Full Paper

Carbon Nanotube Composite as Novel Platform for Microbial Biosensor

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Abstract
The presented work includes the development of a microbial biosensor based on a carbon-nanotube epoxy composite (CNTEC) platform used as supporting electrode for cell immobilization. For this purpose, cells of *Pseudomonas fluorescens* were immobilized on the surface of the CNTEC electrode by means of gelatin which was then cross linked with glutaraldehyde. After optimization of experimental parameters like cell amount, pH and temperature, the system was calibrated for glucose. From the calibration graph the linear range was estimated as 0.5 – 4.0 mM with a response time of 100 s. Furthermore, substrate specificity and operational stability were investigated. Finally, the results that were obtained with CNTEC electrodes were compared with conventional graphite epoxy composite electrode (GECE) and as a result, higher current values (2 to 3 folds) were observed with CNTEC microbial biosensor.

Keywords: Microbial biosensor, *Pseudomonas fluorescens*, Carbon nanotube, Graphite-epoxy composite

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1. Introduction

Since their rediscovery in 1991, carbon nanotubes (CNT) have attracted considerable interest due to their remarkable properties [1]. Besides their high surface area, ability to accumulate analyte, minimization of surface fouling and electrocatalytic activity, their high electrical conductivity allows the utilization of CNTs as an electrode material [2]. The electrocatalytic activity of CNT has been investigated for a wide range of compounds, such as neurotransmitters [3 – 6], NADH [1, 7 – 9], hydrogen peroxide [2, 3, 8, 10], ascorbic [2 – 5] and uric acid [2], cytochrome *c* [11], hydrazines [12], hydrogen sulfide [13], amino acids [14] and DNA [15]. The reported catalytic property of CNT is claimed to originate from the open ends and possible defects of the structure [9, 16].

Various types of CNT modified electrodes were prepared including physical adsorption of CNT onto electrode surfaces, like glassy carbon [1, 3 – 5, 7, 10, 11, 13, 14] and composite paste electrodes [9, 15] while in some case, it was consolidated into Teflon [8].

Recently CNT was incorporated into an epoxy polymer, forming an epoxy composite hybrid material as a new electrode with improved electrochemical sensing properties. The performance of this electrode in electrochemical and bioanalytical processes were investigated [2]. As a result, lower peak to peak separations and well defined peaks were obtained with this electrode. On the other hand, multi wall carbon nanotube (MWCNT) biosensor was obtained by means of GOx immobilization through physical entrapment inside an epoxy resin matrix and its performance was examined for glucose determination. As a result a biosensor that has high sensitivity and good stability was fabricated [17].

Following these studies, present work contains the results obtained with a MWCNTEC modified with bacterial cells for future applications as a microbial biosensor.

Microbial biosensors are devices incorporating a biological sensing element (microorganism) that can specifically recognize species of interest, either intimately connected to or integrated within a suitable transducing system. The transducer is the responsible for the quantitative conversion of the biochemical signal into an electronic signal that can be suitably processed and outputted. In this way, analytes of interest may be measured by using the assimilation capacity of the microorganism as an index of the respiration activity or of the metabolic activity [18] The major application of microbial biosensors is in the environmental field [19 – 21]. Real-time analysis, simplicity of operation, portability, sensitivity and specificity of the microbial biosensor make these tools very interesting.

In this study, the effect of CNT on microbial sensor response in terms of electron transportation was aimed to search. For this purpose, *Pseudomonas fluorescens* was used

as a biological material where the immobilization of the cell was done via gelatin membrane that was then cross-linked with glutaraldehyde. The characterization and optimization of the system were performed by using glucose as a substrate. Amperometric measurements were based on the respiratory activity of the cells which means, in the presence of glucose, oxygen consumption due to the metabolic pathway of the *P. fluorescens* was followed by means of a potentiostat. In order to investigate the contribution of CNT on the biosensor response, obtained results under the optimum conditions were compared with that of conventional GECE based bacterial sensor.

2. Experimental

2.1. Apparatus

Chronoamperometric experiments were carried out with a Radiometer electrochemical measurement unit (France). The electrodes were inserted into the cell through its Teflon cover. Ag/AgCl (including 3 M KCl with saturated AgCl as an internal solution, Radiometer Analytical, REF321) and Pt (Radiometer Analytical, M241PT) were used as reference and counter electrodes, respectively. Micrographs of the CNTEC surfaces without any modification were obtained by scanning electron microscopy (SEM) using Hitachi S-570DATA while the SEM image for the microorganism was obtained by using a Philips XL30SFEG SEM.

2.2. Reagents

Glucose was purchased from Merck AG (Darmstadt, Germany) while 300 bloom calf skin gelatin and glutaraldehyde were obtained from Sigma Chem. Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Mineral salt medium (MSM) with the following composition was used as a growth medium for *Pseudomonas fluorescens*; 0.244% Na_2HPO_4, 0.152% KH_2PO_4, 0.050% $(NH_4)_2SO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.005% $CaCl_2 \cdot 2H_2O$ and trace element solution (10 mL/L) [22] were prepared from reagent grade chemicals. The pH of the growth media was adjusted to 6.9.

2.3. Biological Material

Pseudomonas fluorescens (*Pseudomonas putida* DSM 6521) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and subcultured on Nutrient Agar. Cell were inoculated into 50 mL of MSM containing 250 mg/L glucose and incubated at 28 °C on an orbital shaker at 150 rpm. After 24 h, when cells were grown, the biomass was harvested by centrifugation at 10000 rpm and suspended in MSM and then recentrifuged. The supernatant was removed and the cellular paste was used for making biosensor. Bacterial cells

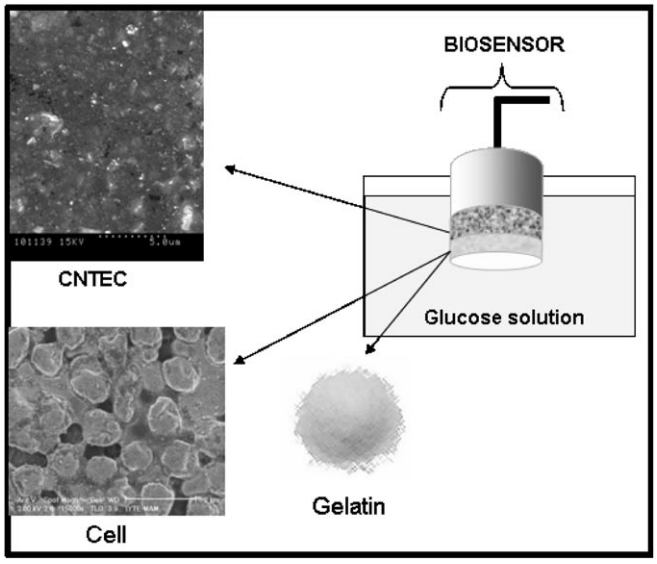

Fig. 1. Schematic diagram (not in scale) of the CNTEC based microbial biosensor including the SEM images of CNTEC and *P. fluorescens*. The acceleration voltage for CNT and CNTEC was 5.00 kV and for *P. fluorescens* it was 3.00 kV.

in logarithmic phase were used during the experiments and cell growth was followed spectrophotometrically by measuring optical density at 560 nm. The standard graph of optical density versus living cells were used to find the optimum cell amount for the preparation of biosensor [22]. Daily inoculated bacteria containing biosensors were prepared daily. Also, microbial electrodes were immersed into MSM containing glucose between the measurements.

2.4. Preparation of Biosensor

CNTEC and GECE electrodes were prepared as described previously [17]. *Pseudomonas fluorescens* cells which have 1.34×10^9 cell titer (25 μL) and 300 bloom gelatin (10 mg) were mixed at 38 °C in phosphate buffer (50 mM, pH 7.5, 25 μL). Mixed solution was placed onto the CNTEC working electrode surface and allowed to dry at 4 °C for 45 minutes. Finally, it was immersed in 2.5% glutaraldehyde in phosphate buffer (50 mM, pH 7.5) for 5 min. The schematic representation of biosensor preparation was given in Figure 1 with the SEM images of CNT and micro organism.

2.5. Measurements

All measurements 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 50 mM phosphate buffer (pH 7.0) solution at 30 °C for 10 min. The current change that was resulted from glucose addition into the medium was recorded at 100 – 120th s. Since the oxygen concentration in the bioactive layer decreases related to

substrate addition, 100 – 120 s. is needed to reach the new steady state. The current density ($\mu A/cm^2$) changes were registered with a potentiostat at – 0.7 V and the results were expressed in terms of % biosensor response. The current density that was obtained at the optimal working conditions was assumed as 100% and other measured values were calculated relative to this value.

3. Results and Discussion

Pseudomonas fluorescens is an aerobic, gram-negative bacterium which use organic compounds as their only source of carbon and energy and has shown to be an interesting model to study the biochemical impact of environmental stress on cellular metabolism [23]. The measurement was based on the respiratory activity of the cells. It is known that simple carbon sources like sugars are preferentially utilized by microorganisms. In pseudomonads, glucose utilization follows two routes: (i) the direct oxidative pathway, which converts glucose to gluconate, 2-ketogluconate and then subsequently to 6-phosphogluconate by extracellular, high affinity, glucose dehydrogenase and gluconate dehydrogenase and (ii) the intracellular, low affinity, nucleotide-dependent phosphorylative pathway where glucose is converted to 6-phosphogluoconate by glucokinase and glucose 6-phosphate dehydrogenase. Depending on the physiological conditions, one or other of the pathways predominates. In the previous works, in order to elucidate the glucose metabolic pathway, O_2 uptake and enzyme activity studies were carried out [24]. In our study, oxygen consumption in the presence of glucose due to the metabolic pathway of the *P. fluorescens* was monitored amperometrically.

The carbon nanotube based biosensors combine the bioselectivity of redox enzymes with the inherent sensitivity of amperometric transductions, and have proven to be very useful for the quantification of glucose [17]. It is expected to have lower oxidation potential and higher sensitivity due to electrocatalytic properties of CNT [1 – 17]. In the previous work, CNTEC based glucose biosensor was developed and the results were compared with that of GECE [17]. In this work, for the first time a CNTEC based microbial sensor was developed by using *Pseudomonas fluorescens* as biological material. For this study GECE based *P. fluorescens* was utilized as control experiment for the verification of electrocatalytic benefit of CNT.

3.1. Optimization of Experimental Parameters

3.1.1. Effect of pH

According to the optimization studies, the effect of pH on the electrode response was investigated by using phosphate buffer systems (50 mM) between pH 6.0 – 8.2 for 2.0 mM glucose (Fig. 2). As clearly can be seen from the Figure, the response current of the electrode to glucose increases significantly from pH 6.5 to 7.5, and then a sharp decrease is obtained at pH values higher than 7.5. As a result pH 7.5 was chosen as optimum pH and used for further studies.

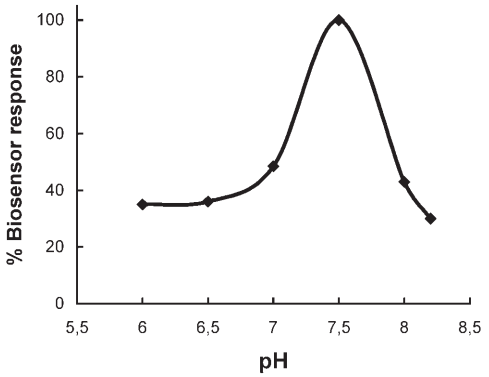

Fig. 2. Effect of pH on the biosensor response (pH 6.0 – 8.2; phosphate buffer (50 mM), – 700 mV, 30 °C).

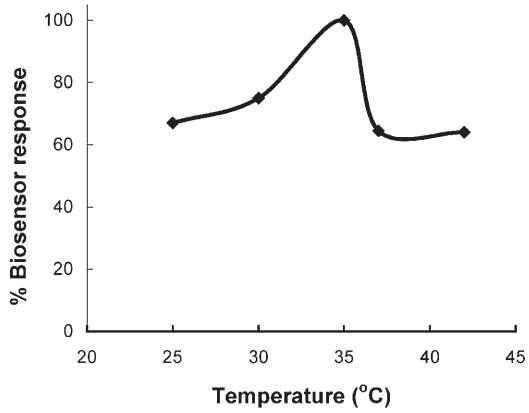

Fig. 3. Effect of temperature on the biosensor response (in phosphate buffer, 50 mM, – 700 mV, pH 7.5).

3.1.2. Effect of Temperature

The amperometric response of the microbial electrode to 2.0 mM glucose was measured at different temperatures varying from 25 to 42 °C and the results are shown in Figure 3. As best current value was observed at 35 °C, further experiments were conducted at this temperature.

3.1.3. Effect of Cell Amount

For this purpose 12.5 μL, 25 μL, and 37.5 μL of bacterial cell which have the same cell titer were used to prepare three immobilization mixture. To investigate the effect of cell amount three separate calibration graphs were obtained by using each amount. The highest responses were obtained with 25 μL cell amount. As the cell activity of 12.5 μL was inadequate and 37.5 μL bacterial cells caused diffusion problem to the substrate, both amounts has tended to decrease the resulting signal. Further experiments were conducted by using 25 μL cell amount (Fig. 4).

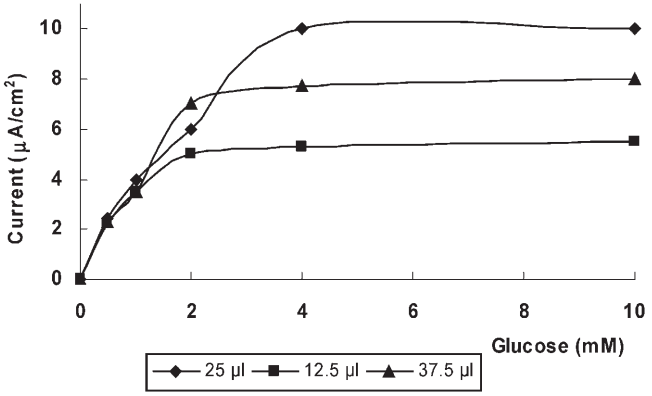

Fig. 4. Effect of cell amount (12.5 μL(■), 25 μL(◆), 37.5 μL(▲)) on the electrode response in phosphate buffer (50 mM at pH 7.5, 30 °C, −700mV).

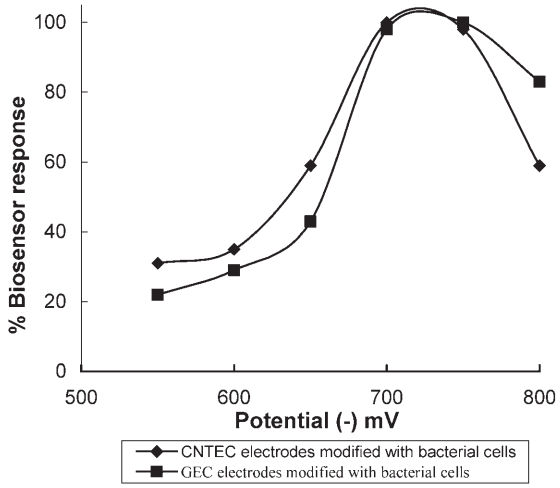

Fig. 5. Effect of working potential on the electrode response in phosphate buffer (50 mM at pH 7.5, 30 °C).

3.1.4. Stability

The stability of cell based biosensor was investigated at working conditions (30 °C in phosphate buffer, pH 7.5) by using 2 mM glucose and a 13% decrease of activity is observed after 4 hours (data not shown). During this period approximately 16 measurements have been made and it could be possible to make more measurements in a longer time period. Moreover to be sure of the life time of bacterial cells in bioactive layer and to obtain reproducible results, the sensors including daily inoculated cells were prepared freshly for each day and utilized through the experiments.

3.1.5. Effect of Working Potential

The effect of working potential was searched by measuring the amperometric responses of two types of microbial electrode based on CNTEC and GECE to 2.0 mM glucose at different potentials vs. Ag/AgCl between −550 and −800 mV. As it is mentioned before, the measurement was based on the respiratory activity of the cells. At lower potentials (between −550 and −650 mV), CNTEC showed almost 1.5 fold higher biosensor response value than traditional GECE that was in agreement with the fact that CNTs promote electron-transfer reactions at low potentials. However maximum currents were obtained at −700 and −750 mV for both systems (Fig. 5) and for this reason −700 mV was chosen as the operating potential for further experiments.

At the previous work, glucose biosensor was fabricated by dispersing multiwall CNT inside the epoxy resin [17] and as a result, lower detection potential (+0.55 V) than for GOx-GECE (+0.90 V; difference $\Delta E = 0.35$ V) was obtained.

Due to loss of metabolic activity of microbial cell, it is inappropriate to disperse bacterial cell into composite materials like epoxy. For this reason they were immobilized onto the electrode surface by means of gelatin membrane [22]. Both gelatin membrane and the structure of bacterial cell membrane act like a diffusion barrier for electron transfer and tend to slow down the reaction kinetics. Though better current values were observed with CNTEC, in our opinion, due to these complex mechanisms, significant lower values can not be obtained in terms of operating potential. The effect of CNT on electron transfer kinetics at microbial systems will be under investigation with the help of monomeric and polymeric mediators.

3.1.6. Analytical Characteristics

CNTEC electrode provides linear relationship between biosensor response (y) and substrate concentration (x) in the range of 0.5–4.0 mM glucose under the response times of 100 s with the equation of $y = 2.1\,x + 1.67$ ($R^2 = 0.995$, y in mM, x in μA/cm^2). At higher concentrations, standard curve showed a deviation from linearity. On the other hand, with GECE same linearity was obtained while the equation of linear graph was estimated as $y = 0.795\,x + 0.897$ with $R^2 = 0.983$ (y in mM, x in μA/cm^2) respectively. Besides, it was observed that CNTEC possess higher current values (2 to 3 folds) compared to GECE. This can be attributed to unique properties of CNT that promotes the electron transfer. [25, 26]. Though the electrocatalytic properties of the CNT have not been completely explained yet, it was suggested that the open ends of the nanotubes might be responsible from this attractive behavior [2].

The repeatability of the biosensor was tested for 2 mM of glucose ($n = 7$) and the standard deviation (SD) and variation coefficient (cv) were calculated as ±0.06 mM and 2.7%, respectively.

Moreover, the substrate specificities of proposed biosensor to different utilizable substrates (galactose, mannose, phenol, ethanol and methanol) were also tested and given in Table 1. No signal was obtained for phenol as well as methanol and ethanol. Since nonadapted bacterial cell were used, they didn't metabolize phenol and alcohols as expected.

Table 1. Substrate specificity of CNTEC microbial biosensor. N.D: Not determined.

Substrate	Concentration (mM)	Current ($\mu A/cm^2$) [a]
Glucose	1 mM	3.58 ± 0.09
	2 mM	6.03 ± 0.05
Mannose	1 mM	0.56 ± 0.05
	2 mM	1.20 ± 0.05
Galactose	1 mM	1.6 ± 0.16
	2 mM	4.8 ± 0.18
Phenol	1 mM	N.D
	5 mM	N.D
Methanol	1 mM	N.D
	5 mM	N.D
Ethanol	1 mM	N.D
	5 mM	N.D

[a] Measurements were performed 4 – 5 times and data were given as ± SD.

4. Conclusions

In the previous work, carbon nanotube epoxy composite (CNTEC) electrodes have been developed, characterized and compared with graphite-epoxy composite (GEC) electrodes prepared from the same epoxy resin. According to data, the CNTEC electrode has been possessed as an improved electrochemistry for ferricyanide, NADH and hydrogen peroxide. It is also stated that the resulting CNTEC electrode might offer a great promise for biosensing by incorporating biomolecules [2]. Present work represents the first example of modification of CNTEC with bacterial cells. Resulted microbial biosensor was characterized for glucose and compared with conventional GECE based microbial biosensor. As the electron transfer mechanism in the case of bacterial cells is more complicated than as it is for enzymes, lower operating potential could not be obtained with CNTEC based microbial biosensor. Although the immobilization method used in this work provides mild conditions in terms of protecting microbial activity nevertheless the usage of gelatin membrane for immobilization procedure rather than dispersing the cells into the epoxy [2] might have affected the overall response mechanism. On the other hand, higher current values (2 to 3 folds) were observed with CNTEC microbial biosensor when compared with GECE based microbial biosensor.

Microbial biosensors are good alternative to monitor some global parameters such as bioavailability and toxicity which cannot be probed with molecular recognition or chemical analysis since complex reactions including bacterial metabolic pathways can only exist in an intact functioning cell [23]. In view of the direct relevance of bioavailability and toxicity to the presence of pollutants, many of the efforts at the development of whole-cell biosensors were directed towards environmental applications. The microbial cell used in our work is well known phenol-degrading bacteria. However, adaptation process is required before using this kind of microbial cells as a specific degrader organisms. Adaptation process may be operationally defined as an increase in the ability of a microbial community to degrade a chemical after prolonged exposure to the material. This phenomena could be due to the several alterations in structure and function of microbial species such as induction or depression of enzymes, genetic change etc. [27]. It could be also possible to use the same bacteria, as the one used in the present study, after an adaptation process to obtain microbial sensors for the environmental monitoring of other analytes [28, 29].

Design of CNT based arrays might also be promising as a good platform for the bacteria and such arrays can serve for high-throughput screening of chemicals and drugs. Utility of different immobilization matrices, as well as electron transfer mediators to overcome the possible diffusion problems and to get more efficient biosensor systems are under investigation.

5. Acknowledgements

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6. References

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