Development of a microbial biosensor based on carbon nanotube (CNT) modified electrodes

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Abstract

Pseudomonas putida DSM 50026 cells were used as the biological component and the measurement was based on the respiratory activity of the cells estimated from electrochemical measurements. The cells were immobilised on carbon nanotube (CNT) modified carbon paste electrodes (CPE) by means of a redox osmium polymer, viz. poly(1-vinylimidazole) 12-[Os-(4,4'-dimethyl-2,2'-dipyridyl)2Cl2]2+/+. The osmium polymer efficiently shuttles electrons between redox enzymes located in the cell wall of the cells and promotes a stable binding to the electrode surface. The effect of varying the amounts of CNT and osmium polymer on the response to glucose was investigated to find the optimum composition of the sensor. The effects of pH and temperature were also examined. After the optimisation studies, the system was characterised by using glucose as substrate. Moreover, the microbial biosensor was also prepared by using phenol adapted bacteria and then, calibrated to phenol. After that, it was applied for phenol detection in an artificial waste water sample.

Keywords: Biosensor; Osmium redox polymer; Carbon nanotube (CNT); Pseudomonas putida

1. Introduction

The recent discovery of the carbon nanotube (CNT) has attracted considerable attention due to their dimensions and structure sensitive properties. The high electrical conductivity of these nanoparticles allows the utilisation of CNTs as electrode material and in combination with its strong electrocatalytic activity offer the ability to mediate electron transfer reactions [1,2]. The facility of electron transfer between the electroactive species and the electrode offers great promise for fabricating chemical sensors or biosensors [3,4]. Besides using CNTs, the introduction of redox mediators (RM) i.e., redox active compounds able to shuttle electrons between the active site of redox enzymes and an electrode replacing the natural co-substrate of the enzyme and when incorporated into biosensor structures, reagentless second generation amperometric biosensors can be obtained.

RMs based on various Os2+/3+-complexes have been successfully introduced into high molecular weight, yet highly flexible and aqueous soluble polymers [5] that form electrostatic complexes with redox enzymes to form hydrogels allowing both very efficient charge transfer reactions between the enzyme and the mediators as well as between the dispersed Os2+/3+ redox centres and additionally allow fast diffusion of both enzyme substrate and product within the hydrogel. These hydrogels can be further stabilised by forming covalent linkages between the redox polymer and the protein promoting a stable immobilisation of both mediator and enzyme as well as the possibility for multiple layers of immobilised protein molecules on the electrode surface [6]. Moreover, it is possible to control through the use of different ligands the formal potential of the Os2+/3+ redox functionality and hence the electron transfer properties [7,8]. For these reasons, since the first usage of...
osmium redox polymers for reagentless mediated biosensors [9–11], polymeric mediators still attract attention.

Whole microbial cells as well as isolated enzymes are frequently used for the construction of biosensors and transformation of organic compounds in bioreactors [12,13]. It is well-known that microbial cells are able to catalyse the oxidation of such organic compounds as glucose and ethanol via redox compounds acting as electron acceptors [14]. Mediated electron transfer from microbial systems to electrodes represents a promising alternative to the use of Clark electrodes [15,16]. The same basic principle can also be used in the operation of microbial fuel cells (MFCs), which are bio-electrochemical transducers that convert microbial reducing power (generated by the metabolism of organic substrates), into electrical energy [17,18]. Moreover, 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 are the basis for a number of devices [19–24].

Mediated whole-cell biosensors based on *Gluconobacter oxydans* and poly(1-vinylimidazole)12-[osmium-(4,4'-dimethyl-2,2'-dipyridyl)2Cl]2+/+/+ [25] and also based on the bacteria *Pseudomonas putida* ATCC 126633 and *Pseudomonas fluorescens* were previously developed using two different flexible osmium redox polymers; poly(1-vinylimidazole)12-[osmium-(4,4'-dimethyl-2,2'-dipyridyl)2Cl]2+/++ and poly(vinylpyridine)-(osmium-(N,N'-methylated-2,2'-biimidazole)3)2+/+/3+ by our group and the efficiency of these polymers for “bacterial wiring” was investigated [26]. In this work, CNT and poly(1-vinylimidazole)12-[osmium-(4,4'-dimethyl-2,2'-dipyridyl)2Cl]2+/+/+ (Fig. 1) were utilised together as a part of a microbial biosensor and the possibility to facilitate electron transfer reactions by these two systems were explored for the first time as far as we know. The integration of an osmium redox polymeric mediator in combination with CNTs as parts of a mediated microbial biosensor has not been accomplished before to the best of our knowledge.

In the present work, whole viable *P. putida* DSM 50026 cells were immobilised on CNT modified carbon paste electrode (CPE) using the osmium redox polymer. After investigating the effect of the amount of CNT and Os redox mediator on the sensor response, the biosensor was characterised using glucose as substrate. Moreover, the system was also calibrated for phenol using bacterial cells adapted to phenol as the biological material.

2. **Material and methods**

2.1. **Reagents and materials**

Poly(1-vinylimidazole)12-[Os-(4,4'-dimethyl-2, 2'-dipyridyl)2Cl]2+/+/+ was generously provided as a gift from Thermosense (Alameda, CA, USA). Multiwall CNT (diameter; 110–170 nm, length; 5–9 μm), mineral oil and graphite powder were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without any pre-treatment since further purification with hot strong acids and treatment with ultrasonic bath had no significant effect on the sensor response. Standard solutions of D-(+)-glucose, D-(+)-galactose, and phenol were of analytical grade and were prepared by dissolving appropriate amounts in the working buffer solutions. Dialysis membranes with a cut-off of 6000–8000 Da were used.

Mineral salt medium (MSM) with the following composition was used as growth medium for *P. putida*; 0.1% NH₄NO₃, 0.05% (NH₄)₂SO₄, 0.05% NaCl, 0.05% MgSO₄·7H₂O, 0.15% K₂HPO₄, 0.05% KH₂PO₄, 0.0014% CaCl₂·2H₂O, 0.001% FeSO₄·7H₂O and trace element solution (1 ml/L). The pH of the growth medium was adjusted to 6.9 [27]. *P. putida* DSMZ 50026 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and was sub-cultured in nutrient agar.

Then, the cells were inoculated into 50 mL of MSM containing 250 mg/L glucose and incubated at 28°C on an orbital shaker at 150 rpm. Cells adapted to 250 mg/L phenol were obtained 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 10000×g, suspended in MSM and then re-centrifuged. The cellular paste was used for making biosensors [28]. All optimisation studies were performed with non-adapted cells using glucose as substrate. However, the adapted cells were used only to calibrate the system to phenol and for sample application. Cell growth was followed spectrophotometrically by measuring the optical density at 560 nm and the relationship between the optical density and bacterial mass was also investigated [25]. In all experiments, log-phase bacterial cells were used. Daily prepared electrodes with fresh cells were used in all experimental steps.

Artificial waste water of a highly acidic and salty nature included 50 g/L NaCl and 100 g/L phenol in 1.0 M HCl solution [27].

![Fig. 1. Chemical structure of osmium redox polymer [poly(1-vinylimidazole)12-[Os-(4,4'-dimethyl-2,2'-dipyridyl)2Cl]2+/+/+].](image-url)

*Fig. 1. Chemical structure of osmium redox polymer [poly(1-vinylimidazole)12-[Os-(4,4'-dimethyl-2,2'-dipyridyl)2Cl]2+/+/+].*
2.2. Apparatus

Chronoamperometric experiments were carried out with a Radiometer electrochemical measurement unit (Lyon, France), where the CNT modified CP electrode was used as the working electrode. An Ag/AgCl electrode (with a 3.0 M KCl solution saturated with AgCl as the internal solution, Radiometer Analytical, REF321) and a Pt wire (Radiometer Analytical, M241PT) were used as reference and counter electrodes, respectively. The electrodes were inserted into a conventional electrochemical cell (25 mL) through its Teflon cover. Cyclic voltammograms of electrodes prepared without bacterial cells were obtained with a PalmSens electrochemical measurement system (Palm Instruments, Houten, The Netherlands) in a three-electrode configuration, as described above.

2.3. Preparation of biosensor

Proper amounts of graphite powder, CNT and mineral oil (66%:8%:26%) were mixed manually to obtain the CNT modified CP electrode. Then, a 2 µL portion of a solution of the osmium redox polymer (10 mg/mL) in distilled water was spread over the surface of the CNT modified CP electrode and water was allowed to evaporate at ambient conditions (20–25 min). In the following step, a 2 µL aliquot of the cellular paste (with a bacterial mass of 0.3 g/L), was evenly spread on top of the electrode and dried-up for 1 h at room temperature. The modified layer was covered with a dialysis membrane, pre-soaked in water. The membrane was fixed tightly with a silicone rubber O-ring.

2.4. Measurement

Amperometric measurements were performed at an applied potential of +0.3 V [26] and with constant magnetic stirring. The microbial sensor was initially equilibrated in MSM (mineral salt medium) solution and phosphate buffer, respectively. After 30 min, substrates were added individually to the reaction cell. Nitrogen was passed through all solutions before use. The biosensor responses were registered as current densities (nA/cm²) using the geometrical surface of the CNT-modified carbon paste electrode for calculations.

Control experiments were done using entrapped cells behind a dialysis membrane on the electrode, however, without osmium polymer and no response signal was observed at the working potential in the presence of substrate.

3. Results and discussion

An electrochemical mediator was employed to make electron transfer from the microbial cells to the electrode possible. In the absence of mediator there is no response current registered in this system. There is, however, currently no general agreement on the mechanism of the individual reactions behind this electron transfer, however, the most common belief is that monomeric mediators penetrate the bacterial cell wall in their oxidised form and interact with reducing agents within the cell becoming reduced themselves [18]. The reduced mediator is then capable of diffusing out of the cells to the electrode surface, where it is electrochemically reoxidised. The oxidised mediator is then free to repeat this cycle. The cycling continually drains off the portion of metabolic reducing power (electrons) to give electrical power at the electrodes [29,30]. However, bacteria can also produce redox mediators themselves, which can occur in two ways: through the production of organic, reversibly reducible compounds (secondary metabolites) and through the generation of oxidisable metabolites (primary metabolites) [31]. Several microbial species have been reported to release electrons to an electrode directly or with the use of their electroactive metabolites [32,33]. Different types of microbial fuel cells, which are evolving to become a simple, robust technology, were designed without mediators especially for wastewater treatment [34]. Polymeric mediators such as the osmium polymer have been successfully used to wire a series of different redox enzymes and applied for construction of a variety of biosensors [6–8,10,11,25,26,35]. We have, however, shown that proper mediators like Os-based polymeric mediators may assist in shuttling electrons between the intracellular bacterial space and an electrode [25,26].

In the present work, the effect of the electron transfer efficiency of the Os polymer was expected to be promoted by adding CNTs into the composite structure. One of the main reasons for using CNTs in sensor fabrication is that they promote electron transfer reactions [4]. For this reason before the characterisation of the system, the effect of the amounts of CNT and Os polymer on the current response was examined.

In order to observe this effect, two different electrodes, one with CNT and the other without CNT were prepared. Fig. 2 shows cyclic voltammograms corresponding to (A) a CNT modified CPE and (B) a plain CPE. Though the highest peak to peak separation was obtained with the CNT modified electrode, \( \Delta E_p = 46 \text{ mV} \) and \( \Delta E_p = 60 \text{ mV} \) for plain and CNT modified CPE respectively, substantially higher current values were observed with the CNT modified CPE (for plain CPE \( i_{\text{anodic}} = 28.00 \mu\text{A} \) and \( i_{\text{cathodic}} = 33.84 \mu\text{A} \) while for CNT modified CPE \( i_{\text{anodic}} = 51.83 \mu\text{A} \) and \( i_{\text{cathodic}} = 46.39 \mu\text{A} \)). Higher current values can be attributed to the efficient electron transfer property of the CNTs. Besides, analysis of the faradic currents as a function of the scan rate resulted in a linear \( I_p \) versus \( v^{1/2} \) relationship over 0.01–0.15Vs⁻¹ range (better slope with CNT) indicating that the current is controlled by a semi-infinite linear diffusion (data not shown). The slightly higher \( \Delta E_p \) for the CNT modified paste may also be due to a somewhat higher ohmic drop as the current is higher.
3.1. Effect of amount of CNT

The amount of CNT present in the CPE electrode has a direct and expected effect on the resulting current values. This was shown using three different biosensors that contain 0%, 8%, 16% CNT and utilised for glucose detection. Fig. 3 demonstrates the influence of the amount of CNT upon the current density. The current values increased almost 2.5-fold when 8% CNT was introduced into the composite. However, when this amount was doubled (16%), a decrease was obtained. These results are in correspondence with previous work [36]. The enhanced current values can be attributed to the high local density of the electronic states in CNT. On the other hand, the decrease in current density is in accordance with a large surface area of CNT that increases the background current [36]. Moreover, changes in composite structure and hence charge transfer properties could lower the current values. Similar findings were obtained in previous work [37] in which a polypyrrole-multiwall carbon nanotube-glucose oxidase biosensor was optimised and the influence of the amount of CNT on the glucose response was investigated. The signal was found to increase gradually with the amount of MWCNT up to a defined level and then, a decrease was observed at higher levels.

3.2. Effect of pH

The effect of pH on the electrode response was investigated through the use of three different 50 mM buffer systems; phosphate (pH 6.0–8.2), tris–HCl (pH 8.0–9.0) and Na-borate (pH 9.5, 10.0). The current response of the microbial electrode to glucose increases significantly from pH 6.0 to 9.0 (especially from 7.5 to 8.0) and then a sharp decrease is obtained at pH values higher than 9.0, (Fig. 4). As a result, pH 9.0 was chosen as optimum pH and further studies were conducted by using the tris–HCl buffer. Furthermore, in order to estimate the influence of CNT on the optimum pH, a microbial electrode was prepared without CNT and as can be seen in Fig. 4, virtually the same pH profile was obtained with the same optimum pH proving that the presence of CNT did not cause any change in this value. In our previous work, the optimum pH of a biosensing system based on the Os redox polymer and P. putida immobilised on a cysteamine modified gold electrode was obtained at 7.0 using glucose as substrate. The difference from the current system might be due to differences in surface properties [38]. In addition, a difference in pH optima depending on the structure of the redox polymers was also observed in our previous study on wiring pyranose oxidase [39].

3.3. Effect of temperature

The amperometric response of the microbial sensor to glucose was measured under optimum working conditions (in tris–HCl buffer, pH 9.0), at different temperatures...
varied from 20 to 40 °C and the results are shown in Fig. 5. From 20 to 35 °C, the steady state current increases with temperature. At temperatures higher than 35 °C, a decrease in the amperometric response was observed. This drop was a result of the loss of activity caused by the higher temperatures. On the other hand, as can be seen from Fig. 5, the amperometric response of the biosensor at 30 °C and at 35 °C was very similar to each other. Hence, in order to keep the system more stable, 30 °C, which is very close to the growth temperature of the bacterium (28 °C), was used in further studies.

3.4. Effect of amount of Os polymer

Biosensors that contained 1, 2 and 5 μL of Os polymer were prepared and their responses to glucose were investigated (Fig. 6). As can be seen from the figure, the current density values were increased as the amount of Os redox polymer was increased. However, when this amount was increased from 2 to 5 μL, a sharp decrease was observed. This might be due to the diffusion problem of electrons to the Os centres from the bacteria. Similar data were also observed in our previous work when wiring pyranose oxidase with Os polymers [39] and by Heller et al., when wiring glucose oxidase [40].

3.5. Analytical characteristics

The analytical characteristics of the developed microbial sensor were examined under optimised conditions. A linear calibration graph between current density and substrate concentration was obtained in the range between 0.05-2.0 mM glucose with a response equation of \( y = 131.32x + 17.46 \) (\( R^2 = 0.993 \)) and a response time of 35 s. Moreover, the biosensor response was also investigated for galactose and a linear calibration graph was observed in the range of 0.5–6.0 mM with a response equation of \( y = 42.68x + 3.02 \) (\( R^2 = 0.999 \)) and a response time of 180 s.

Phenol adapted bacterial cells were also used as the biological material for phenol detection, a linear relationship between the sensor response (nA/cm²) and phenol concentration was observed in the range of 0.5–4.0 mM. A linear graph defined by the response equation \( y = 21.87x + 11.53 \) with a correlation coefficient \( R^2 = 0.991 \) was obtained with a response time of 50 s.

The reproducibility of the biosensor was tested for 1.0 mM glucose (n = 8) and the standard deviation (SD) and variation coefficient (c.v) were calculated as 1.018 ± 0.03 mM and 2.8%, respectively.

3.6. Sample application

The developed biosensor was used for phenol detection in waste water samples. Synthetic waste water samples with a highly acidic and salty nature were prepared according to a previous work [28]. Artificial waste water samples were used instead of stock substrate phenol solutions and known amounts of the sample (1 and 2 mM) were added into the reaction cell containing working buffer solution respectively and then the signal was measured. From the calibration curve, the amount of phenol was calculated as 1.05 ± 0.21 μM and 2.08 ± 0.48 μM, respectively. Results are expressed as the mean ± SD, (n = 3). Furthermore, recoveries were calculated and found to be 105% and 104%, respectively. Apparently, we can conclude that no sample matrix effect due to the salty and acidic nature interfered in the measurements. The developed biosensor shows promising results to be used also in the analysis of similar waste water samples without requiring previous pretreatment.

3.7. Stability

The operational stability was investigated for 1 mM glucose by measuring the sensor response for 6.5 h at
optimised conditions. During 5 h, no decrease was observed in the response and after 6.5 h, only a 20% decrease was obtained whereas the biosensor without CNT lost 30% of its activity during the same period. The improved operational stability of the CNT-based electrode can be attributed to a faster charge transfer at the CNT [41]. In addition, the fast diffusion of species away from the CNT and the protection of the bacterial cells by the Os polymer matrix also contribute to the improved stability of the CNT-Os polymer biosensor.

4. Conclusion

Whole cell \textit{P. putida} biosensors using Os-redox polymers could be good alternatives for the analysis of different substrates such as glucose as well as xenobiotics in the absence of oxygen with high sensitivity because of the fast electron collection efficiency between the Os-redox polymer and the bacterial cells. In this work, a microbial biosensor was developed by introducing CNT with an Os polymer and a living microorganism. Although the introduction of redox mediators into the CNT-based sensors is defined as redundant and complex by some researchers [41,42], the benefit of this concept has to be explored. The use of optimum amounts of CNT and the Os redox mediator provides better sensor sensitivity by promoting the electron transfer within the structure of the biosensor. The main disadvantages are the high surface area of CNT that increases the background current [41] and the diffusion problem of electrons that occurs due to overlapping of the diffusion layers formed at closely spaced CNT in the film. However, these problems may be overcome by optimising the CNT and polymer amounts.

The proposed system does not require any complicated immobilisation procedure for the construction of the biosensor. The integration of CNT to the system provides more sensitive results that can be very important for the usage of the developed sensor in microbial fuel cell studies and BOD measurements.

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