Biosensors and Bioelectronics 25 (2009) 482–487

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Leptospirillum ferrooxidans based Fe^{2+} sensor

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ARTICLE INFO

Article history:
Received 20 May 2009
Received in revised form 9 August 2009
Accepted 12 August 2009
Available online 22 August 2009

Keywords:
Bacterial sensor
Leptospirillum ferrooxidans
Ferrous ions

ABSTRACT

A novel electrochemical biosensor integrating the strictly autotrophic bacterial strain *Leptospirillum ferrooxidans* as a recognition element and a Clark type oxygen probe as a transducer was designed, metrologically and analytically characterized and applied for the specific Fe^{2+} determination. The bacterial Fe^{2+} oxidation involves O_2 consumption, thus the quantification was performed registering the decrease of the oxygen reduction current. The limit of detection was found to be 2.4 μmol L^{-1} and the sensitivity of the determinations—3.94 nA L μmol^{-1}. The response time of the biosensor is 18 s for Fe^{2+} concentrations of 10^{-5} to 10^{-4} mol L^{-1}. The biosensor was applied as well for the indirect determination of Fe^{2+} oxidizing species such as $Cr_2O_7^{2-}$, reaching a sensitivity of 2.47 nA L μmol^{-1}.

The transducer characteristics were evaluated and optimized to obtain short response time and high sensitivity.

The analytical performances of the biosensor subject of the present work were found to be similar to that of the *At. ferrooxidans* based one developed by the authors earlier, avoiding however the sulfur compounds interference, because of the substrate specificity of the applied bacterial strain.

1. Introduction

Leptospirillum ferrooxidans is known as an acidophilic chemolithotrophic iron-oxidizing bacterium (Balashova et al., 1974; Harrison, 1984; Harrison and Norris, 1985; Hippe, 2000; Johnson, 2001; Markosyan, 1972; Norris, 1983). Its metabolic activity, similar to that of the most extensively characterized iron-oxidizing acidophil *Acidithiobacillus ferrooxidans* (Colmer and Hinkle, 1947; Harrison, 1984; Kelly and Wood, 2000; Leduc and Ferroni, 1994; Temple and Colmer, 1951; Trudinger, 1971), involves ferrous iron oxidation using molecular oxygen as electron acceptor:

$$4Fe^{2+} + O_2 + 4H^+ = 4Fe^{3+} + 2H_2O \quad (1)$$

Hence, this process could be exploited for Fe^{2+} determination measuring the bacterial oxygen consumption. However, while Fe^{2+} sensors based on *At. ferrooxidans* have been already designed, characterized and described (Mandl and Macholan, 1990; Zlatev et al., 2006a,b), a *L. ferrooxidans* based Fe^{2+} sensor has not been reported until now.

Unlike *At. Ferrooxidans*, growing in both ferrous iron and sulfur media, *L. ferrooxidans* is a strictly autotroph and uses Fe^{2+} as a sole source of energy. The substrate specificity of *L. ferrooxidans* promotes the expectation that the Fe^{2+} sensor based on this bacterium will provide no sensitivity to sulfur compounds, thus overcoming the main drawback of the *At. ferrooxidans* based Fe^{2+} sensors (Mandl and Macholan, 1990; Zlatev et al., 2006a). In addition, *L. ferrooxidans* displays greater affinity to ferrous iron, combined with about 10 times higher tolerance to ferric iron inhibition, compared to *At. ferrooxidans* (Norris et al., 1988).

The goal of the present work is the development and the analytical and metrological characterization of a *L. ferrooxidans* based electrochemical sensor for direct Fe^{2+} determination. Additionally, its application for indirect Fe^{2+} mediated $Cr_2O_7^{2-}$ determination is presented, as well as the characteristics of the Clark type oxygen probe used as a sensor transducer (Zlatev et al., 2006a), which were evaluated and optimized to obtain a maximal sensor response.

2. Experimental

2.1. Instrumentation

A special construction Clark type oxygen probe with a flat front end and uniformly distributed gold microcathodes equipped with a bacterial membrane holder (Zlatev et al., 2006a) connected to an appropriately modified Model LC-4B Amperometric Detector (BAS, USA) was applied for the sensor response registration. An especially developed noise suppression unit (subject of another publication) was connected between the current to voltage converter and the ADC, allowing about 50-fold suppres-

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doi:10.1016/j.bios.2009.08.019

sion of the noise without any signal distortion and response time degradation.

Model Unimax 1010 DT turntable (Heidolf, Germany) and Model 64R temperature controller (Brookfield, USA) were used for the bacterial culture development. Model 350 pH/ion analyzer (Corning, USA) connected to a PC was applied for pH measurements and redox potential monitoring. The Fe^{2+} photometric determinations were carried out applying Stasar III photometer (Gilford, USA).

2.2. Reagents

All the reagents were of analytical grade and were purchased from Merck and Sigma. Deionized water produced by MilliQ system of Milipore was used for the experiments and bacterial culture media preparation.

2.3. Bacterial strain and culture media

The bacterial strain *L. ferrooxidans*, DSM 2705^T, isolated and described by Markosyan (1972) was cultured in DSM medium 882 with the following composition: (i) solution A: $(NH_4)_2SO_4$—132 mg, $MgCl_2{\cdot}6H_2O$—53 mg, KH_2PO_4—27 mg, $CaCl_2{\cdot}2H_2O$—147 mg, H_2O—950 mL; (ii) solution B: $FeSO_4{\cdot}7H_2O$—20 g, H_2SO_4 0.25N—50 mL; (iii) trace element solution: $MnCl_2{\cdot}2H_2O$—62 mg, $ZnCl_2$—68 mg, $CoCl_2{\cdot}6H_2O$—64 mg, H_3BO_3—31 mg, Na_2MoO_4—10 mg, $CuCl_2{\cdot}2H_2O$—67 mg, H_2O—1000 mL. The medium, with final pH adjusted to 1.8, was prepared by mixing solution A and solution B and adding 1 mL of trace element solution. Prior to mix, the solutions were autoclaved separately at 112 °C for 30 min.

2.4. Bacterial cultures development

L. ferrooxidans bacterial cultures were developed in 0.5-L flasks mounted on a turntable at 100 rpm, ensuring thus a moderate bacterial culture oxygenation. The turntable was placed in air thermostated reactor maintaining 30 °C. Photometric determinations of the Fe^{2+} concentration applying the ortho-phenantroline method (Charlot, 1961) were carried out periodically and the redox potential was continuously monitored in parallel. The bacteria development was stopped at the end of the exponential—the beginning of the stationary growth phase (90% of the ferrous ions oxidized), and followed by culture centrifugation. The concentrated bacterial mass was washed, resuspended in deionized water, obtaining protein concentrations between 5 and 10 $\mu g\,L^{-1}$, and kept at 4 °C. It was used for bacterial membrane preparation.

2.5. Fe^{2+} determination and monitoring

2.5.1. Photometric method

Samples of 10 μL were taken from the bacterial culture periodically for Fe^{2+} determination by the ortho-phenanthroline spectrophotometric method. The formed extremely stable over the time red–orange complex absorbs light in the range of 380–580 nm (Charlot, 1961) with a maximal absorption at 505 nm in the pH interval from 2 to 9. The Fe^{2+} concentration was evaluated using a preliminary built calibration curve: light absorption vs. Fe^{2+} concentration.

2.5.2. Potentiometric method (Pesic et al., 1989)

Unlike the photometric method, the potentiometric one allowed continuous monitoring of the Fe^{2+} concentration during bacterial culture development. Very precise control of the bacterial culture development can be achieved, since the redox potential E depends on the Fe^{3+}/Fe^{2+} concentration ratio, according to the Peters–Nernst equation:

$$E = E^{\circ} + \left(\frac{RT}{F}\right) \ln\left(\frac{[Fe^{3+}]}{[Fe^{2+}]}\right) \quad (2)$$

where E° is the standard redox potential, R is the gas constant, T is the temperature, F is the Faraday constant, and $[Fe^{3+}]$ and $[Fe^{2+}]$ are the ionic concentrations at equilibrium.

A Pt wire sensing electrode and Ag, AgCl/3 M KCl reference electrode was applied connected to the PC controlled Corning pH/ion analyzer for Fe^{2+} concentration monitoring.

2.6. Determination of the protein concentration

The Bradford method (Bradford, 1976) was applied initially for the resuspended bacterial mass protein concentration determination. Since this method is complicated and time-consuming, it was replaced by the simpler and more rapid turbidimetric method (Layne, 1957) based on the preliminarily built calibration plot: light absorption–protein concentration determined by the Bradford method.

It was found experimentally that the maximal light absorption by the *L. ferrooxidans* concentrated bacterial mass occurs at 275 nm, in the ultraviolet light range. The absorption value however remained sufficiently high and usable even for wavelengths up to 400 nm. Thus, a standard curve was built at 340 nm using a simple visible photometer, by dilution of an initial concentrated bacterial mass whose protein concentration was previously determined by the Bradford method. The curve appeared to be linear in the concentration range under study up to 25 $mg\,L^{-1}$ of protein.

2.7. Sensor construction and bacterial membrane preparation

The biosensor, including a Clark type oxygen probe as a transducer and a bacterial membrane as a sensing element was fabricated like described in a previous work (Zlatev et al., 2006a).

The bacterial membrane was prepared by filtration of a defined volume of the resuspended bacterial mass through a 0.15 μm pore size Sartorius cellulose filter. After the filtration, the cellulose filter charged with bacteria was attached onto the plastic membrane of the Clark oxygen probe forming a sandwich type bacterial membrane. The bacterial mass was confined between the Clark probe plastic membrane and the cellulose filter, permeable for the oxygen and the analyte.

The biosensor construction was schematically presented and discussed by the authors earlier (Zlatev et al., 2006a; Stoytcheva et al., 2009).

Even kept at 4 °C, the bacteria mortality caused a diminution of the resuspended bacterial mass activity with time, resulting in decrease of the sensor sensitivity and LOD. Although the deviations could be corrected by sensor calibration, the proposed method for bacteria membrane preparation allowed avoiding the great dispersion of the initial sensor characteristics. For this purpose the filtered volume of the resuspended bacterial mass was determined as a function of the bacterial activity. A two steps procedure was applied for the bacterial membranes preparation: (i) determination of the response R_0 to 50 $\mu mol\,L^{-1}$ Fe^{2+} of the sensor with bacterial membrane prepared by filtering of 1 mL or known volume (V_0) of the resuspended bacterial mass; (ii) calculation of the volume V_1 to be filtered to achieve the desired sensor response R_1 applying the equation:

$$V_1 = \frac{(R_1/R_0)}{V_0}. \quad (3)$$