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Reagentless amperometric formaldehyde-selective biosensors based on the recombinant yeast formaldehyde dehydrogenase

Olha Demkiv[a], Oleh Smutok[a], Solomiya Paryzhak[a], Galyna Gayda[a], Yusif Sultanov[b], Dmitrii Guschin[c], Halyna Shkil[c], Wolfgang Schuhmann[c], Mykhailo Gonchar[a,*]

[a] Department of Analytical Biotechnology, Institute of Cell Biology, Drahomanov Street 14/16, 79005 Lviv, Ukraine
[b] Institute of Chemical Problems, National Academy of Sciences of Azerbaijan, H. Javid avenue 29, AZ1143 Baku, Azerbaijan
[c] Anal. Chem. – Elektroanalytik & Sensorik, Ruhr-Universität Bochum, Universitätsstr. 150, D-44780 Bochum, Germany

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ABSTRACT

Novel formaldehyde-selective amperometric biosensors were developed based on NAD^+- and glutathione-dependent formaldehyde dehydrogenase isolated from a gene-engineered strain of the methylotrophic yeast *Hansenula polymorpha*. Electron transfer between the immobilized enzyme and a platinized graphite electrode was established using a number of different low-molecular free-diffusing redox mediators or positively charged cathodic electrodeposition paints modified with Os-bis-*N*,*N*-(2,2′-bipyridil)-chloride ([$Os(bpy)_2Cl$]) complexes. Among five tested Os-containing redox polymers of different chemical structure and properties, complexes of osmium-modified poly(4-vinylpyridine) with molecular mass of about 60 kDa containing diaminopropyl groups were selected. The positively charged cathodic paint exhibited the best electron-transfer characteristics. Moreover, the polymer layers simultaneously served as a matrix for keeping the negatively charged low-molecular cofactors, glutathione and NAD^+, in the bioactive layer. Additionally, covering the enzyme/polymer layer with a negatively charged Nafion membrane significantly decreased cofactors leakage and simultaneously enhanced the sensor' stability. The developed sensors revealed a high selectivity to formaldehyde (FA) and a low cross-sensitivity to other substances (such as, e.g. butyraldehyde, propionaldehyde, acetaldehyde, methylglyoxal). The maximum current value was $34.2 \pm 0.72\ \mu A/mm^2$ (3.05 mm diameter electrode) and the apparent Michaelis–Menten constant (K_M^{app}) derived from the FA calibration curves was 120 ± 5 mM with a linear detection range for FA up to 20 mM. The best observed sensitivity for reagentless sensor was 1.8 nA μM^{-1} (358 A m^{-2} M^{-1}). The developed sensors had a good operational and storage stability. The laboratory prototype of the sensor was applied for FA testing in some real samples of pharmaceutical (formidron), disinfectant (descoton forte) and industrial product (formalin). A good correlation was revealed between the concentration values measured using the developed FdDH-based sensor, an enzymatic method and standard chemical methods of FA determination.

1. Introduction

Formaldehyde (FA) has found broad application in chemical synthesis of phenol-, urea-, and melamine–formaldehyde resins which are used in the manufacturing of building plates, plywood and lacquer materials. FA is additionally necessary for the production of different consumer goods such as detergents, soaps, and shampoos, as well as in pharmacology and medicine as a sterilising agent [1]. Advanced technologies of water pre-treatment which include ozonization lead to FA formation by the reaction of ozone with natural humus traces [2,3] or with contaminations of chlorinated benzene derivatives [3]. It was reported that FA is found in more than 2000 commercial products to which many industrial workers are exposed on a daily basis. This calls for a continuous control of possible contamination processes [4]. The permissible level of FA in industrial areas was set to 0.5–2.0 ppm. FA is produced also in the atmosphere as a product of photo-chemical oxidation of automobile exhaust and combustion processes.

FA has a negative influence on human's health, especially on the central nervous, blood and immune systems. It is a potent nasal irritant, causes stunted growth, blindness and respiratory diseases. FA is one of the chemical mediators of apoptosis and is considered as a mutagenic and a possibly human carcinogenic compound [5]. However, FA is not only of artificial origin. It is a natural metabolite found in tissues, cells, and body fluids. It is present in fruits, vegetables, meat, and fish. In extreme cases, some frozen fishes,

* Corresponding author. Tel.: +380 32 2612144; fax: +380 32 2611648.
E-mail address: gonchar@cellbiol.lviv.ua (M. Gonchar).

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especially of the gadoid species, can accumulate up to 200–780 mg of FA per kg of moist weight due to the enzymatic degradation of trimethylamine oxide which is a natural fish component [6,7].

All these facts convincingly demonstrate the requirement of reliable analytical devices for an accurate FA determination in testing consumer goods, the environment, as well as biological samples. A number of attempts to develop biosensors for the detection of FA were reported [8–13] including amperometric sensors [14–16], potentiometric detection schemes [10,17–21] and optical sensors [12,22]. However, some serious problems remain unsolved such as a low sensitivity of the potentiometric sensors, insufficient stability, and poor selectivity. These limitations provoke the development of new biosensors based on novel enzymes including recombinant proteins isolated from genetically modified microbial cells.

We have described the fabrication and properties of a reagentless bienzyme amperometric biosensor based on alcohol oxidase/peroxidase in combination with Os-complex modified electrodeposition paint [23]. Although the developed alcohol biosensor showed a good co-sensitivity for the detection of FA, the poor selectivity of alcohol oxidase used as biological recognition element limited the application of this sensor for FA determination.

As new approach to development of highly selective FA assays, formaldehyde dehydrogenase (FdDH), a key enzyme of FA metabolism in microorganisms, was proposed to be used as biorecognition element in biosensors [10,16,24–30]. However, the broad application of FdDH in analytical practice is hampered by its insufficient activity, as well as by relatively high costs of the commercially available enzymes' preparations. Recently, we have reported about the construction of a recombinant yeast clone originated from the recipient strain of thermotolerant methylotrophic yeast *Hansenula polymorpha* which is a NAD^+- and glutathione-dependent FdDH over-producer [31]. A simple scheme for the isolation and chromatographic purification of the target enzyme from the over-producing yeast cells was proposed and highly purified FdDH preparations were obtained [32].

Recently, we have described the construction of highly selective biosensors [28–30] using, on the one hand, commercial bacterial FdDH [28] and, on the other hand, the yeast FdDH from the recombinant over-producer [29–30]. Although in the case of the amperometric biosensor [30] a good sensitivity was achieved ($22\,A\,m^{-2}\,M^{-1}$), the procedure of FA measuring was complicated by the need for addition of the yeast FdDH cofactors to the analyte solution.

In this communication, we propose an improved sensor architecture for the development of yeast FdDH-based amperometric biosensors providing the secure fixation of all sensor components in a bioactive layer on the transducer surface. Specifically, the sensor architecture was designed to prevent any leakage of the low-molecular and free-diffusing cofactors (NAD^+ and glutathione), thus avoiding the need for any addition of these cofactors to the analyte solution. For the design of the electron-transfer pathway between electrode surface and enzyme, besides standard free-diffusing and low-molecular weight redox mediators, different positively charged cathodic electrodeposition polymers, synthesized on the basis of 4-vinilpyridine and butylmethacrylate as monomers and modified with Os-bis-*N*,*N*-(2,2′-bipyridil)-chloride ($[Os(bpy)_2Cl]$) were used as mediators and supporting carriers for enzyme and its cofactors.

The obtained biosensors were successfully applied for FA determination in real samples of commercial chemical product (formalin), pharmaceutical (formidron) and disinfectant (descoton forte).

2. Experimental

2.1. Materials

DEAE–Toyopearl 650 M was from Toyo Soda (Tokyo, Japan); EDTA and nitrotetrazolium blue (NTB) were from Merck (Darmstadt, Germany); hexachloroplatinum(IV)-acid-hexahydrate, ferrocene, formalin and methanol were from Merck-Schuchardt (Hohenbrunn, Germany). Methylene blue was obtained from Riedel-de Haën (Seelze, Germany); dithiothreitol (DTT), *para*-formaldehyde, phenylmethylsulfonyl fluoride (PMSF), potassium hexacyanoferrate(III), potassium hexacyanoferrate(II), triton X-100, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), phenazine ethosulfate and methylglyoxal were from Sigma (Deisenhofen, Germany). Glutathione (reduced) and phenazine methosulfate (PMS) were from Fluka (Buchs, Switzerland), NAD^+ and NADH were obtained from Gerbu Biotechnik (Gailberg, Germany). Nafion, butyraldehyde, propionaldehyde and acetaldehyde were from Aldrich (Deisenhofen, Germany).

Poly(4-vinylpyridines) (P4PV), poly(4-vinylpyridine-co-butyl methacrylate) (CP4PVBMA), epichlorhydrin (99%), and 4(5)-imidazol-carboxaldehyde (ICA) (98%) were obtained from Aldrich (Taufkirchen, Germany). Potassium hexachloroosmate (K_2OsCl_6), 2,2′-bipyridine (99%; bpy), 1,3-diaminopropane (DAP) (99%), 1,8-diaminooctane (DAO) (98%) were from Acros (Geel, Belgium), osmium-bis-*N*,*N*-(2,2′-bipyridil)-chloride was synthesized according to Ref. [33]. Descoton forte was from Desomark (Novoyavorivsk, Ukraine), formidron was obtained from Ternofarm (Ternopil, Ukraine).

All chemicals were of analytical reagent grade and all solutions were prepared using HPLC-grade water. FA solution (1 M) was prepared by hydrolysis of the corresponding amount of *para*-formaldehyde in water (300 mg; 10 ml water) by heating the suspension in a sealed ampoule at 105 °C for 6 h.

2.2. Synthesis of Os-containing redox polymers

As a polymer base for mediators, poly(4-vinylpyridines) with a molecular mass 60 kDa (P4VP60) and 160 kDa (P4VP160) and copolymers of 4-vinylpyridine with butylmethacrylate (poly(4-vinylpyridine-co-butyl) methacrylate; CP4VPBMA) have been chosen. P4VP60, P4VP160 and CP4VPBMA were alkylated with epichlorhydrin and the resulting products were treated with DAP or DAO. After this, ICA was added to the reaction mixture. The obtained polymers [34] were modified by coordinating osmium-complexes by means of a ligand exchange reaction of osmium-bis-*N*,*N*-(2,2′-bipyridil)-chloride with suitable ligands at the polymer chains. Molecular structures of the synthesized Os-containing redox polymers are shown in Fig. 1.

The redox properties of the synthesized Os-modified polymers were studied using differential pulse voltammetry.

2.3. Formaldehyde dehydrogenase (FdDH)

As a microbial source of NAD- and glutathione-dependent FdDH, the gene-engineered yeast strain Tf 11-6, derivative of the recipient *H. polymorpha* strain NCYC 495, was chosen [31]. Cells of recombinant strain Tf 11-6, over-producing thermostable FdDH, were cultivated and a cell-free extract was prepared according to Ref. [31].

FdDH was isolated from the cell-free extract by a two-step ion-exchange chromatography on DEAE–Toyopearl 650 M [32]. FdDH activity was determined by the rate of NADH formation [35]. One