Bacterial sensors based on chitosan matrices

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A B S T R A C T

Bacterial cells were entrapped together with chitosan matrix onto the surface of graphite electrode to form microbial biosensors. Two types of bacterial sensors were constructed by using Pseudomonas fluorescens (Pseudomonas putida DSM 6521) and P. putida DSM 50026 cells as the biological components and the measurements were based on the respiratory activity of the cells. Carbon nanotube (CNT)-modified chitosan membrane was also prepared to test nanoparticle effect on the efficiency of biosensor performances. As well as the response characteristics, stabilities and substrate specificities were investigated. Data were given as the comparison of CNT-modified and -unmodified systems.

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1. Introduction

Microbial cells have been immobilized to use in bioreactors, and production of useful compounds such as amino acids, organic acids, antibiotics, hydrogen, steroids, exopolysaccharides and enzymes [1–7]. And also a promising approach in constructing biosensors is the use of microbial cells as the basis of the recognition elements [8]. Microbial biosensors have some advantages as follows: The enzyme does not need to be isolated, microbial cells are more tolerant to inhibition by solutes and sub-optimal pH or temperature. Moreover, they are durable than enzyme electrodes because enzymes are more stable in their natural environment in the cells [9–11]. Furthermore, microbes are susceptible to genetic modifications through mutation or through recombinant DNA technology and serve as an economical source of intracellular enzymes [12].

Bacteria belonging to the genus Pseudomonas are in the γ subclass of the Proteobacteria and contain mostly fluorescent Pseudomonas sp. as well as a few non-fluorescent species. Pseudomonas fluorescens and Pseudomonas putida are ubiquitous bacteria. They are frequently present in water, in soils and especially in the plant rhizosphere [13] and have been extensively used for biosensor construction [14–18].

Carbon nanotubes (CNTs) are a new type of carbon materials and can be considered as the result of folding graphene layers in to carbon cylinders [19]. CNTs are utilized as an electrode material because of the high electrical conductivity [20].

Recent studies demonstrated that these nanoparticles enhance the electrochemical reactivity of important biomolecules and can promote the electron transfer reactions of proteins [21]. A major problem is the insolubility of CNTs in solvents. Therefore, polymeric materials such as nafion, poly(propionylethylenimine-co-ethylenimine), tocopheryl polyethylene glycol succinate, chitosan, poly(metaphenylenevinylene) and helical amilose are used in the preparation of CNT solutions [22–27]. In this study, bacterial cells were immobilized onto the graphite electrode via chitosan matrix. Two types of bacterial sensors were assembled by using P. fluorescens and P. putida cells. The response characteristics, stabilities and substrate specificities were investigated as well as the effect of CNT as a modifier in the biosensing system.

2. Experimental

2.1. Reagents

d-Glucose, d(+)mannose, d(+)galactose, d(+)xylose, β-lactose, chitosan and glutaraldehyde were purchased from Sigma Chem. Co. (St. Louis, MO, USA, www.sigmaaldrich.com), multi-walled carbon nanotube (diameter; 110–170 nm, length; 5–9 μm, 90%) were obtained from Aldrich (Dorset, UK, www.sigmaaldrich.com), d(−)-fructose, N-acetyl d-glucosamine, maltose monohydrate were purchased from Fluka (Steinheim, Germany, www.sigmaaldrich.com). All other chemicals were analytical grade.

Mineral salt medium (MSM) with the following compositions was used as growth media for P. fluorescens (A) and P. Putida (B), respectively:
2.2. Apparatus

Chronoamperometric experiments were carried out with a Radiometer electrochemical measurement unit (Lyon, France, www.radiometer.com) where the modified graphite electrode was used as a working electrode, an Ag/AgCl electrode (with 3 M KCl saturated with AgCl as the internal solution, Radiometer Analytical, REF321) and platinum electrode (Metrohm, Switzerland, www.metrohm.com) were used as reference and counter electrodes, respectively. The electrodes were inserted into a conventional electrochemical cell (20 mL) through its Teflon cover.

Scanning electron microscope (JEOL JSM 5200 SEM, Tokyo, Japan) was used for surface imaging of the microbial electrodes.

2.3. Biological material

Preparation of biological material includes the following steps; microbial cells were sub-cultured in nutrient agar. Then, transferred to MSM containing 1 g/L glucose. After 16 h, the biomass was harvested by centrifugation and suspended in MSM and then re-centrifuged. The supernatant was removed and the cellular paste was used for the construction of biose.sor. Bacterial cells in logarithmic phase were used during the experiments and the cell growth was followed spectrophotometrically via measuring optical density at 560 nm [33,34]. Daily prepared microbial electrodes including daily inoculated fresh cells were used in all experimental steps.

2.4. Biosensor preparation

For the construction of the bioactive layer on the top of the graphite electrode, first the spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, 3.05-mm diameter and 13% porosity) were polished on wet emery paper (Tufback Durite, P1200) to obtain a smooth surface, washed thoroughly with distilled water and sonicated for 2 min, and then rinsed again with water and dried in an oven at 105 °C [35].

20 µL CHIT or CHIT–CNT solutions were placed on top of the polished end of the electrode and allowed to dry at +4 °C for 18 h. Then 20 µL of the cellular paste and glutaraldehyde solution (with the proper dilution with potassium phosphate buffer, pH 7.0) were spread evenly onto the chitosan-modified graphite electrode and allowed to stand at ambient conditions for 30 min. Then, it was washed with distilled water and phosphate buffer several times to remove unbound cells.

2.5. Measurements

The principle of measurement was based on the following of oxygen consumption due to the respiratory activity of microorganisms in the presence of glucose. Oxygen was reduced electrochemically by the working electrode by applying a potential of −700 mV versus the Ag/AgCl reference electrode. All experiments were carried out at 30 °C under continuous and constant magnetic stirring. After each run, the electrode was washed with distilled water and kept in working buffer solution at 30 °C for 10 min and working buffer solution has been changed after each measurement.

When the current density reached a steady-state, the substrate solution was added to the working buffer solution (50 mM potassium phosphate buffer, pH 6.0) until reached a new steady-state. The sensor response was defined as the difference between first and second steady-state currents and registered as current density (µA/cm²).

3. Results and discussion

Different types of microbial sensors were presented in our previous works in which P. fluorescens and P. putida cells were immobilized on the surface of different transducers by means of various polymeric membranes such as gelatin and osmium redox polymers to construct mediated and non-mediated microbial systems [12,17,18,28,29,34]. Different matrices such as conductive polymers were also tested as a new immobilization platform to fabricate efficient microbial sensors for different analytes [33]. In the present study, microbial sensors based on intact P. putida and P. fluorescens cells were characterized and the effect of CNT on the electrode responses was investigated.

Carbon nanotubes are new materials with unique properties. To expand and optimize the application of CNTs in biotechnological areas, it is required to functionalize CNTs with various biomaterials. Biomolecules, biopolymers and other biomaterials have been attached to CNTs successfully [31]. Chitosan, the primary derivative of chitin, is a natural polymer [36] and obtained by N-deacetylation to a varying extent that is characterized by the degree of deacetylation, and is consequently a copolymer of N-acetylglucosamine and glucosamine [37]. Chitin and chitosan are used in a wide range of applications, such as in food and beverage, agriculture, water and waste treatment, cosmetics, material science, biotechnology, drugs and biopharmaceuticals, and recently in gene therapy too [38,39]. It has been widely applied due to its good biocompatibility, film-forming availability, biodegradability to harmless products, non-toxicity, physiologically inertness and hydrophilicity [40,41]. Compared with other solvents, chitosan can prevent biological molecules from denaturation [41].

It was previously reported that the controlled decoration of CNTs with chitosan created new chitosan–CNT nanomaterials with combined features [31]. Many papers dealing with CNT-modified chitosan–based enzyme biosensors have been published [42,43,41,44–46]. However, to our knowledge the chitosan and CNT combination has not been used to prepare any electrochemical bacterial biosensors. According to our findings it can be said that chitosan could be a good alternative to keep the microbial cells alive onto the electrode surface during the operational conditions. Additionally, morphologies of microbial electrodes reveal the most precise information about the cells and matrices used in the system. Scanning electron microscopy technique is used to detect the surface characteristics of the membrane and to show the interaction between biological materials and immobilization platforms. In this part, morphologies of both modified and unmodified matrices for both different types of cells were detected (Figs. 1 and 2). In
order to remove unbound cells, the electrode surfaces were washed before analysis. As can be seen from the micrographs, CNT modification caused to create more efficient immobilization platform for the cell immobilization due to the larger surface areas [47]. It is also clear that the presence of CNT provided more compact structure compared to the other one so that higher cell amount could be kept on to the surface (Figs. 1B and 2B) which caused higher sensor responses.

3.1. Optimization studies

3.1.1. Optimization of preparation conditions of microbial biosensors

In terms of optimization, firstly the effect of cell amount on biosensor response was investigated. Biosensors containing 10, 20 and 30 μL of bacterial cells were prepared and their responses to glucose were measured. As a result of these experiments, 20 μL of bacterial cells (which equivalent to $1.07 \times 10^9$ cell titer for *P. fluorescens* and $0.72 \times 10^9$ cell titer for *P. putida*) was found to be optimum amount for both *P. fluorescens*- and *P. putida*-based microbial sensors. Furthermore, all measurements were conducted by using this cell amount.

Chitosan amount was also examined and 1.0% was found to be optimum for both types of bacterial sensors. When, chitosan amount was increased from 1.0% to 2.0%, a drop was observed on the biosensor responses. This could be due to the improper matrix structure to keep the cells on the surface.

As far as the crosslinker amount was concerned, different microbial sensors were prepared by using various amounts of glutaraldehyde (0.25%, 0.5%, 1.0% and 1.5%) and the highest current signals were obtained with 0.5% glutaraldehyde for *P. fluorescens* and 1.0% for *P. putida* biosensors.

Finally, effect of CNT amount on the response was tested. For this trial, various biosensors containing 0.1, 0.2 and 0.5 mg/mL CNT were prepared and rather high responses for glucose in comparison with the CNT-free biosensors were observed with 0.2 mg/mL for both systems. It could be due to the fact that CNTs with large surface areas could provide efficient matrices to keep higher cell amount on the electrode which was mentioned before and also shown in Figs. 1 and 2, respectively, and facilitate the electron transfer due to the electroactive properties between the electrode and the cell throughout the metabolic activity in the presence of the substrate [48,49].

3.1.2. Effect of pH

The pH dependence of both CNT-modified and -unmodified *P. fluorescens* and *P. putida* sensors was examined to 2.0 mM glucose.
at the range of 5.0–7.0 in sodium acetate and potassium phosphate buffer systems (50 mM) and shown in Fig. 3. It can be easily seen that the optimum pHs for both bacterial sensors were determined as 6.0. It could also be stated that CNT modification of the membrane did not affect the pH optima of these systems. Similar data were also observed in our previous work [29]. On the other hand, we have obtained different pH optima when different matrices were used for the immobilization in previous works [12,28,29,34]. This could be due to the ionic properties of the matrices which caused a shift in optimum pH values.

3.1.3. Effect of temperature

The amperometric response of the microbial sensors to glucose (2.0 mM) was followed at different temperatures varying from 20 to 40 °C. From 20 to 25 °C, an increase was observed till 30 °C and then the signal started to decrease at 35 °C (Fig. 4). As a result, the optimum temperature was found to be about 30 °C. And further experiments were conducted at this temperature.

3.1.4. Effect of applied potential

Hydrodynamic voltammograms using CNT-free and -modified chitosan on graphite microbial electrodes were given in Fig. 5. The working potential was found to be optimum at −0.700 mV for both types of bacterial biosensors in which oxygen consumption due to the metabolic activity of intact cells was followed. As can be seen from the figures CNT modification was not effective on working potential.

3.2. Analytical characteristics

The analytical characteristics of the developed microbial sensors were examined under optimized conditions. For *P. fluorescens* sensors (modified and unmodified with CNT), linear graphs were obtained in the range of 0.1–1.0 mM for the glucose and defined by the equation of $y = 1.84x + 0.166$ ($R^2 = 0.996$) and $y = 2.245x + 0.223$ ($R^2 = 0.993$), respectively. In case of *P. putida* sensors, the linearities were observed in the range of 0.5–2.0 mM and the linear graphs were defined by the equations of $y = 0.652x − 0.0995$ ($R^2 = 0.996$) and $y = 0.749x + 0.017$ ($R = 0.988$). Linear ranges for both bacterial systems were not changed by CNT modification while slightly higher current responses were registered.

Repeatability was also tested. According to the results of 10 replicates of trials variation coefficients (c.v) were calculated as 2.9% and 4.5% for CNT-free and -modified *P. fluorescens* biosensors (for 0.4 mM glucose). In case of *P. putida* biosensors c.v values were calculated as 2.9% and 1.2% for CNT-free and -modified electrodes (for 0.7 mM glucose), respectively.

As far as stability was concerned, microbial sensors were kept in the reaction cell containing working buffer with the temperature adjusted to 30 °C. For *P. fluorescens*-based biosensors, 19% and 8% decreases were observed up to fourth hour and in case of *P. putida*-based biosensors, 15% and 11% drops were observed up to third hour for CNT-free and -modified electrodes.
P. putida
P. fluorescens

Cell type Analytes Linear range (mM) Without CNT CNT-modified (0.2 mg/mL)

- **P. fluorescens**
  - Galactose 0.2–1.0
  - Mannose 0.2–1.0
  - Xylose 0.4–1.0

- **P. putida**
  - Galactose 1.0–3.0
  - Mannose 1.0–3.0
  - Xylose 1.0–3.0

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**Table 1**

Substrate specificity of the microbial biosensors (in phosphate buffer, 50 mM, pH 6.0, 30 °C, −0.7 V)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Analytes</th>
<th>Linear range (mM)</th>
<th>Without CNT Equation*</th>
<th>R²</th>
<th>CNT-modified (0.2 mg/mL) Equation*</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. fluorescens</td>
<td>Galactose</td>
<td>0.2–1.0</td>
<td>y = 0.538x + 0.004</td>
<td>0.995</td>
<td>y = 0.598x + 0.041</td>
<td>0.984</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>0.2–1.0</td>
<td>y = 1.001x + 0.033</td>
<td>0.989</td>
<td>y = 1.267x + 0.047</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>0.4–1.0</td>
<td>y = 0.257x – 0.013</td>
<td>0.976</td>
<td>y = 0.329x – 0.003</td>
<td>0.983</td>
</tr>
<tr>
<td>P. putida</td>
<td>Galactose</td>
<td>1.0–3.0</td>
<td>y = 0.118x + 0.008</td>
<td>0.993</td>
<td>y = 0.138x + 0.023</td>
<td>0.979</td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>1.0–3.0</td>
<td>y = 0.132x + 0.029</td>
<td>0.960</td>
<td>y = 0.201x + 0.023</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>1.0–3.0</td>
<td>y = 0.137x – 0.019</td>
<td>0.986</td>
<td>y = 0.250x – 0.042</td>
<td>0.960</td>
</tr>
</tbody>
</table>

* x and y show concentration in mM and current density as μA/cm², respectively.

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**Fig. 5.** Hydrodynamic voltammograms of CNT-modified and unmodified microbial biosensors in potassium phosphate buffer (50 mM, pH 6.0) for *P. fluorescens* (A) and *P. putida* (B) based microbial biosensors (30 °C, 1.0 mM glucose, CNT-free (●), CNT-modified (■)).

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**4. Conclusion**

*P. fluorescens* and *P. putida* cells were entrapped together with chitosan matrix onto the surface of graphite electrodes to construct microbial biosensors and the measurements were based on the respiratory activity of the intact cells. CNT-modified chitosan membrane was also prepared to test nanoparticle effect on the biosensor performances. The proposed systems do not require any complex immobilization procedures and showed a good linearity and repeatability with a high operational stability. It can be concluded that the proposed systems could also be adapted to detect different analytes as well as sugars by the addition of an adaptation step to various compounds into the microbial growth. Moreover, combining the properties of carbon nanotubes and the versatility and biocompatibility of chitosan created chitosan surface-decorated carbon nanotubes. Apart from microbial sensing, these systems could be good alternatives also in biofuel cell applications, toxicity measurements as well as gene, drug delivery and other chemical and biological applications as reported previously [31].

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**References**

Biographies

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