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ScienceDirect
Bioelectrochemistry 71 (2007) 38–45
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Electrical wiring of *Pseudomonas putida* and *Pseudomonas fluorescens* with osmium redox polymers

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Received 9 March 2006; received in revised form 14 August 2006; accepted 16 August 2006
Available online 18 August 2006

Abstract

Two different flexible osmium redox polymers; poly(1-vinylimidazole)$_{12}$-[Os-(4,4′-dimethyl-2,2′-di'pyridyl)$_2$Cl$_2$]$^{2+/+}$ (osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$ (osmium redox polymer II) were investigated for their ability to efficiently "wire" *Pseudomonas putida* ATCC 126633 and *Pseudomonas fluorescens* (*P. putida* DSM 6521), which are well-known phenol degrading organisms, when entrapped onto cysteamine modified gold electrodes. The two Os-polymers differ in redox potential and the length of the side chains, where the Os$^{2+/3+}$-functionalities are located. The bacterial cells were adapted to grow in the presence of phenol as the sole source of organic carbon. The performance of the redox polymers as mediators was investigated for making microbial sensors. The analytical characteristics of the microbial sensors were evaluated for determination of catechol, phenol and glucose as substrates in both batch analysis and flow analysis mode.

Keywords: Osmium redox polymers; Microbial biosensor

1. Introduction

Microbial cells have a number of advantages as biological sensing materials in the fabrication of biosensors. They are present ubiquitously, are able to metabolise a wide range of chemical compounds and have a great capacity to adapt with unfavourable conditions and to develop the ability to metabolise new chemicals. Microbes are also susceptible for genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes. Purified enzymes, biological elements with high specific activities and high analytical specificity, are expensive and unstable and in this regard, the utilisation of whole cells as a source of intracellular enzymes avoids the lengthy and expensive operations of enzyme purification, preserves the enzyme in its natural environment and protects it from inactivation by external toxicants. Whole cells also provide a multipurpose catalyst especially when the process requires the participation of a number of enzymes in sequence [1–4]. The major limitation of microbial biosensors as compared to enzyme sensors is the slow response, which has been attributed to diffusional problems associated with the cell membranes. It is well known that microbial cells are able to reduce small redox compounds such as ferricyanide, dichlorophenolindophenol, and other organic dyes in the presence of organic compounds such as glucose and ethanol [5]. This indicates that microbial cells are able to catalyse the oxidation of the mentioned substrates using redox compounds as electron acceptors [6]. To follow electrochemically such processes through mediated electron transfer from microbial systems to electrodes represents a promising alternative to the use of a Clark electrode [7,8]. Perturbations in microbial respiration due to changes in substrate or microbial concentration have previously been detected via the interaction of redox mediators at electrochemical transducers and found the basis for a number of devices. Establishing a method for quantitative evaluation of intact cells as biocatalysts is beneficial for developing more advanced biofuel cell systems, whole cell-based

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doi:10.1016/j.bioelechem.2006.08.001

biosensors and bioreactors [5] and also provides useful information concerning the enzymatic reactions proceeding within the intact cells under physiological conditions. Rapid detection of the concentration of bacteria was achieved using redox-mediated amperometry [9], which is free from influence by the turbidity of the bacterial cell suspension and allows the measurements of the consumption rates of artificial dyes by bacterial catalysis [10]. Mediated whole-cell biosensors have also been developed for on-line pesticide screening [11]. The reduction of a range of redox mediators by bacteria [12], including the reduction of ferricyanide by *E. coli* [13], have been studied to identify the most effective mediator-microorganism combinations for utilising substrates in microbial fuel cells [5,14,15]. Electron mediators perform a special function in biosensors; their role is to replace the natural electron acceptor usually oxygen, thus preventing the process from the problem of having a low oxygen concentration. An advantage of applying mediators is that the amperometric measurement can be performed at a less drastic potential, which reduces the possibility of interfering reactions to contribute to the response signal and thus enhancing selectivity. A notable number of mediator type biosensors based on either enzyme or whole microbial cells have been developed. Aqueous freely soluble mediators such as ferricyanide and *p*-benzoquinone, as well as less aqueous soluble mediators including ferrocenes have successfully been used in these systems [16,17].

Since the first applications of osmium redox polymers for reagentless mediated biosensing were described [18–20], polymeric mediators still attract attention due to the efficient electron shuttling properties combined with the polymeric structure promoting a stable adsorption as well as a possibility for multiple layers of immobilised enzymes as well as microbial cells on the electrode surface [21]. In developing biosensors, polymers containing dispersed redox centres are promising because of their synthetic flexibility and the ability to control the formal potential ($E^{\circ\prime}$), and hence the electron transfer properties [19,21].

In this work, *Pseudomonas fluorescens* (*Pseudomonas putida* DSM 6521) and *P. putida* ATCC 126633, which are well-known phenol degrading organisms, were wired with two different Os-polymers, one polymer with a high $E^{\circ\prime}$-value but with a restricted length of the side chains (poly(1-vinylimidazole)$_{12}$-[Os(4,4-dimethyl-2,2-di'pyridyl)$_2$Cl$_2$]$^{2+/+}$, osmium redox polymer I) [19], and one with a low $E^{\circ\prime}$-value but with long-side chains and with a much higher flexibility (poly (vinylpyridine)-[Os-(*N*,*N*-methylated-2,2-biimidazole)$_3$]$^{2+/3+}$, osmium redox polymer II), [22,23]. The positive effect of increasing the chain length of the side chain containing the mediator by the end of the side chain, was shown by Mao et al. in a recent publication [23], where the efficiency of wiring glucose oxidase by two different Os-polymers having the same $E^{\circ\prime}$-value was compared. One of the Os-polymers contained the Os-functionality at the end of a short length side chain and the other at the end of a long side chain. The two types of osmium redox polymers (osmium redox polymer I and II) were used recently, for electrical wiring of pyranose oxidase to graphite [22]. Osmium redox polymer I was recently shown also to wire whole living gram-negative bacteria *Gluconobacter oxydans* cells [24].

In this work the bacterial cells were entrapped together with either of these two redox polymers behind a dialysis membrane onto the surface of cysteamine modified gold electrode to form microbial biosensors. The response characteristics for catechol, phenol and glucose of the biosensors were investigated in both batch and flow mode.

2. Material and methods

2.1. Reagents

Poly(1-vinylimidazole)$_{12}$-[Os(4,4′-dimethyl-2,2′-di'pyridyl)$_2$ Cl$_2$]$^{2+/+}$(osmium redox polymer I) and poly(vinylpyridine)-[Os-(*N*,*N*′-methylated-2,2′-biimidazole)$_3$]$^{2+/3+}$(osmium redox polymer II) were generously provided as gifts from TheraSense (Alameda, CA, USA). Phenol, catechol, glucose, and cysteamine were purchased from Merck AG (Darmstadt, Germany). A 5 mM cysteamine solution was prepared by dissolving the appropriate amount in ethanol. All other chemicals were of analytical grade and used without further purification. Solutions used for immobilisation were prepared in ultrapure distilled water (Millipore, Milford, CT, USA) and the others used as substrate were in working buffer. Dialysis membranes with a cut-off of 6000–8000 Da were used.

Mineral standard medium (MSM) with the following compositions were used as growth media for *P. putida* (A) and *P. fluorescens* (B), respectively;

A. 0.1% NH_4NO_3, 0.05% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4{\cdot}7H_2O$, 0.15% K_2HPO_4, 0.05% KH_2PO_4, 0.0014% $CaCl_2{\cdot}2H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$ and trace element solution (1 ml/L), [25,26].
B. 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4{\cdot}7\ H_2O$, 0.005% $CaCl_2{\cdot}2H_2O$ and trace element solution (10 ml/L), [27]. The pH of the growth media was adjusted to 6.9.

2.2. Biological materials

P. fluorescens (*P. putida* DSM 6521) and *P. putida* ATCC 126633 were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and CCM (Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic), respectively, and were sub-cultured in nutrient agar. Adaptation of the cells to phenol (250 mg/L) was performed by gradually increasing the phenol and decreasing the glucose (250 mg/L) concentrations by daily inoculations until the medium contained 250 mg/L phenol. When the cells were grown, the biomass was harvested by centrifugation at 10,000 *g*, suspended in MSM and then re-centrifuged. The cellular paste was used for making the biosensor [25].

Cell growth was followed spectrophotometrically by measuring the optical density at 560 nm and the relationship between optical density and the living cells was also investigated [25]. In all experiments, log-phase bacterial cells were used. Daily prepared enzyme electrodes including fresh cells were used in all experimental steps.