

Improvement of the Storage Stability for Biosensors Based on Alcohol Oxidase Immobilized by Entrapment in a Photopolymerisable Matrix

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One important impediment for successful commercial application of enzymatic biosensors is their limited storage stability caused to the instability of the used biocomponent. The storage stability of alcohol oxidase (AOX) immobilized onto screen-printed electrodes by entrapment was improved by the use of three different stabilizers (sorbitol, Tween 20 and PEG 6000) in comparison with the enzyme immobilized without stabilizers. The most appropriate stabilizer proved to be PEG 6000 that allowed the increase of the storage time of the biosensors by more than 3 times.

Keywords: alcohol oxidase (AOX) biosensor, screen-printed electrodes, PVA-SbQ photopolymer

The enzymatic biosensors are widely used in clinical analysis [1], food control [2], environmental monitoring [3] etc. Besides satisfactory analytical figures of merit for a successfully commercial application, the storage stability of the biosensors is essential as biosensors may often be stored for weeks or months prior to use. The storage stability is given by the stability of the immobilized enzymes and is an issue often neglected in the papers reporting new biosensors development.

Protein denaturation is a complex phenomenon caused by external stress that induces the alteration of its shape in such a way that it will no longer be able to carry out its cellular function. There are various ways of protein denaturation e.g. changes in the quaternary structure like protein sub-units dissociation; tertiary structure denaturation involves the disruption of covalent or dipole-dipole interactions or even in the secondary structure [4].

The storage stability of the enzymes is dependent on the working conditions: dry protein extract, solubilized in aqueous solutions or immobilized on supports. The modern biosensors use immobilized enzymes. There are numerous immobilization protocols e.g. adsorption, entrapment, (multi-point) attachment, reticulation [5] each one with its advantages and shortcomings. Usually, the immobilized enzymes are more stable than the soluble enzyme, but the immobilization is accompanied by a loss of activity [6]. The encapsulation of enzymes in microenvironments and especially in liposomes, has proven to greatly improve enzyme stabilization against unfolding, denaturation and dilution effects [7].

The usually long storage conditions of biosensors or proteins are dry at low temperature. Unfortunately, it is not always possible to keep the biosensors at low temperature, e.g. the *in situ* environmental monitoring and more the drying itself leads to dehydration stress of proteins [8]. Unfolding of proteins can be prevented by using additives that remain in the amorphous phase with the protein and hydrogen bond to the protein in the place of water during drying [8]. The stability of the enzyme extracts or preparations was intensively studied and in the literature are reported numerous stabilizers like: lactitol [9],

poligalacturonic acid, sucrose [10], carbohydrates [11] or even products used to prevent the microorganism development like sodium azide [12].

This paper investigates the possibilities to improve the storage stability of an alcohol oxidase (AOX) biosensor obtained by the entrapment of the enzyme in photopolymerisable PVA on the surface of the working electrode. In literature are presented viable AOX based biosensors coupled with different transduction methods (spectrometric, electrochemical, chemiluminescence, etc.), but this enzyme is known to be relatively unstable [13]. As stabilizers there were tested: a carbohydrate-sorbitol [14], a long chain -OH graphed polymer polyethylene glycols-PEG [15] and a non-ionic surfactant-Tween [16] (fig. 1).

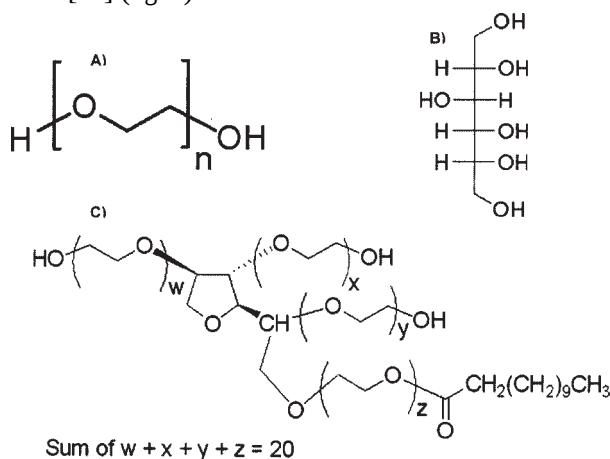


Fig. 1. The structure of the investigated stabilizers: A) PEG, B) Sorbitol and C) Tween 20

Materials and methods

Chemicals

Alcohol oxidase (AOX) 1600 IU/mL solution from *Pichia pastoris* (Sigma-Aldrich) was immobilized by entrapment in a photocrosslinkable polyvinyl alcohol containing stilbazolium groups (PVA-SbQ), type SPP-S-13 (bio) (polymerization degree 1700) provided by Toyo Gosei

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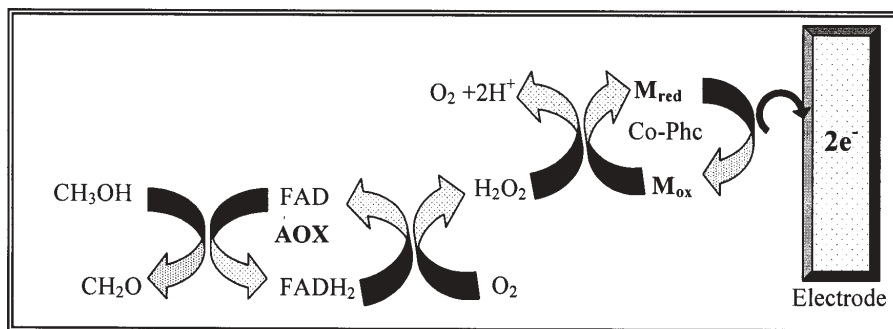


Fig. 2. The electrochemical detection principle of an AOX biosensors based on the mediated H_2O_2 quantification. Co-Phc: Cobalt phtalocyanine

Kogyo Co (Japan). The stabilizers investigated were polyethylene glycol –PEG 6000 (Fluka), Tween 20 (SigmaUltra) and D(-) sorbitol (Merck). Enzymatic substrates were methanol ($\geq 99.9\%$) and ethanol (99%) (Sigma-Aldrich). The supporting electrolyte was a 0.1 M phosphate buffer solution pH=8.0, supplemented with 0.1 M KCl for the proper functioning of the screen-printed pseudoreference electrode.

Apparatus

The screen-printed electrodes were produced in 24 sets of working/auxiliary/reference electrodes per sheet using a DEK 248 printing machine at BIOMEM-University of Perpignan (France) according to a procedure previously described [17]. Finally on the working electrode was deposited a final layer with an ink C2030408D3 (Gwent Electronic Materials, UK) that contains an electrochemical mediator: Co-phtalocyanine. A potentiostat PGZ100 All-in-one (Radiometer) controlled by a PC with Voltalab v4 software was used for chronoamperometric measurements.

Immobilization procedure

The enzymes were immobilized directly on the working electrode (WE) surface by entrapment in PVA-SbQ using a slightly modified previously reported procedure [18]. There were manufactured biosensors with stabilizers and compared with blank biosensors produced in the same manner, but without stabilizers. For AOX blank biosensors (without stabilizers): 20 μL of AOX stock solution diluted with 20 μL distilled water were mixed with 40 μL of PVA-SbQ. For AOX biosensors with stabilizers instead of 20 μL distilled water there were used: 20 mL solution of 50 mg/mL PEG 6000, 20 μL solution of 1M D(-) sorbitol and respectively 20 μL solution 10 % (v/v) Tween 20. Then, the enzyme-stabilizers-PVA-SbQ mixture was homogenised by mixing with a vortex. 2 μL of this solution were carefully spread on the WE surface. The SPE were exposed to neon light for 4 h at 4°C to allow the entrapment of the enzymes by photopolymerization. The electrodes were then stored in sealed plastic bags at -20°C .

Chronoamperometric measurement

The chronoamperometric measurements were performed using the SPE kept horizontally. All three electrodes were covered with 90 mL phosphate buffer solution. A constant potential +600 mV vs. the screen-printed Ag/AgCl pseudoreference electrode was applied. After current stabilization (baseline), 10 mL of sample was injected and the intensity of the current was recorded until stabilization on a new plateau. The difference of the current intensity was measured between the baseline and the plateau. The time necessary to obtain the stabilization of the base line was 2-3 min and to reach the plateau was 4-

6 min. The SPE was washed with distilled water between measurements.

Results and discussions

Chronoamperometric measurement principle

The AOX is an oxidase and thus, theoretically there are three measurement possibilities: (i) the use of the disappearance of the dissolved O_2 into the analysis medium or the H_2O_2 produced during the alcohol oxidation; (ii) the use of electrochemical mediators to avoid the use of O_2 and (iii) the direct transfer of electrons between the electrode and enzyme. The AOX was classified as a “true oxidase”, which means that the enzyme is able to catalyse only in the presence of O_2 and it is not able to accept any other oxidant. This odd behaviour for an oxidase suggests that the catalytic mechanism of AOX involves a ternary intermediate, where both the substrate (alcohol) and O_2 are bound to the same active site of the enzyme [19]. This is in contrast with other oxidases, such as glucose oxidase, whose reaction mechanism occurs via a “ping-pong mechanism” that implies the sequential binding of the substrate and oxidant in two distinct active sites of the enzyme and the subsequent shuttle of the electrodes. The direct transfer of electrons is an interesting option, but unfortunately it is possible only for a reduced number of enzymes that have the active site at the exterior of the protein. Thus for analytical signal quantification remains only the possibility to use $\text{O}_2/\text{H}_2\text{O}_2$ system. The quantification of O_2 was used from the first biosensors developed [20], but has some drawbacks: low response and the oxygen dependence that reduces the accuracy and reproducibility [21]. The mediated detection of H_2O_2 is the alternative used in this study (fig 2).

Biosensors characterization

The prepared AOX biosensors presented a satisfactory operational stability; at least 10 successive measurements are possible with a single biosensor. Each time a new lot of biosensors was produced it was verified the operational stability to confirm a good immobilization of the enzyme on the surface of the WE.

The AOX biosensors respond to a large variety of aliphatic alcohols that may contain different moieties (amino, halogen, mercapto, cyano, etc.). For different substrates at the same concentration, the magnitude of the analytical signal decreased with the increase of the aliphatic chain or with the presence of different moieties [22]. AOX based biosensors are generally used to develop ethanol analyzers [13], but the magnitude of the analytical signals is bigger for the methanol. The difference between the responses of the manufactured AOX biosensors to the methanol and respectively ethanol is suggestively shown by the LODs for both substrates: 1 mM for methanol and only 10 mM ethanol. The fundamental study reported in

this paper was devoted to the investigation of the enzyme stability and kinetic properties of the AOX enzyme and thus methanol as substrate was chosen.

The calibration graph determined by successive methanol injections for the AOX blank biosensors was linear from 3 to 70 mM methanol ($I(nA) = 1.66 \cdot \text{Conc}_{\text{methanol}}(\text{mM}) + 18.884$; $n=5$; $R^2=0.9927$). The reproducibility of the analysis for a single AOX blank biosensor was determined for a solution of 50 mM methanol and was determined to be 99 ± 6.8 nA ($n=10$, $RSD=6.8\%$). The reproducibility between the responses of different AOX biosensors produced in the same lot measured for the analysis of 50 mM methanol was $RSD = 16.2\%$ (105 ± 17 nA; $n=10$).

Stabilizers effect on the biosensors

The use of different stabilizers did not change the response time or the shape of the analytical signal of the biosensors. More, the measurement reproducibility for the single biosensor and between different biosensors remains at the same order of magnitude with the one determined for the AOX blank biosensors at the section 3.2.

For the biosensors that contained Tween 20 it was observed an important increase of the base line. This base line has a negative effect on the sensibility of the measurements and has the tendency to decrease from one measurement to another, but despite washing the biosensor thoroughly with distilled water and phosphate buffer it was not possible to obtain the same base line as the one of the blank biosensors. Thus, the baseline values during the first 5 measurements with the same biosensor were: (i) 2.14 μA ; (ii) 1.23 μA ; (iii) 0.94 μA ; (iv) 0.78 μA and respectively (v) 0.64 μA . For comparison, the baseline for a biosensor without stabilizers was 0.2 μA , for a biosensor with PEG 6000 the baseline was 0.27 μA and for biosensors with sorbitol the baseline value is 0.14 μA .

The use of PEG 6000 for the manufacturing of the biosensors had a secondary advantage that facilitates the homogenous mixing of the AOX solution with the PVA-SbQ photopolymer. The PVA-SbQ is homogeneously mixed with AOX dissolved in buffer by vortex, a process that for the blank biosensors took up to 4 min and produced some air bubbles in the mixture that are difficult to remove. To produce biosensors that have a good operational stability, it is essential to use a homogenous enzyme with PVA-SbQ mixture that has no air bubbles. The presence of PEG 600 into the AOX solution reduced these mixing difficulties encountered during the biosensors manufacturing, the enzymatic solution being easily homogenized with PVA-SbQ by vortex mixing in less than 1 minute.

There were observed no particularities for the biosensor manufactured using an AOX solution that contains sorbitol in comparison with the blank biosensors.

Stabilizers effectiveness

The storage stability was studied by periodically measuring the response of different biosensors to methanol injection. It was chosen a methanol concentration of 70 mM as the highest point on the calibration graph in order to have a maximum analytical signal and to correlate any decrease of the current intensity with enzyme denaturation. For each stability measurement of the biosensors, there were tested three different electrodes. The response of each tested biosensor was measured five times.

There were investigated three different stabilizers: one with a low molecular weight (sorbitol), one with medium molecular weight (Tween 20) and the other one with high molecular weight (PEG 600). Depending on their volume, the stabilizers may leak into the measuring solution or be

washed away by diffusing through the pores of the PVA-SbQ structure that entraps the enzyme. The biosensors were discarded after measurements and the subsequent determinations are performed with biosensors produced in the same lot. This has the disadvantage that the storage stability is not measured with the same biosensor and thus the errors are relative higher, but it was done in order to avoid the stabilizer loss during the measurements that will reduce their efficiency.

It was noticed that the activity of the blank biosensors rapidly decreased, after three weeks the biosensors losing their entire activity. The use of the stabilizers dramatically improves the storage stability. The most effective stabilizer proved to be PEG600 followed by sorbitol and respectively Tween 20. The biosensors with Tween 20 lost their activity after one month while the biosensors stabilized by sorbitol lost their activity after 6 weeks. The PEG 600 succeeds to improve the storage stability of the biosensors, the complete loss of activity occurring only after 10 weeks (fig. 3). This represents a substantial improvement of the storage stability of the biosensors based on AOX.

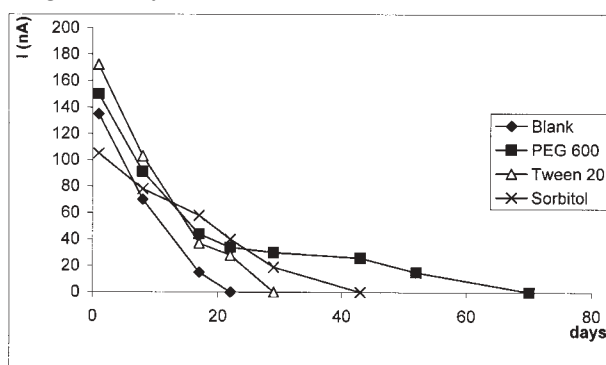


Fig. 3. Stability of biosensors based on immobilized AOX in PVA-SbQ with different stabilizers. AOX was immobilised on the screen-printed electrodes in the presence of various stabilizers, dried at room temperature and stored at 20 °C in sealed plastic bags. Activity was measured as described in Section 2.4 by injecting 70 mM methanol final concentration. Data points are mean $\pm$ S.E. ($n = 3$ biosensors each one tested 5 times).

Conclusions

Three different stabilizers were successfully used to improve the storage stability of AOX immobilized on electrochemical surface. All stabilizers have a positive effect on the enzyme stability. The increase of storage time in comparison with biosensors without stabilizers was by 25% for Tween 20 while the sorbitol allow a double storage time of the biosensors. The PEG 6000 permitted to increase more than 3 times the storage time of the biosensors. The PEG 6000 had a secondary advantage that it make easier the mixture of the PVA-SbQ with the enzyme solution and thus the biosensor manufacturing process is substantially facilitate. More PEG 6000 does not have a negative affect on baseline as it was noticed for Tween 20 and due to its overall performances was considered to be the most appropriate in the tested system.

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