared with producer companies' alcohol values. Table 2 shows the results belonging to the sample application.

In Table 2, the recovery data are demonstrated. It is shown that the values found via the biosensing system are closer to 100%, which means the alcohol biosensor offers a good, precise, and accurate method without any matrix effect for the alcohol analysis in real samples.

Fermentation Experiments. It is well known that *Saccharomyces cerevisiae* is extensively used in fermentations to convert sugars to ethanol. So, the optimized biosensor was applied for off-line monitoring of continuous ethanol

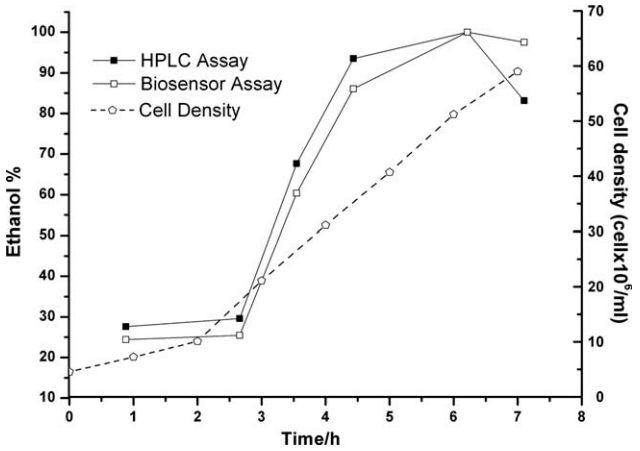


Figure 10. Time depending alcohol concentration in fermentation medium (in sodium phosphate buffer, 50 mM, pH 7.0; 25°C; −0.7 V).

fermentation. Taking into account that the concentration of ethanol in fermentation media is far outside the working range of the biosensor, a 100-fold dilution of the sample is required to adjust the sample concentration to the linear range of the PAMAM/AOX biosensor. Obtained results represent time characterization of ethanol production (Figure 10). Ethanol production is growth associated; as we expected the amount of ethanol produced by yeast during anaerobic fermentation was increased equivalently to substrate consumption. Additionally, ethanol was assayed by HPLC as a reference method. Obtained results were very good agreement with the biosensor measurements. Consequently, we can claim that the possibility of applying the developed biosensing system to real samples analysis is really high.

As illustrated in Figure 10, the slope of the measured cell density was exponential for the first 9 h and became stationary after 10 h. During exponential growth period, ethanol had accumulated, after 6 h, concentration of ethanol did not change. High concentration of ethanol is inhibitory for cell growth, so cells are most active at the earliest times of fermentation. The fermentative activity decreased after the accumulation of ethanol.

Conclusions

In this work, the use of PAMAM/AOX biosensor for alcohol analysis is described in both batch and FIA system. As well as optimization and characterization studies, the system has been used to monitor alcoholic beverages and fermentation media for off-line detection. It has been reported that after providing appropriate sampling and samples, biosensors can be adapted to the bioprocess monitoring.[86] In our case, the proposed biosensor could be a useful alternative as a device with high stability as well as sensitivity to be integrated as an online process monitoring sensor via flow injection analysis system (FIA).

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