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Sol-Gel Immobilization of Aldehyde Dehydrogenase and NAD^+ on Screen-Printed Electrodes for Designing of Amperometric Acetaldehyde Biosensor*

by Th. Noguer[1], D. Szydlowska[2], J.-L. Marty[1] and M. Trojanowicz[2]**

[1]*Université de Perpignan, Centre de Phytopharmacie, UMR CNRS 5054, 52, Avenue de Villeneuve, 66860 Perpignan Cedex, France*
[2]*University of Warsaw, Department of Chemistry, Pasteura 1, 02-093 Warsaw, Poland*

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The stability of response of amperometric biosensors based on entrapment of aldehyde dehydrogenase (AlDH) and nicotinamide adenine dinucleotide (NAD^+) in a sol-gel matrix on the surface of screen-printed electrodes modified with the mediator Meldola Blue-Reinecke salt was studied. The operational stability of biosensors was tested using acetaldehyde as substrate by applying a potential of –150 mV *vs.* pseudo Ag/AgCl screen-printed electrodes. The type and ratio of silicium alkoxides precursors, the amounts of AlDH and NAD^+, as well as the influence of drying and storage temperature on the stability of electrodes were examined. Among the tested biosensors, the most stable devices were those incorporating a sol-gel layer containing both AlDH and NAD^+, which was covered by an additional sol-gel layer in order to avoid the co-enzyme leaching.

Key words: acetaldehyde, aldehyde dehydrogenase, NAD^+, sol-gel entrapment, screen-printed electrode, Meldola Blue

Effective immobilization of enzymes onto screen-printed electrodes surface is one of the key features for the successful operation of electrochemical biosensors. The objectives of this important step is to provide a simple mean for protein attaching, allowing to retain its affinity, catalytic properties and stability over long periods, while providing accessibility towards the target analytes and an intimate contact with the electrode surface.

Dehydrogenases represent the largest group of redox enzymes and are thus of considerable interest for the development of amperometric biosensors, especially in the field of agrifood analysis. Aldehyde dehydrogenase (AlDH, EC 1.2.1.5) has been already used immobilised in biosensor configurations by various methods. For example Noguer *et al*. [1] immobilized AlDH together with diaphorase in a photocrosslinkable polymer poly(vinyl alcohol) bearing styrylpyridinium groups (PVA-SbQ). For biosensors construction, a piece of polymeric membrane was fixed on a platinum anode and covered with a semi-permeable membrane of cellophane. An

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* Dedicated to Prof. Dr. Z. Galus on the occasion of his 70th birthday.
** Corresponding author; trojan@chem.uw.edu.pl

advantage of screen-printing technology compared to traditional electrodes manufacturing is the possibility to incorporate electronic mediators in the working electrode matrix. Based on this technology, reusable sensors have thus been designed using direct deposition of a mixture of AlDH and PVA-SbQ on the surface of Meldola Blue (MB)-modified screen-printed graphite electrodes (SPE) [2]. Single-use biosensors have also been described based on simple deposition of enzyme and cofactor (NAD^+) on the surface of Meldola Blue-modified electrodes, the detection being based on a chronoamperometric method. Finally, sensors based on sol-gel entrapment of AlDH on screen-printed carbon containing a MB insoluble salt was developed. In this case, the sensor response was possible providing that the coenzyme NAD^+ was added in the reaction medium [3].

The current tendency in development of dehydrogenase-based biosensors is to provide reagentless devices based on the co-immobilisation of the enzyme and the NAD^+ cofactor. The following three conditions are desirable for NAD^+ entrapment. First, entrapped NAD^+ should be active and highly concentrated. Secondly, the entrapped NAD^+ has to be mobile in the enzyme-immobilized membrane or matrix, because the NADH produced by an enzymatic reaction is detected electrochemically on an electrode surface. Finally, the release of NAD^+ in the solution must be prevented, this in order to optimise the operational stability of the sensor.

Various methods for retaining NAD^+ cofactor in/on the surface of working electrodes were reported. In this aim, macromolecular NAD^+ derivatives have been synthesized avoiding the leaching of coenzyme from electrode surface. As an example, co-enzymatically active dextran-bound NAD^+ was retained on the electrode together with glutamate dehydrogenase and lactate dehydrogenase on the bulb of an ammonium-sensitive glass using a dialysis membrane [4]. NAD-PEG (polyethylene glycol enlarged NAD^+) macromolecule was also used in a similar device in association with alcohol dehydrogenase and formate dehydrogenase. In this work NMP^+TCNQ (N-methylphenazium and tetracyanoquinodimethane) was used like a mediator [5]. A more elaborated method based on the covalent binding of an NAD-analogue to a dehydrogenase has been described by coupling of an N^6-[N-(6-aminohexyl)carbamoylmethyl]-NAD to alcohol dehydrogenase, leading to an enzyme-cofactor complex [6].

Another alternative consists in NAD^+ immobilization in conductive polymers matrix. A mediator-coexisting alcohol dehydrogenase-NAD conductive membrane was prepared by electrochemical polymerization in a pyrrole solution containing alcohol dehydrogenase, NAD^+ and Meldola Blue. The resulting polypyrrole membrane allows to retain enzyme activity as well as electroconductivity [7]. Some studies have also described the incorporation of free cofactor in Meldola Blue modified screen-printed carbon electrodes [8,9], the electrodes being further covered by a cellulose acetate membrane.

The use of immobilized cofactor in enzyme electrodes considerably extends the range of potential substrates that can be assayed and with careful optimization of the parameters, efficient and stable enzyme electrode could be developed. In search for

suitable matrices for biological active molecule immobilization, there has been growing interest in the application of sol-gel technology [10,11]. The sol-gel reactions proceed by hydrolysis of an alkoxide precursor under acid or basic conditions and condensation of the hydroxylated monomers to form a porous gel. Typically, a low-molecular weight metal alkoxide precursor molecule (usually tetramethoxysilane; TMOS; $Si(OCH_3)_4$, methyltrimethoxysilane; MTMOS; $Si(OCH_3)_3CH_3$ or tetraethoxysilan; TEOS; $Si(OC_2H_5)_4$ is hydrolyzed first in the presence of water acid. Hydrolysis results in the formation of silanol groups (Si–OH) which react further to form siloxane (Si–O–Si) polymers in a condensation reaction.

Acetaldehyde is commonly found in alcoholic beverages as a result of the oxidation of ethanol by alcohol dehydrogenase. It is also produced during fermentation by decarboxylation of pyruvate by pyruvate decarboxylase. Due to its toxic effects it plays a central role in the manifestation of alcohol intoxication. The usual concentrations of acetaldehyde in beverages are between 60 μM (2.64 mg$\cdot$L^{-1}) and 300 μM (13.2 mg$\cdot$L^{1}) in beers and between 0.7 mM (30.8 mg$\cdot$L^{-1}) and 1.8 mM (79.2 mg$\cdot$L^{-1}) in wines. Acetaldehyde is a very important parameter in wine elaboration as it prevents sulphur dioxide to exercise its antioxidant and antiseptic function.

The determination of acetaldehyde in beverages can be carried out based on enzymatic oxidation by an NAD^+-dependent aldehyde dehydrogenase (AlDH), according to the quasi-irreversible reaction:

$$\text{acetaldehyde} + NAD^+ + H_2O \xrightleftharpoons{AlDH} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed is stoichiometric with the amount of acetaldehyde and can be determined by monitoring its absorbance at 340 nm. An important problem related to the cofactor NAD^+ ($E^{o}_{NAD/NADH}$ = –560 mV *vs*. SCE) is its low oxidising power which drives the equilibrium of most dehydrogenase-catalysed reactions to the reactant side. Many attempts to shift the equilibrium towards the product side have been reported based on the use of additional coupled reactions (chemical or enzymatic) combined or not with the use of large amounts of NAD^+ and alkaline pH. One of the main drawbacks of the previously described system is due to the use of two enzymes and to the mandatory addition of hexacyanoferrate in the working medium [1]. The most convenient alternative to the use of two-enzyme systems seems to use electron transfer mediators that undergo a spontaneous oxidoreduction reaction with NADH. The most common mediators for NADH oxidation are phenazine, phenothiazine and phenoxazine derivatives [12,13]. Among these compounds, Meldola Blue, a phenoxazine dye, has been extensively used due to its fast rate of electron transfer with NADH [8,9]. The incorporation of electronic mediators on the electrode surface may involve covalent binding of the electrochemically active molecule, chemisorption, chemical or physical deposition of polymeric films containing active redox group, as well as physical immobilization in electrode material [1,14]. The transduc-

ers used in this work are based on incorporation of an insoluble salt of Meldola Blue into the screen-printed carbon electrode [2,15,16,17,18]. The reactions that are taking place in these conditions are:

$$NADH + MB^{+} \longrightarrow NAD^{+} + MBH$$

$$MBH \longrightarrow MB^{+} + H^{+} + 2e^{-}$$

The screen-printed electrodes, although basically considered as a single use measuring devices should allow at least two stable consecutive measurements of analyte for standard and sample. The aim of this work was to investigate SPE biosensors with immobilized AlDH, which are prepared using different protocols. This paper reports studies of response stability of biosensors obtained by the co-entrapment of aldehyde dehydrogenase (AlDH) and nicotinamide adenine dinucleotide (NAD^{+}) in sol-gel matrices.

EXPERIMENTAL

Material and reagents. Aldehyde dehydrogenase (E.C.1.2.1.5.) (AlDH) from Baker's Yeast (1 IU/mg solid), Meldola Blue (8-dimethylamino-2,3-benzophenoxazine), Reinecke's salt (ammonium tetrathio-cyanodiammono chromate), tetramethoxysilane (TMOS), polyethylene glycol 600 (PEG_{600}), bovine serum albumine (BSA), poly(ethylenimine) (PEI) and nicotinamide adenine dinucleotide (NAD^{+}) were obtained from Sigma (St.Louis, MO). Hydroxyethylcellulose (HEC) and methyl-trimethoxysilane (MTMOS) were supplied by Fluka (Buchs, Switzerland).

The inks for the fabrication of screen-printed electrodes were provided by Acheson France: silver ink (Electrodag PF 410), graphite ink (Electrodag 423 SS), silver/silver chloride ink (Electrodag 6037 SS), and Timcal – Timrex T15 graphite (Timcal, Bodio, Switzerland).

Apparatus and measurements. A DEK 248 screen-printing machine (DEK, Weymouth, Dorset, UK) was used to fabricate the electrodes. A simple two-electrode system was used in the assays. A carbon ink incorporating Reinecke's salt of Meldola Blue (MBRS) was used as working electrode material, while the Ag/AgCl electrode was both reference and counter electrode. A Metrohm 641 VA Detector potentiostat, connected with a Servotrace recorder (Sefram, Paris, France) were used to perform amperometric measurements.

The supporting electrolyte used in this work was 0.2 M phosphate buffer pH 8.0, containing 0.1 M sucrose and 0.1 M KCl. The sensor was immersed in a cell containing 5 mL of supporting electrolyte. Operational stability was performed by injections of acetaldehyde at final concentration 50 μM. Between assays, the cell was rinsed with distilled water. In these experiments, the working potential was fixed at –150 mV *vs*. Ag/AgCl screen-printed reference electrode.

Fabrication of screen-printed electrodes. Transparent PVC sheets (200×100 mm, 0.5 mm thick) (SKK, Germany) were used as the support for the screen-printing electrodes. A chemically-modified ink was prepared by mixing 23 ml of hydroxyethylcellulose (3% w/v) with 3.5 g of Timrex T15 and 140 mg of an insoluble salt of the mediator Meldola's Blue (MBRS). This salt was obtained by precipitating Meldola's Blue with Reinecke's salt (by mixing equimolar solutions in the ratio 1:1 v/v), according to the method reported earlier [19].

The electrodes were prepared in 5 steps, by screen-printing different consecutive layers on clear PVC sheets (64-electrodes/sheet, corresponding to 32 working and 32 reference electrodes). A first conducting track was deposed using silver-loaded ink. A graphite layer was deposed to cover the bottom part of the silver track. The role of this layer is to avoid that electrochemical reactions take place on the silver-conducting track. The third layer consisted in silver/ silver chloride ink for the reference/ counter

electrode and graphite ink containing Meldola Blue for the working electrode. This layer allowed to define a geometric working area of 8.5 mm×2 mm. Finally an insulating ink was deposited by screen-printing a commercial paint in order to leave uncovered only a 9 mm long portion of the silver track, which is necessary for the electrical contact.

Immobilization of enzyme in sol-gel matrix. The influence of drying and storage temperature on the stability of electrodes were tested. The sol solution was mixed with enzyme solution and water. A 1.5 μl of mixtures were spread on the surface of a screen-printed carbon electrode incorporating Mendola Blue-Reinecke's salt (MBRS). The electrodes were lead to dry in different conditions.

Immobilization of enzyme and coenzyme in sol-gel matrix. Enzyme activities were those given by the suppliers and were expressed in IU (micromole of substrate transformed per minute) throughout the text. The enzyme and cofactor were immobilized in different sol-gels matrices using various mixtures of two sol-gel precursors: methyltrimethoxysilane (MTMOS) and tetramethoxysilane (TMOS). The reactant solutions containing various amounts of tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMOS), 1 mM HCl, distilled water and PEG_{600} (Table 1) were mixed, sonicated for 15 min to homogenize the solutions, and let overnight at 4°C to allow hydrolysis of the precursors. Mono- and bi-layer immobilization matrices were prepared according to the following methods.

Table 1. Composition of the reaction mixtures used for the sol-gel immobilisation procedure according to methods 1 to 5.

Components	Method 1	Method 2	Method 3	Method 4	Method 5
TMOS	0	0	2	5	10
MTMOS	20	20	20	20	20
HCl	20	20	20	20	20
H_2O	40	64	64	64	64
PEG_{600}	4	4	4	4	4

Monolayer matrix. 50 μl of sol solutions obtained according to data in Table 1 were mixed with 40 μl of aldehyde dehydrogenase (100 IU/mL) and 10 μl of NAD^+ 0.05 M. The 1.5 μl aliquot of the obtained mixture was spread with a micropipette on the surface of a screen-printed carbon electrode containing MBRS and allowed to dry (horizontally). The enzyme and cofactor theoretical loading were 0.06 IU and 7.5 nmol per electrode.

Bilayer matrices.

Procedure a. Volume of 1.5 μl of 0.05 M NAD^+ solution was spread on the working electrode surface and let to dry for half an hour at 4℃ to allow adsorption. Then 1.5 μL of a 50% (v/v) mixture of enzymatic solution and sol-gel made according to method 4 (see Table 1) was deposited on the electrode. The enzyme and cofactor loading were 0.06 IU and 75 nmol per electrode.

Procedure b. The first enzyme and coenzyme-containing layer was deposited on the working electrode surface as described for monolayer matrix device (method 4). After 1 day drying, this catalytic layer was covered by spreading 1.5 μL of a mixture (volumetric ratio 1:1) of BSA solution at 2.5 or 5 mg /mL in 0.2 M phosphate buffer pH 8 and sol-gel prepared according to method 4.

Procedure c. A catalytic layer containing enzyme and coenzyme was first deposited on the working electrode as described for monolayer matrix device (method 4). After five days drying at 4°C, this catalytic layer was covered by spreading 1.5 μL of a mixture (volumetric ratio 1:1) of PEI 1% and sol-gel prepared according to method 2. The measurements were done after 1, 2, 3 and 5 days of drying of the second layer at 4°C.

Procedure d. A catalytic layer containing enzyme and coenzyme was first deposited on the working electrode as described for monolayer matrix device (method 4). This layer was covered by 1.5 μM of a mixture of sol-gel made according to method 5 and 0.2 M phosphate buffer pH 8 (volumetric ratio 1:1). The biosensors were let to dry from 1 to 6 days. The influence of drying on the stability of biosensors was tested.

RESULTS AND DISCUSSION

Biosensors with immobilized enzyme with cofactor in solution. As preliminary attempt the stability of signal in consecutive measurements using screen-printed electrodes with immobilized enzyme AlDH, only, was examined. The effect of drying and storage was examined in 10 consecutive measurements using 0.06 units of enzyme per electrode. As it can be seen in Fig. 1, in all different conditions used the stability of response was satisfactory. In further studies of joint immobilization of enzyme and cofactor, the electrodes were dried and stored at 4°C.

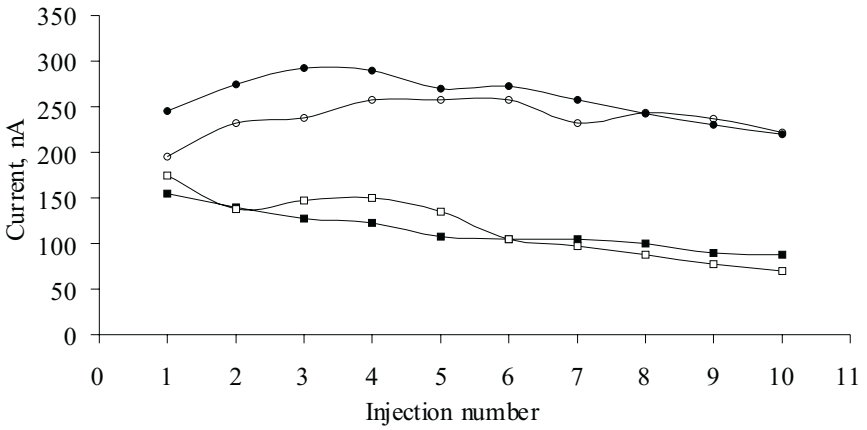

Figure 1. The influence of drying temperature and storage temperature on response stability of electrodes. Immobilization using method 2. The finale cofactor and acetaldehyde concentration loading were respectively 0.5 mM and 50 μM o • – electrodes dried at 4℃, ■ □ – electrodes dried at 25°C, • ■ – electrodes stored at –18°C, o □ – electrodes stored at 4°C.

Biosensors with joint immobilization of enzyme and cofactor. As it was reported in Introduction there were several reports in the literature about joint immobilization of both components of enzymatic system – enzyme and cofactor. In this studies the effect of several factors was studied on stability of SPEs in the systems with a single layer (monolayer systems) containing both species, and also for multi-layers obtained in different ways.

Monolayer matrix. As the first examined factor the composition of sol solution on stability of SPE was considered, according to Table 1.The influence of the polymer was studied using different ratio of MTMOS and TMOS as the sol-gel precursor. We found that a polyhydroxyl compound can form strong hydrogen bonds in the sol-gel hybrid material and its biocompatibility enables the reproducible and efficient confinement of enzyme inside silica. We used PEG_{600} according to our previous experience [20]. Data plotted in Fig. 2 show that the most stable SPE biosensors were obtained with sol-gel prepared according to method 4. The obtained results, however, were not satisfactory, as about 50% of the initial response was lost already after first measurement. Therefore, in the next step, the amount of entrapped NAD^+ was increased up to 31.5 nmol/electrode, while the enzyme loading remained constant (0.06

IU/electrode). Even using such a high cofactor loading, the response decreased rapidly and continuously, exhibiting the progressive leaching of the soluble coenzyme (Fig. 3).

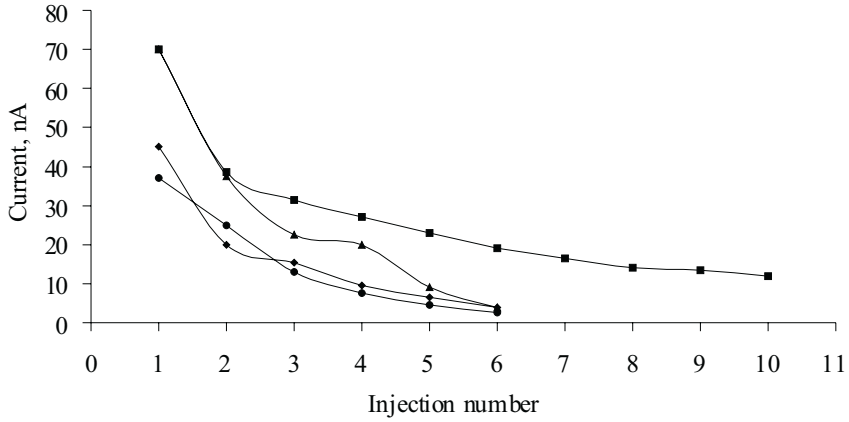

Figure 2. Response stability of sensors based on sol-gel monolayer configuration, using different methods of sol-gel preparation: ◆ method 1, ▲ method 2, • method 3, ■ method 4. AlDH 0.06 IU/electrode, NAD^+ 10.5 nmol/electrode. Response of the sensor to acetaldehyde 50 μM.

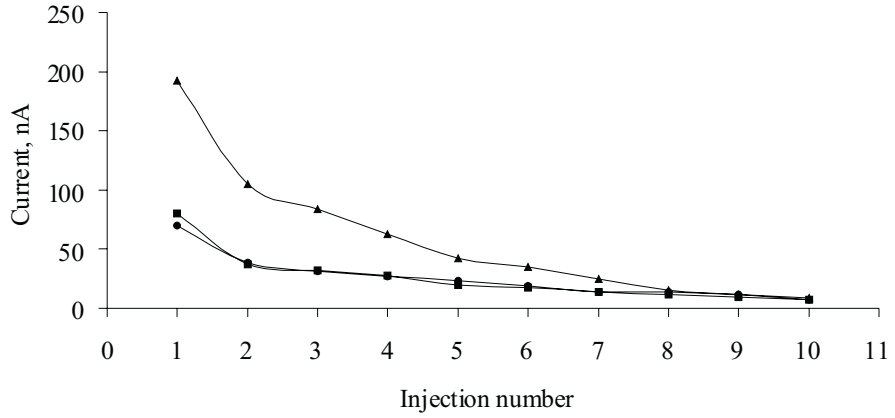

Figure 3. Influence of the amount of entrapped NAD^+ on the stability of response for sensors based on monolayer configuration: • 7.5 nmol/el, ■ 10.5 nmol/el, ▲ 31.5 nmol/el. Final acetaldehyde concentration 50 μM.

An incomplete hydrolysis of sol-gel precursors may lead to insufficiently cross-linked network, allowing a rapid and irreversible coenzyme leaching. The time of hydrolysis of MTMOS and TMOS (at 4°C) were thus varied from 12 to 48 hours to optimize the polymer formation. As it is shown in Fig. 4, the hydrolysis time affects the signal magnitude of SPE biosensor, but procedure used was still not sufficient to obtain stable response.

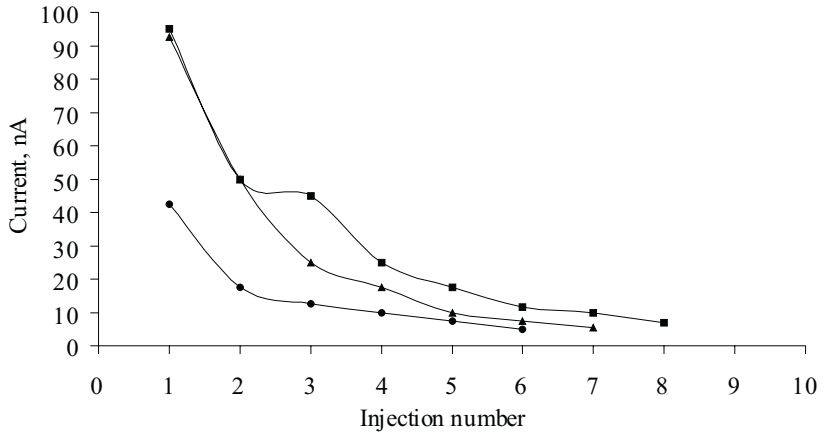

Figure 4. Influence of hydrolysis time on the stability of response for sensors based on sol-gel monolayer configuration: • 12 h, ■ 36 h, ▲ 48 h. Final acetaldehyde concentration 50 μM.

Bilayer matrices. Because of poor operational stability obtained for monolayer SPE biosensors, being related to NAD^+ leaching, several different configurations were investigated involving the deposition of an additional sol-gel layer over the enzyme-cofactor matrix.

Procedure a. In this configuration NAD^+ was simply adsorbed and covered by a AlDH-containing sol-gel layer, made according to method 4 (Table 1). Unfortunately, this sensor allowed to perform only one measurement. The following initial current values were obtained for each new electrode: 65, 57.5, 62.5, 63, 63 nA. Reproducibility of the signal obtained for this configuration is compared to other ones in Fig. 5. The reproducibility of sensor response was the best for method a, however, they could be used in a single measurement, only.

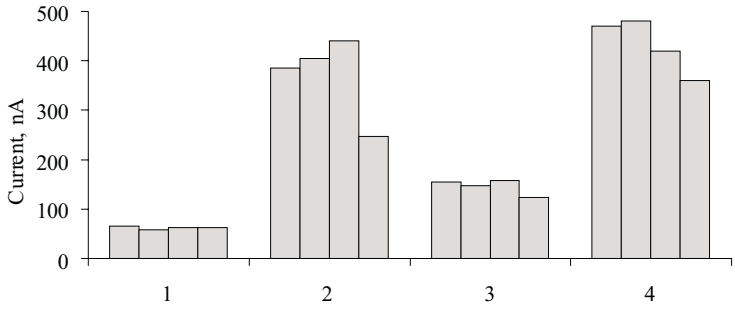

Figure 5. Reproducibility of the signal for the same configuration of electrodes obtained for all procedure of immobilization employed: 1 – procedure a, 2 – procedure b, 3 – procedure c, 4 – procedure d.

Procedure b. In this configuration the catalytic layer, described above, was covered by an additional sol-gel layer doped with BSA. Even, if the initial sensor response was improved when compared with the previously described devices (Fig. 5), the additional layer did not allow to prevent coenzyme leaching (Fig. 6). The reproducibility of preparation of SPEs using this procedure was worse than the procedure a.

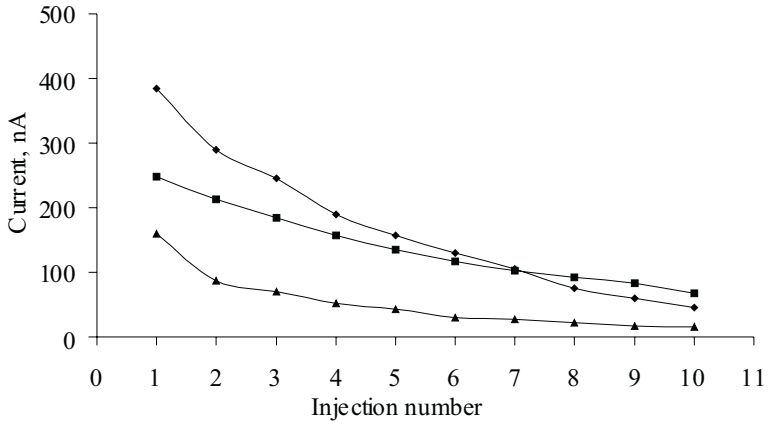

Figure 6. Response stability of sensors based on sol-gel bilayer configuration. The catalytic layer (method 4) was covered by a BSA-doped additional sol-gel layer made according to method 4. The final enzyme and cofactor loading were respectively 0.06 IU and 10.5 nmol per electrode. Final acetaldehyde concentration 50 μM. Sensors were testing after ◆ 1, ■ 2, ▲ 4 days.

Procedure c. This procedure was similar to the previous one, except that the second layer contained PEI, of which global positive charge was supposed to prevent NAD^+ leaching. The sensor operational stability was comparable to that one achieved using a BSA additional layer (Fig. 7), and reproducibility much better (Fig. 5). The increase of the drying time led to a significant increase of the initial response of biosensor, probably, due to enhancing diffusion of substrates through better dried bilayer (Fig. 7).

Procedure d. In this configuration the initial catalytic layer was allowed to dry and then covered by an additional sol-gel layer made according to method 5 (Table 1), *i.e.* using a higher amount of TMOS that is known to give more dense gel network. The biosensors were let to dry from 1 to 6 days. Fig. 8 shows the influence of the second layer drying time on the stability of the sensors. It can be noted, however, that despite the initial response decrease of about 60% compared to 1 day drying (the sensitivity decreasing from 9 to 3.5 mA/M), a 6-days drying time of the additional layer allows finally to obtain a quite stable reagentless sensor with stable response for at least 10 assays. The reproducibility of manufacturing this configuration is also reasonable (Fig. 5).

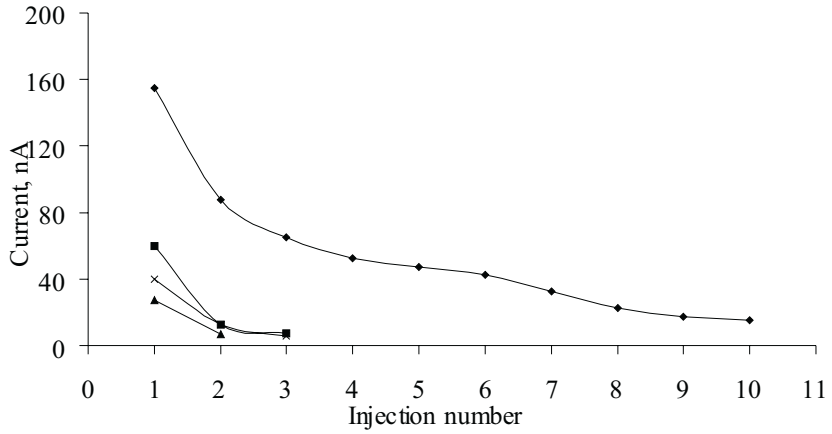

Figure 7. Response stability of sensors based on sol-gel bilayer configuration. The catalytic layer (method 4) was covered by a PEI-containing additional sol-gel layer made according to method 2. The final enzyme and cofactor loading were respectively 0.06 IU and 10.5 nmol per electrode. Final acetaldehyde concentration 50 μM. Sensors were tested after × 1, ▲ 2, ■ 3, ◆ 5 days of draying of the outer layer.

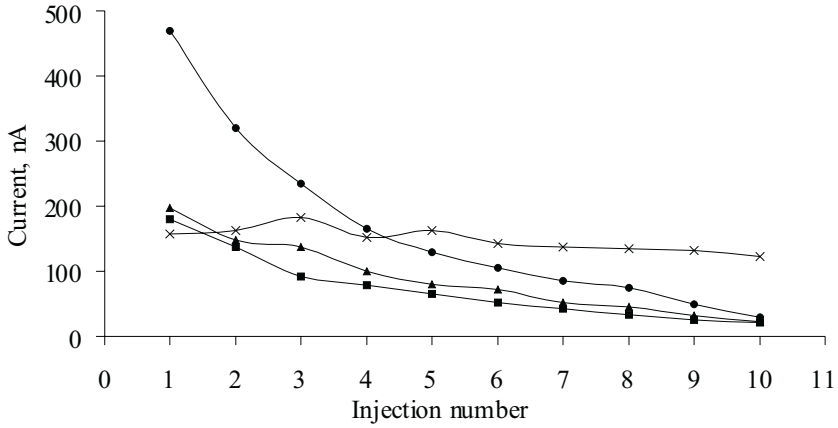

Figure 8. Operational stability of sensors based on sol-gel bilayer configuration. The catalytic layer (method 4) was covered by a sol-gel layer made according to method 5. The final enzyme and cofactor loading were respectively 0.06 IU and 10.5 nmol per electrode. Final acetaldehyde concentration 50 μM. Sensors were tested after • 1, ■ 2, ▲ 4, × 6 days of draying.

CONCLUSIONS

This work describes the development a reagentless acetaldehyde biosensor with enzyme (AlDH), and cofactor (NAD^+) immobilized in the same sol-gel matrix on the surface of a Meldola Blue-modified screen-printed electrode. Among all the conditions tested to obtain a stable and sensitive sensor response, successful results were achieved by deposing an additional sol-gel layer on the catalytic layer and let it dry at

least 6 days. This second layer, incorporating increased amount of TMOS, allows to retain efficiently the coenzyme, thus preventing its leakage in the measuring medium.

Acknowledgments

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