Biosensors and Bioelectronics 50 (2013) 235–238

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

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

Short communication

Microbial monooxygenase amperometric biosensor for monitoring of Baeyer–Villiger biotransformation

Andrea Schenkmayerová, Marek Bučko, Peter Gemeiner, Jaroslav Katrlík *

Department of Glycobiotechnology, Institute of Chemistry, Slovak Academy of Sciences, Dúbravská cesta 9, 84538 Bratislava, Slovakia

ARTICLE INFO

Article history:
Received 3 April 2013
Received in revised form
27 June 2013
Accepted 28 June 2013
Available online 8 July 2013

Keywords:
Baeyer–Villiger oxidation
Microbial biosensor
Genetically modified bacteria
Oxygen microelectrode
Biotechnology
Biotransformation

ABSTRACT

A whole-cell amperometric biosensor consisting of genetically engineered *Escherichia coli* immobilised in polyelectrolyte membrane onto a miniaturised oxygen electrode was developed and used for monitoring of biotransformation based on Baeyer–Villiger oxidation. Baeyer–Villiger oxidation is commonly performed using microorganisms overexpressing Baeyer–Villiger monooxygenase enabling the production of enantiopure lactones or esters used in pharmaceutical industry. The biorecognition element, genetically modified *E. coli* overexpressing either cyclopentanone monooxygenase or cyclohexanone monooxygenase was immobilised in the form of solid polyelectrolyte complex gel membrane made of cellulose sulphate, sodium alginate and poly(methylene-co-guanidine) and attached to the surface of miniaturised oxygen electrode. The time response of the biosensor was 30 s, the linear range of the calibration curve (R^2=0.9993) was 8–130 μM and the sensitivity was 1.8 nA μM^{-1} (RSD=5.0%) for substrate of Baeyer–Villiger oxidation (±)-cis-bicyclo[3.2.0]hept-2-en-6-one as analyte. The biosensor sensitivity was assessed for two other commercially available substrates, 4-methylcyclohexanone and 3-methylcyclohexanone. No interferences from ampicillin, citric acid, acetic acid, ethanol, methanol, glucose and products of Baeyer–Villiger oxidation (1R, 5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one and (1S, 5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one were detected. After 1 week of storage at 4 °C the biosensor sensitivity was without changes. The biosensor was employed for monitoring of Baeyer–Villiger biotransformation and the results were correlated with gas chromatography. Till now, this is the first described biosensor based on Baeyer–Villiger monooxygenase and the first reported application of biosensor for monitoring of biotransformation based on Baeyer–Villiger oxidation.

1. Introduction

Baeyer–Villiger (BV) oxidations are widely studied since their discovery more than 110 years ago (Baeyer and Villiger, 1899). They permit monooxygenation of wide range of ketone substrates by the oxidative cleavage of a C–C bond adjacent to a carbonyl, which converts ketones to esters and cyclic ketones to lactones. BV oxidation can be carried out with strong oxidisers (e.g. peroxy acid or hydrogen peroxide) or enzymatically by BV monooxygenases (BVMOs). The monooxygenases catalyse the incorporation of one oxygen atom into the organic substrate whilst the other oxygen atom is reduced to water thus enabling regio- and enantioselective oxidations of ketone substrates to the highly valuated products serving as intermediates for pharmaceuticals (Zhang et al., 2013) or aroma compounds synthesis (Fink et al., 2011). The reactants and products of BV oxidation are often volatile, therefore they are usually analysed *via* gas chromatography (GC).

Oxygenases can be defined as oxidoreductases oxidising substrate by transferring one or two atoms of oxygen from the molecular oxygen to the substrate. Most of oxygenases are classified with EC numbers EC 1.13.x.x and EC 1.14.x.x and overwhelming majority of oxygenases based biosensors use enzymes from cytochrome P450s' family (Schneider and Clark, 2013). Other examples include enzymatic organic field effect transistor biosensor with flavin-containing monooxygenase (1.14.13.8) dedicated to the detection of trimethylamine (Diallo et al., 2009), biosensor for catechol and its derivatives using 2,3-dihydroxybiphenyl 1,2-dioxygenase (EC 1.13.11.39) modified glassy carbon electrode (Zhang et al., 2011) and use of whole-cell fibre optic enzymatic biosensor for the measurement of toluene with toluene ortho-monooxygenase (1.14.13.-) (Zhong et al., 2011).

BVMOs used for BV oxidation can act as well as biocatalyst in the biosensors for determination of substrates of BV reactions. The enzymes of BVMO family are naturally present in many microbial species, e.g. *Comamonas*, *Acinetobacter*, *Arthrobacter* and *Pseudomonas*. However, the use of isolated BVMOs has significant drawbacks (Kayser, 2009), as (i) the majority of BVMOs have poor stability, (ii) need of NAD(P)H cofactor regeneration system, (iii)

* Corresponding author. Tel.: +421 2 59410258.
E-mail addresses: jaroslav.katrlik@savba.sk, katrlik@yahoo.com (J. Katrlík).

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http://dx.doi.org/10.1016/j.bios.2013.06.061

the isolation of enzymes is time and money consuming and (iv) isolated BVMOs are expensive or not commercially available. On the other hand, the natural strains have low enzyme activity, their metabolism represent possibility of product degradation and one of the most frequently used natural sources of BVMOs *Acinetobacter sp.* is pathogenic. Another approach is the use of cell engineering. The genetic modification of cells is well described and routinely used method as well as their use in biosensing (Song et al., 2012). Modern recombinant methods enabled construction and utilisation of genetically modified cells overexpressing BVMOs including *E. coli* as a host microorganism (Mihovilovic, 2006). These highly productive cells with BVMOs are able to catalyse BV biooxidations of broad range of natural and synthetic ketones for nearly enantiopure production of corresponding lactones and esters as potential drug precursors (De Gonzalo et al., 2010). Taking it into account, the use of genetically modified cells for BVMOs based biosensors is much more promising option than the use of either isolated enzymes or naturally occurring microorganisms.

For immobilisation of whole cells we chose the entrapment into the solid polyelectrolyte complex (PEC) gel membrane made of cellulose sulphate, sodium alginate and poly(methylene-co-guanidine). This PEC matrix was primarily developed to encapsulate Langerhans islets with focus on diabetes curing (Lacík, 2006). In the context of biotechnological use, it was proved that the matrix preserves stability of immobilised biocomponent and the chemical stability is higher comparing to alginate gels (Bučko et al., 2005, 2011).

We focused on development of whole-cell BVMO biosensors based on miniaturised oxygen electrode for determination of the substrates of BV oxidation and their application in the monitoring of biotransformation process based on BV oxidation. To our knowledge, no biosensor has been reported neither based on BVMOs nor used for BV oxidation monitoring until now. The solid PEC gel membrane based on poly(methylene-co-guanidine) has never been used in biosensor construction before.

2. Material and methods

2.1. Material

(±)-cis-bicyclo[3.2.0]hept-2-en-6-one (CBCH) from Fluka (Sigma-Aldrich) was used as both the substrate for BV biotransformation and the substrate standard for biosensor and GC measurements. 4-methylcyclohexanone (4MCH) from Merck and 3-methylcyclohexanone (3MCH) from Fluka were used as substrate standards, (1R, 5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one (1R5SOBO) from Fluka and (1S, 5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one (1S5ROBO) from Aldrich were used as product standards. LB_{amp} medium for cell growth consisted of (g dm^{-3}): peptone 10; yeast extract 5; NaCl 10; ampicillin 0.2. TB_{amp} medium for cell growth consisted of (g dm^{-3}): tryptone 12; yeast extract 24; glycerol 5; $K_2HPO_4 \cdot 3H_2O$ 16.4; KH_2PO_4 2.3; ampicillin 0.2. High viscosity sodium alginate from ISP Alginates (Girvan, Ayrshire, UK) and cellulose sulphate, sodium salt, from Acros Organics (New Jersey, NJ, USA) were used as received. Poly(methylene-co-guanidine) hydrochloride from Scientific Polymer Products Inc. (Ontario, NY, USA) supplied as 35% (w/v) aqueous solution was lyophilised prior to use (Bučko et al., 2005).

2.2. Cultivation of cells

Glycerol stocks of recombinant *E. coli* overexpressing cyclopentanone monooxygenase (CPMO, EC 1.14.13.16) from *Comamonas sp.* NCIMB 9872 and *E. coli* overexpressing cyclohexanone monooxygenase (CHMO, EC 1.14.13.22) from *Acinetobacter sp.* NCIMB 9871 were stored in refrigerator at −80 °C. The recombinant *E. coli* strains were kindly donated by Prof. Marko D. Mihovilovic (VUT, Vienna, Austria). Cells were streaked on LB_{amp} agar Petri dish and let grow overnight at 37 °C. A single colony was inoculated to 10 ml liquid LB_{amp} medium and cultivated 12 h at 37 °C on a rotary shaker 200 rpm. 1% (v/v) inoculum was pipetted to TB_{amp} media (37 °C, 200 rpm) and let grow till $OD_{620}=0.6$. Induction of bacteria was carried out by addition of IPTG to final concentration 0.25 mM for 2 h. Biomass was centrifuged for 15 min at 4000xg, 25 °C and used for biotransformation experiment and immobilisation into polyelectrolyte membranes for further biosensor experiments.

2.3. Shake flask Baeyer–Villiger biooxidations

1 g of wet cell weight (0.2 g dry mass) was added to 100 mL of biotransformation medium (phosphate buffer 50 mM, glucose 4 g dm^{-3}, pH=7.0). Biooxidation was initialised by addition of substrate CBCH (1:2, v/v in ethanol) to the final concentration 0.5 g dm^{-3} stirred at 175 rpm at 25 °C. The contents of substrate and products in samples were analysed by GC and biosensor.

2.4. Oxygen electrode instrumentation

Oxygen electrode was provided with a polytetrafluoroethylene membrane attached to the terminal end of the electrode body with an O-ring. The electrode body was filled with 1 M KCl. Electrode was connected with potentiostat and PC with software Bioanalyzer. After each measurement the reactor was thoroughly washed with distilled water. The potential of Pt working electrode was set to −650 mV vs. internal Ag/AgCl reference electrode. The oxygen electrode system including miniaturised oxygen electrode, potentiostat BA1, software Bioanalyzer and reaction chamber was purchased from BVT Technologies (Brno, Czechia).

2.5. Biosensor construction

The biomass was immobilised into PEC matrix forming solid PEC gel membrane. Wet biomass (5% w/w) was resuspended into polyanione solution consisting of (g dm^{-3}): cellulose sulphate 0.9; sodium alginate 0.9; NaCl 0.9. 5 μL of this mixture was pipetted onto a glass Petri dish forming a disk of 5 mm in diameter. The gelation process was performed by pouring over the PEC matrix with the solution consisting of (g dm^{-3}): poly(methylene-co-guanidine) 18; $CaCl_2 \cdot 2H_2O$ 13.25 and NaCl 0.9 for 8 min. Gelated membrane was washed with physiological salt solution, poured with solution of sodium citrate 14.8 g dm^{-3} and NaCl 0.9 g dm^{-3} for 5 minutes and stored in biotransformation medium at 4 °C. The membrane was attached to the calibrated oxygen electrode before measurement by elastic porous polyamide membrane fastened by rubber O-ring. The scheme of biosensor system including electrochemical setup is shown in Fig. S1.

2.6. Biosensor measurement

BVMO-based biosensor experiments were performed in 10 ml of biotransformation medium at 25 °C and stirring speed 950 rpm. After signal stabilisation, aliquots of 1 μL standard solution (95 mM substrate in methanol) were added each 100 s till signal saturation to obtain calibration curve. Real samples from biotransformation were deprived of biomass by centrifugation after sampling from reactor and measured in triplicates undiluted without any further treatment using the method of standard addition.

2.7. Gas chromatography

Concentration of substrates and products was measured by Trace GC Ultra with MS detector ITQ 900 at column Rt®-2330NB, length 105 m, I.D. 0.32 mm, d_f 0.1 μm (Thermo Scientific). Samples from biotransformation were extracted with dichloromethane (1:1, v/v) containing 0.5 mg mL^{-1} methyl benzoate as internal standard and the organic layer was analysed by GC.

3. Results and discussion

3.1. Preparation of biocatalytic solid gel membrane

The various cell concentration of the wet biomass within PEC gel matrix (2.5, 5, 10 and 20%, w/w) and volume of the gel (3, 4 and 5 μL) spread on the circle surface of 5 mm diameter were investigated in relation to the biosensor sensitivity, effective current range and background noise. The highest biosensor sensitivities were achieved for all three gel volumes with biomass content of 5 and 10% and for 3 μL of gel also with biomass content of 20% (Fig. S2). The effective current range decreased with the increase of biomass content for all three tested volumes of gel (Fig. S3). Moreover, the initial current after signal stabilisation of the membrane prepared from 5 μL of gel with biomass content of 20% was zero. We conclude that the cells within membrane were able to consume all of the oxygen before its penetration to the oxygen electrode surface. The noise of biosensor was independent on tested biomass content and for the membrane prepared with 5 μL of the gel was 2 times lower than for the membrane prepared with 3 μL of the gel. Based on these observations the composition of 5 μL 5% (w wet biomass /w matrix) was chosen as optimal for the membrane preparation.

The PEC membrane was made of sodium alginate and cellulose sulphate as polyanions, poly(methylene-co-guanidine) as polycation, calcium chloride as gelling and sodium chloride as anti-gelling agents. The suitability of PEC based membranes for immobilisation of biocatalyst in biosensors was already proved (Yabuki, 2011). Our type of PEC previously appeared in the form of capsules with thin semipermeable PEC membrane and liquid core, originally invented for encapsulation of Langerhans islets (Lacík, 2006), bacterial cells (Hucík et al., 2010) and enzymes (Bučko et al., 2012). The main advantages of PEC membrane, in comparison with ordinary hydrogels such as calcium alginate, include defined adjustment of the membrane size, molecular weight cut-off and mechanical resistance. Moreover, the PEC membrane was proved to be chemically more resistant than calcium alginate against such compounds as sodium L-tartrate, cis-epoxysuccinate and phosphate buffer (Bučko et al., 2005, 2011). PEC matrix used in this work enables preparation of planar PEC membranes that might be stored and easily placed on the electrode just before use.

3.2. Biosensor characteristics

The amperometric transducer fits well with oxygenases based biosensors and the use of miniaturised oxygen electrode system allows not only to increase the detection sensitivity but also to minimise consumption of both samples and standards. The used oxygen electrode system allows setting the gap between the active membrane of the oxygen electrode and the rotating part of disc stirrer resulting in rapid electrode response and noise reduction. Both the laminar flow and the thin diffusion layer assure the stability and reproducibility of the measurement. Typical biosensor response and calibration curve of biosensor based on CPMO are shown in Fig. 1. Time of signal stabilisation after the standard solution addition was about 30 s. The following standard addition

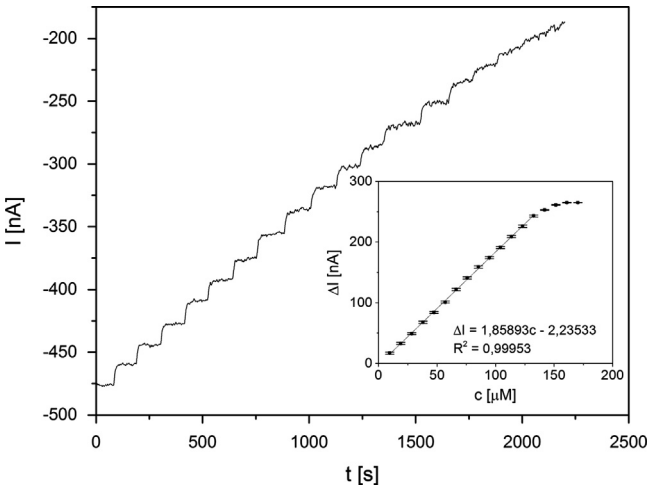

Fig. 1. CPMO biosensor signal as a function of CBCH concentration within the reaction chamber. Measured in phosphate buffer 50 mM, glucose 4 g dm^{-3}, pH=7.0. Inset: calibration curve of CPMO biosensor processed by the least squares method. n=3, error bars shown in the graph.

Table 1
Sensitivities of CPMO and CHMO biosensors to the selected substrates. n=3, RSD=5.0%.

Substrate	CPMO biosensor sensitivity [nA/μM]	CHMO biosensor sensitivity [nA/μM]
CBCH	1.8	1.9
4MCH	1.4	1.1
3MCH	1.2	1.5

was added after 100 s to ensure that the equilibrium was achieved. The linear range of the CPMO biosensor was 8–130 μM of substrate CBCH. In this range the sensitivity was 1.8 nA/μM (RSD=5.0%), the limit of detection was 2 μM (S/N=3) and the noise was only 1 nA.

BVMO enzymes are known for their broad substrate range (De Gonzalo et al., 2010). The sensitivities of both CPMO and CHMO biosensors were examined towards three commercially available BVMO substrates, CBCH, 4MCH and 3MCH (Table 1). Both CPMO and CHMO biosensors were able to detect all three substrates. Sensitivity towards bicyclic substrate was the highest as expected, since this substrate is the most reactive one.

After one week of storage at 4 °C no change in biosensor sensitivity was observed what can be due to the protecting effect of PEC gel matrix. For example, the sensitivity of whole-cell monooxygenase biosensor reported by Zhong et al. (2011) stored at 4 °C after one week achieved about 70% of its initial sensitivity. The linear range of BVMO biosensor was shortened to one third of the initial value after one week of storage. The reason could lie in the swelling of the gel membrane occurring while its storage in the buffer what might cause the membrane volume increase resulting in a reduction of the effective current range due to limited oxygen diffusion through the membrane. At the same time, increasing the thickness of the membrane does not significantly affect the biosensor sensitivity with biomass contents of 5% (Fig. S2).

3.3. Interferences

Ampicillin and glucose as components of medium, citric acid and acetic acid as common co-products of fermentation process, products of BV oxidation (1R5SOBO and 1S5ROBO), ethanol and methanol were identified as possible interferences. The concentration of tested interferences in the measured solution in the

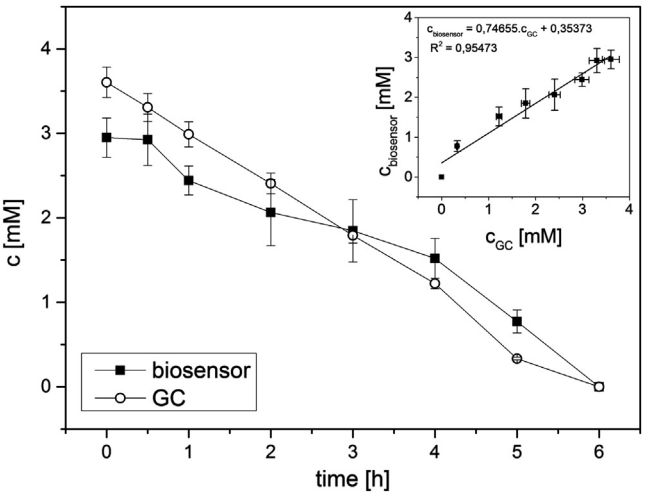

Fig. 2. Real samples measurement. Concentration of substrate CBCH in real samples from biotransformation measured by CPMO biosensor and GC. Inset: Correlation between biosensor and GC measurements of real samples processed by the least squares method. $n=3$, error bars shown in the graph.

reaction chamber was 100 μM for ampicillin, glucose, citric acid, acetic acid and ethanol, and 12 μM for products of BV oxidation, respectively (the stock concentration of tested interferences was 50 mM and 120 mM, respectively, and the stock solution was during addition into reaction chamber diluted 500 times and 10,000 times, respectively). At these conditions, the biosensor response was zero to any of those tested compounds. Pure methanol and ethanol were tested as solvents for standards. There was no response of the biosensor to pure methanol. After addition of 1 μL of pure ethanol into 10 mL of buffer, the biosensor response was 30 nA and there was no response to the following additions of ethanol into the same solution. Pure methanol was used to dilute all standards.

3.4. Analysis of real samples

The measurements were realized at standard conditions, neutral pH=7 and $t=25$ °C, that are typical for both CPMO and CHMO (Schenkmayerová et al., 2012) biotransformation experiments. Real samples were stored in freezer and analysed in triplicates, each measurement with a different membrane. Standard relative deviations were in the range 7–20%. During the measurement of real samples the biosensor sensitivity to the addition of standard solution remained stable within one day. The progress of biotransformation was measured in parallel by BVMO biosensor and GC (Fig. 2).

Given the differences in methods of analyses, sample pretreatment by extraction for GC determination and error of measurement for each method, the obtained correlation is sufficient with respect to the applications in biotechnology and confirms the suitability of biosensor use for rapid monitoring of BV oxidation.

4. Conclusions

In this paper we report development and characterisation of the whole-cell biosensors based on two BVMO enzymatic systems (CPMO and CHMO) and their application in monitoring of BV biotransformation. It is for the first time that the biosensor was used for substrate concentration measurement of BV biooxidation as well as the first use of BVMOs in biosensors. The innovative immobilisation of cells overexpressing BVMO in solid PEC gel membrane made of cellulose sulphate, sodium alginate and poly (methylene-co-guanidine) enabled preparation of disposable biocatalytic membranes with good biocatalyst stability. The biosensor measurement was rapid (up to 30 s) with low sample consumption and the biosensor was successfully used for monitoring of the course of BV biotransformation. Advantages of using miniaturised "mother of biosensors" Clark oxygen electrode was thus demonstrated in the case of BVMOs-based biosensors. The analytical characteristics as time response, linear range, sensitivity, selectivity, reproducibility and stability together with the results of real samples measurement implicate suitability of the developed biosensor for biotransformation monitoring based on BV oxidation as well as for other applications employing detection of substrates of BV biooxidation.

Acknowledgements

This work was supported by the Grants of Slovak Grant Agency for Science VEGA 1/0229/12 and VEGA 2/0127/10 and by the Slovak Research and Development Agency under the Contract No. APVV-0302-10. We thank to Prof. Marko D. Mihovilovic (VUT, Vienna, Austria) for the delivery of the *E. coli* strains overexpressing CPMO and CHMO.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.06.061.

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