Electrochimica Acta 251 (2017) 256–262

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Optimization of an amperometric biosensor array for simultaneous measurement of ethanol, formate, D- and L-lactate

Johanna Pilas[a,b], Yasemen Yazici[a], Thorsten Selmer[a], Michael Keusgen[b], Michael J. Schöning[a,c,*]

[a] Institute of Nano- and Biotechnologies (INB), Aachen University of Applied Sciences, 52428 Jülich, Germany
[b] Institute of Pharmaceutical Chemistry, Philipps-Universität Marburg, 35037 Marburg, Germany
[c] Peter Grünberg Institute (PGI-8), Forschungszentrum Jülich, 52425 Jülich, Germany

ARTICLE INFO

Article history:
Received 17 May 2017
Received in revised form 17 July 2017
Accepted 20 July 2017
Available online 1 September 2017

Keywords:
Dehydrogenase
Diaphorase
Enzymatic biosensor
Simultaneous determination

ABSTRACT

The immobilization of NAD^+-dependent dehydrogenases, in combination with a diaphorase, enables the facile development of multiparametric sensing devices. In this work, an amperometric biosensor array for simultaneous determination of ethanol, formate, D- and L-lactate is presented. Enzyme immobilization on platinum thin-film electrodes was realized by chemical cross-linking with glutaraldehyde. The optimization of the sensor performance was investigated with regard to enzyme loading, glutaraldehyde concentration, pH, cofactor concentration and temperature. Under optimal working conditions (potassium phosphate buffer with pH 7.5, 2.5 mmol L^{-1} NAD^+, 2.0 mmol L^{-1} ferricyanide, 25 °C and 0.4% glutaraldehyde) the linear working range and sensitivity of the four sensor elements was improved. Simultaneous and cross-talk free measurements of four different metabolic parameters were performed successfully. The reliable analytical performance of the biosensor array was demonstrated by application in a clarified sample of inoculum sludge. Thereby, a promising approach for on-site monitoring of fermentation processes is provided.

1. Introduction

In the course of ongoing and rapid development of novel biosensing technologies, the construction of multiparametric biosensors has become the next logical step for fast determination of several analytes [1]. Such devices for simultaneous measurement of various species are of great interest for the application in biotechnological [2], biomedical [3][4], environmental [5] and industrial fields [6]. In this context, the monitoring of parameters such as ethanol, lactate and formate is, for example, crucial for the control of fermentation processes. The determination of ethanol is also relevant in clinical analysis of human body fluids. Lactate, as a key metabolite in metabolic pathways serves, for example, as freshness indicator in the food processing industry [7]. Formate is also an important intermediate in aerobic and anaerobic fermentation processes. Currently, conventional methods for analyses of these compounds are performed by spectrophotometry, gas chromatography and high performance liquid chromatography [8], [9]. These techniques are expensive, laborious, time-consuming and often require extensive sample pre-treatment. In this regard, enzyme-based biosensors are an attractive alternative. Due to their versatility, they fulfill the requirements for multianalyte detection by rapid, sensitive and selective monitoring features.

Amperometric enzyme-based biosensors rely often on the catalytic activity of an oxidase or dehydrogenase. However, most of the devices reported in literature, that are capable of simultaneous measurement, operate with oxidases. A potential drawback in using oxidases for biosensor development, is the low specifity of these enzymes and their cross-talk tendencies. Therefore, application of dehydrogenases in biosensor arrays seems feasible, despite the requirement of the cofactor NAD^+. More than 250 NAD^+-dependent dehydrogenases are known [10] and thus, a huge potential for the development of further biosensors is provided. Numerous examples of dehydrogenase-based sensors for detection of ethanol [11], formate [12], D-lactate [13] and L-lactate [14] have been recently described. However, reports on integration of multiple dehydrogenases into a single sensing device are rare. Miertuš et al. have used solid binding matrices for the development of two multibiosensors for simultaneous amperometric

* Corresponding author at: Institute of Nano- and Biotechnologies (INB), Aachen University of Applied Sciences, 52428 Jülich, Germany. Tel.: +49 241 6009 53215; fax: +49 241 6009 53235.
E-mail address: schoening@fh-aachen.de (M.J. Schöning).

http://dx.doi.org/10.1016/j.electacta.2017.07.119
0013-4686/

detection of glucose, fructose and ethanol with one of the systems, and L-lactate, L-malate and sulfite with the other [15]. The enzyme electrodes were modified with both oxidases and dehydrogenases, according to the analyte and the associated detection principle.

Recently, we have reported on the development of an amperometric multiparameter sensor chip for simultaneous detection of formate, D- and L-lactate in biogas processes [16]. The detection principle is based on a two-enzyme system, consisting of a dehydrogenase and a diaphorase [17]. In a first step, the substrate (formate, D- and L-lactate, respectively) is converted to a product (CO_2 or pyruvate), as presented in reaction (1). This reaction is catalyzed by a specific dehydrogenase (formate dehydrogenase, D- and L-lactate dehydrogenase, respectively). Thereby, the cofactor NAD^+ is reduced to NADH.

$$Substrate + NAD^+ \xrightarrow{Dehydrogenase} Product + NADH \quad (1)$$

In a second enzymatic reaction, the released NADH is regenerated by a diaphorase and the reduction of the cofactor ferricyanide to ferrocyanide (2).

$$Ferricyanide + NADH \xrightarrow{Diaphorase} Ferrocyanide + NAD^+ \quad (2)$$

Amperometric detection of the substrate concentration is realized by anodic oxidation of ferrocyanide at +0.3 V *vs.* Ag/AgCl according to the following reaction (3).

$$Ferrocyanide \xrightarrow{Oxidation} Ferricyanide + e^- \quad (3)$$

The focus of the present study is the improvement of a multiparametric biosensor array for simultaneous determination of ethanol, formate, D- and L-lactate using various specific dehydrogenases. The performance of the biosensor array was optimized with regard to enzyme loading, concentration of cross-linking agent, pH, cofactor concentration and temperature. Compatibility of four different sensing elements, integrated within one biosensor array, was investigated by parallel amperometric measurement.

2. Experimental

2.1. Chemicals and reagents

Alcohol dehydrogenase EC 1.1.1.1 from *Saccharomyces cerevisiae* (ADH, 310 U mg^{-1}), diaphorase EC 1.8.1.4 (also known as dihydrolipoyl dehydrogenase) from *Clostridium kluyveri* (DIA, 51 U mg^{-1}), D-lactate dehydrogenase EC 1.1.1.28 from *Lactobacillus leichmanii* (D-LDH, 213 U mg^{-1}) and L-lactate dehydrogenase EC 1.1.1.27 from *Bacillus stearothermophilus* (L-LDH, 174.5 U mg^{-1}) were obtained from Sigma-Aldrich. Formate dehydrogenase EC 1.2.1.2 from *Candida boidinii* (FDH, 0.49 U mg^{-1}) was purchased from Roche Diagnostics GmbH. Bovine serum albumin (BSA), glutaraldehyde solution (25%), glycerol, potassium ferricyanide, sodium D-lactate and ethanol standard solution were supplied by Sigma-Aldrich, too. Sodium formate, sodium L-lactate and β-nicotinamide adenine dinucleotide (NAD^+) were from AppliChem GmbH. The buffer components K_2HPO_4, KH_2PO_4, Tris, HCl and H_2SO_4 were bought from Roth. Ethanol, formate, D- and L-lactate assay kits used for comparative purposes were purchased from Megazyme International Ireland.

Stock solutions of ADH, FDH, D-LDH and L-LDH were prepared in 100 mmol L^{-1} potassium phosphate buffer (pH 7.5). The DIA solution contained addtionally 0.5 mmol L^{-1} flavin adenine dinucleotide (FAD). In order to fabricate sensors with reproducible enzyme loadings, the activity of the different enzyme solutions was determined photometrically (Ultrospec 2100 pro, Amersham Biosciences). For this reason, the dehydrogenase activity of ADH, FDH, D-LDH and L-LDH was measured by monitoring the NADH production at 340 nm [18], [19]. The DIA activity was evaluated by following the decrease in NADH absorbance, using potassium ferricyanide as substrate [20]. The indicated enzyme loadings (activity in Units per electrode) refer to 1.5 µL of immobilization mixture applied to each electrode.

2.2. Sensor preparation

The biosensor array chips (10×10 mm^2), each consisting of five platinum thin-film working electrodes, were fabricated from a p-Si wafer by photolithographic techniques. Fig. 1 shows an image of the multiparameter biosensor with different enzyme membranes immobilized on each working electrode. Detailed description of the fabrication process was provided earlier [16]. In this work, the sensor chips were additionally modified by spin-coating a 20 µm thick passivation layer of SU-8 photo resist (SU-8 25, micro resist technology GmbH) [21]. Prior enzyme immobilization, the biosensor surface was electrochemically pretreated in 0.5 M H_2SO_4 by applying an anodic current of +2.0 V *vs.* Ag/AgCl for 2 min. Afterwards, potential cycling between -0.2 V and +1.4 V at a scan rate of 100 mV s^{-1} was performed until reproducible voltammograms were obtained [22].

The various enzyme membranes of the biosensor array were constructed by chemical cross-linking using glutaraldehyde 4 vol% (prepared with 10 vol% glycerol). Thereby, enzyme solutions with defined activity of the particular dehydrogenase and DIA (total volume 4 µL) were mixed with 0.5 µL BSA 24%. Finally, 1.5 µL of glutaraldehyde were added and mixed carefully. For adjustment of enzyme loading or glutaraldehyde concentration in the immobilization matrix, solutions were diluted appropriately. For simultaneous measurements an additional blank electrode without catalytic activity was utilized. In this case, 4.5 µL BSA 1.3% were mixed with 1.5 µL glutaraldehyde (1.2 vol%). In each case, the electrode surface was modified by drop-coating 1.5 µL of a particular immobilization mixture. The electrodes were dried overnight and stored at 4 °C when not in use.

2.3. Apparatus and measurement

Amperometric measurements and cyclic voltammetry were executed by using a potentiostat (PalmSens, Palm Instruments BV) in combination with an eight-channel multiplexer (MUX8, Palm Instruments BV). Experiments were performed in a three-electrode setup, consisting of a platinum counter electrode (diameter 7.5 mm), a double junction Ag/AgCl reference electrode (Metrohm, 3 M KCl) and a biosensor array chip with five platinum working electrodes (diameter each 2 mm). The measurement cell was equipped with a magnetic stir bar (200 rpm) and a working potential of +300 mV *vs.* Ag/AgCl was applied to the working electrodes [16]. Optimization of the working conditions was carried out in 12 mL potassium phosphate buffer (100 mmol L^{-1}, pH 7.5) with various concentrations of ferricyanide (0.1-6.0 mmol L^{-1}),

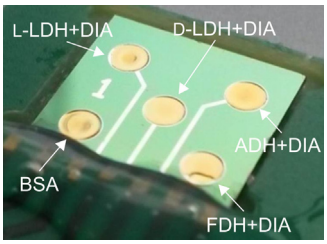

Fig. 1. Multiparameter biosensor chip (10×10 mm^2) with five working electrodes, each modified by immobilization of different enzymes (L-LDH+DIA, D-LDH+DIA, ADH+DIA and FDH+DIA). One electrode without enzymatic activity (only BSA) is serving as a reference.