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Short communication

A microbial biosensor based on bacterial cells immobilized on chitosan matrix

Dilek Odaci, Suna Timur *, Azmi Telefoncu

Ege University, Faculty of Science, Biochemistry Department, 35100 Bornova-Izmir, Turkey

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ABSTRACT

A bio-electrochemical system consisting of *Gluconobacter oxydans* DSM 2343 cells as a biological material and carbon nanotube (CNT)-free and CNT-modified chitosan as immobilizing matrices has been developed. The measurement was based on the respiratory activity of the cells estimated by the oxygen consumption at −0.7 V (versus the Ag|AgCl reference electrode) due to the metabolic activity in the presence of substrates. The system was calibrated and dependence of signal amplitude on the measuring conditions and cell amount was studied as well as the substrate specificity, pH, temperature and working potential. The biosensors (CNT-modified and unmodified) were demonstrated for the quantification of glucose in the range of 0.05–1.0 mM, at 30 °C and pH 7.0 with the 40 s of response time. The linear relationships between sensor response (y; μA/cm^2) and substrate concentration (x; mM) were defined by the equations of $y=1.160x+0.151$ ($R^2=0.990$) and $y=1.261x+0.197$ ($R^2=0.982$), respectively. All other data were also given as comparison of two systems one with CNT-modified and CNT-free.

1. Introduction

Gluconobacter oxydans is an obligate aerobe, having a respiratory type of metabolism using oxygen as the terminal electron acceptor and a gram-negative bacterium belong to the *Acetobacteraceae* [1]. These organisms have been used since ancient times in biotechnological processes like the vinegar and vitamin C production, [2] and are still used for industrial applications [3,4]. *G. oxydans* has been previously used industrially to produce polysaccarides, cellulose, dextran [5], L-sorbose from D-sorbitol [6], dihydroxyacetone from glycerol [7], L-riboluse from ribitol [8], tagatose from D-galactitol [9], gluconic acid from glucose [10]. Moreover, *G. oxydans* has been identified as a prospective biocatalyst for saccharide and alcohol biosensors because of the membrane localization of oxidative enzymes [11]. Most of the reactions are catalyzed by membrane-bound dehydrogenases, whose reactive centers are oriented to the periplasmic space so that transport of substrates into the cell is not necessary [12].

Since cofactor and recycle systems form part of the cells' metabolism, application of whole microbial cells can be very attractive when oxidations are involved [13]. Whole cell microbial sensors have received recent attention; because enzyme purification is unnecessary, whole microbial sensors are simple and inexpensive systems to construct and [14] enzymes are usually more stable in their natural environment in the cell [15]. On the other side, microbial biosensors have some disadvantages in compare to the enzyme biosensors: in many cases they have a low selectivity and longer response time [16]. When viable microbial cells are involved a gentle immobilisation technique should be used to keep the cells in their metabolic active forms. Entrapment into the natural polymers prepared from alginate/pectate, κ-carrageenan, collagen, gelatin, chitosan and agar (agarose) can be performed under mild conditions with high viability of cells entrapped [17].

Chitosan (CHIT), the primary derivative of chitin, is 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 [18,19]. Increasingly over the last decade chitin- and chitosan based materials have been examined and a number of potential products have been developed for areas such as wastewater treatment, the food industry, agriculture, pulp and paper industry, cosmetics and toiletries, medicine and biotechnology, in biosensors as an immobilization platform [20]. Recently, CHIT has been used as an effective dispersant of carbon nanotubes (CNTs). The resulting biocompatible composite of CHIT–CNT has been applied as matrix to immobilize biological materials [21–25].

CNTs are built from sp^2 carbon units and present a seamless structure with hexagonal honeycomb lattices, being several nanometers in diameter and many microns in length [26]. CNTs have attracted tremendous interest in analytical chemistry because of their unique properties, such as high electrical conductivity, high mechanical properties, and ability to grown on different substrates, and nanoscale size with a high aspect ratio [27]. In recent years, many works have been published using CNTs together with biological materials in biosensors, drug and vaccine delivery vehicles and novel biomaterials [28].

* Corresponding author. Tel./fax: +90 232 3438624.
E-mail address: sunatimur@yahoo.com (S. Timur).

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In the present study, *G. oxydans* cells were immobilized onto the graphite electrode via CHIT matrix. The response characteristics, stabilities and substrate specificities were investigated as well as the effect of the presence of CNT as a modifier on the CHIT membrane.

2. Experimental

2.1. Reagents

D(+)-glucose, D(+)-mannose, D(+)-galactose, D(+)-xylose, β-lactose, chitosan (CHIT, from crab shells, minimum 85% deacetylated), glutaraldehyde were purchased from Sigma Chem. Co. (St. Louis, MO, USA), multiwalled carbon nanotube (MWCNT, diameter; 110–170 nm, length; 5–9 μm, 90%) were obtained from Aldrich (Dorset, UK), D(−)-fructose, N-acetyl D-glucosamine, maltose monohydrate were purchased from Fluka (Steinheim, Germany). All other chemicals were analytical grade.

To prepare CHIT–CNT solutions, different amounts of MWCNTs were dispersed in CHIT solutions and the blends were treated under an ultrasonic field for 10 min. This CHIT–CNT blend was then stirred for 1 h. During this step, chitosan macromolecules were adsorbed on the surface of the CNTs thereby acting as polymer cationic surfactants to stabilize the CNTs [29].

2.2. Apparatus

Chronoamperometric measurements were carried out by Radiometer electrochemical measurement unit (Lyon, France) where *G. oxydans* modified graphite electrode were used as working electrode. Ag|AgCl electrode (with 3 M KCl saturated with AgCl as the internal solution, Radiometer Analytical, REF321) and platinum electrode (Metrohm, Switzerland) 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.

2.3. Biological material

G. oxydans DSMZ 2343 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and was sub-cultured in media containing (in g/L): glucose, 100; yeast extract, 10; calcium carbonate, 20; agar, 20. Then, cells were cultivated in shake flasks on a shaker (175 rpm) at 28 °C, in media containing (in g/L): yeast extract, 5; carbon source: glucose, 5 [30]. Cells at late exponential phase were harvested by centrifugation at 6 000 rpm for 15 min. Cells were washed by 0.9% NaCl and re-centrifuged. Obtained cellular paste was used for biosensor construction. Cell growth was followed spectrophotometrically by measuring the optical density at 600 nm [31].

2.4. Biosensor preparation

For the construction of the bioactive layer on the graphite electrode, the spectrographic graphite rods (Ringsdorff Werke GmbH, Bonn, Germany, 3.05 mm diameter and 13% porosity) were polished and washed thoroughly with distilled water and sonicated for 2 min.

20 μL of CHIT or CHIT–CNT solutions were placed on the electrode and allowed to dry at +4 °C for 18 h. Then 10 μL of the cellular paste and 20 μL of glutaraldehyde solution (in potassium phosphate buffer, pH 7.0) 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 to remove unbound cells [32]. During the experimental steps microbial electrodes containing daily inoculated cells were prepared every day.

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 electrodes by applying a potential of −0.7 V (versus the Ag|AgCl reference electrode). After each measurement, the electrodes was washed with distilled water and kept in working buffer solution at 30 °C for 3 min. When the current density reached a steady state, the substrate solution was added to the working buffer solution (50 mM PBS (phosphate buffer saline), pH 7.0) 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/cm^2).

3. Results and discussion

The major motivation behind the use of intact cells as biocatalysts in biosensors is the ease of preparation and the accessibility of enzymatic activities not available otherwise. *Gluconobacter sp.* is particularly suitable for biosensor construction due to the some attractive features that these species grow very fast in simple cultivation media, contain high activities, and relatively stable during immobilization, as well as their PQQ dependent dehydrogenases do not require an external cofactor to be added [33]. In previous works, *G. oxydans* cells were immobilized on the surface of different transducers by means of various polymeric membranes such as osmium redox polymers [34,35], cellulose acetate [30,36], gelatin cross-linked with glutardialdehyde [33] etc. and mediated and non-mediated microbial sensors were fabricated. Additionally, another type of system was characterized by physical adsorption of the bacterial cells to Whatman GF/A chromatographic paper as a support matrix [14,37,38]. On the other hand, a fuel cell assembly was constructed using *G. oxydans* cells

Table 1
Comparison of analytical characteristics of various *G. oxydans* biosensors reported in literature

Electrode	Analyte	Immobilization method	Working potential (mV)	Analytical performances		Reference
				Linear range	Operational stability	
GCE	PDO	DM with a silicone rubber O-ring	+300 (in the presence of the ferricyanide)	0.3 mg/L–1.2 g/L	Stable over the 10 h (0.66% h^{-1})	[35]
GCE	PDO	OP with a silicone rubber O-ring	+150	0.8 mg/L–1.5 g/L	70% decrease after 10 h (0.47% h^{-1})	[35]
CE	Glc	By adsorption onto CP	–	0.0–10.0 mM	–	[38]
CE		By adsorption onto CP	–	0.0–10.0 mM	–	[38]
CE	EtOH	By physical adsorption, using CP	–	0.05–10 mM	Stable for no less than 10–12 h	[37]
GE	Glc	DM with a O-ring	+300	0.002–2.2 g/L	No decrease over 9 consequent measurements	[11]
GDE	EtOH	OP with a silicone rubber O-ring	+300	0.25–2.5 mM	50% decrease after 51 h	[34]
GDE	GOH	OP with a silicone rubber O-ring	+300	2.5–45.0 mM	50% decrease after 68 h	[34]
GDE	Glc	OP with a silicone rubber O-ring	+300	0.25–2.0 mM	50% decrease after 3 h	[34]
CE	Xyl	On CP by simple physical adsorption	–	0.5–20 mM	–	[14]
GCE	EtOH	CA membrane	+300	2–270 μM	75% decrease after 4.5 h,	[30]
GE	Glc	Unmodified CHIT	−700	0.05–1.0 mM	24% decrease after 5 h	This work
GE	Glc	CNT-modified CHIT	−700	0.05–1.0 mM	15% decrease after 5 h	This work

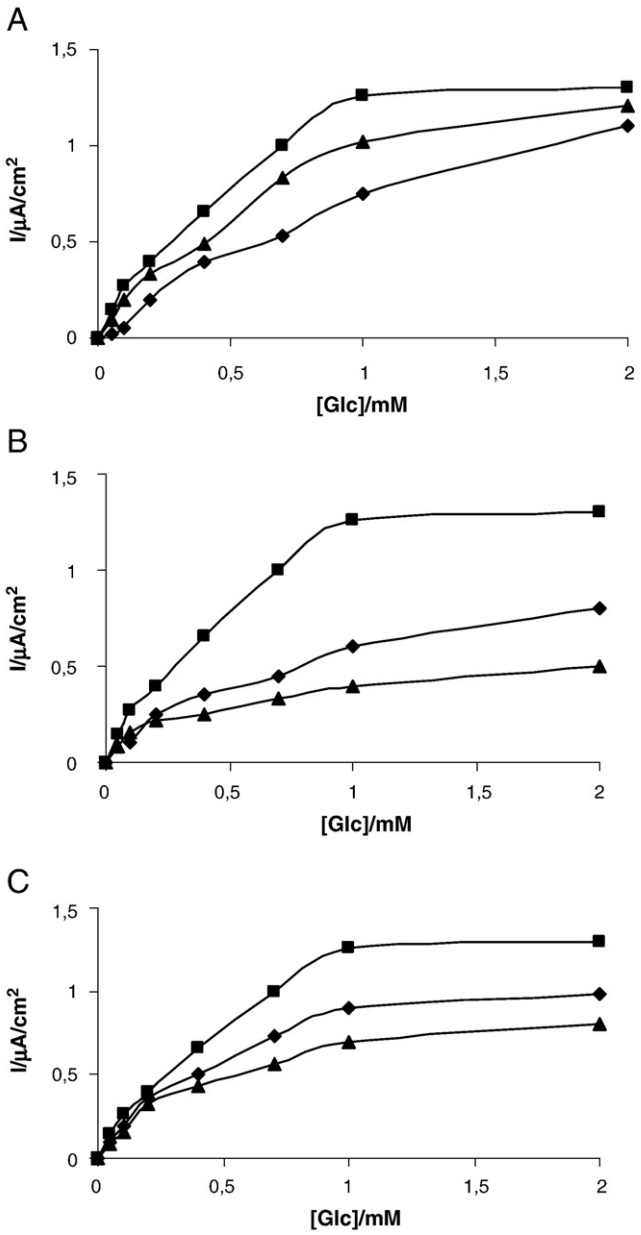

Fig. 1. Optimization of the preparation conditions: Effect of cell loading [(A); ♦ 5 μL, ■ 10 μL, ▲ 20 μL], chitosan amount [(B); ♦ 0.5%, ■ 1.0%, ▲ 2.0%] and glutaraldehyde amount [(C); ♦ 0.5%, ■ 1.0%, ▲ 1.5%], in 50 mM PBS, pH 7.0, 30 °C; for the biosensor *x* and *y* shows glucose concentration, mM and current density, μA/cm^2, respectively).

as a biological material [39,40]. The comparison of some analytical features of these systems is summarized in Table 1.

In the present study, CNT-free and modified CHIT matrices were used as an immobilization platform for whole *G. oxydans* cells to prepare microbial sensor. Proposed bacterial sensors were characterized and the effect of CNT on the electrode responses was investigated.

3.1. Optimization studies

3.1.1. Optimization of preparation conditions of microbial biosensors

For the optimization studies, firstly, the effect of cell amount on biosensor response was investigated (Fig. 1(A)). Biosensors containing 5, 10 and 20 μL of bacterial cells which equals to 7.5×10^9, 15×10^9 and 30×10^9 cell titer were prepared and their responses to glucose were measured. As a result of these experiments, 10 μL of bacterial cell (which is equivalent to 15×10^9 cell titer) was found to be optimum and all measurements were conducted by using this cell amount. It was observed that higher cell amounts caused lower signals. It is an expected result that could be due to the diffusion problem. Especially, limitation in oxygen diffusion to the bioactive layer could be possible and reduced signals could be explained by the lower efficiency of substrate oxidation due to the oxygen deficit in the layer of immobilized cells [37].

Effect of CHIT amount was also tested (Fig. 1(B)). Biosensors containing 0.5%, 1.0% and 2.0% of chitosan solutions were prepared. Then, the biosensor responses were measured and 1.0% was found to be optimum for bacterial sensors. When, CHIT amount was increased from 1.0% to 2.0%, a drop was observed on the biosensor responses. This might be due to the improper matrix structure to keep the cells on the surface. A decreased response with increased CHIT amount might be explained by a restricted diffusion of oxygen towards the electrode as well the substrate.

Additionally, optimum glutaraldehyde amount as the cross-linker was investigated (Fig. 1(C)) and different microbial sensors were prepared by using various amount of glutaraldehyde (0.5%, 1.0% and 1.5%). The highest current signals were obtained with 1% glutaraldehyde. At the lower cross-linker amount cell leakage from the electrode surface could occur while higher amounts might result in excess cross links that restricted metabolic activities of the cells and created diffusion problem.

Finally, effect of CNT amount on the response was examined. Various biosensors containing 0.1, 0.2, 0.5 mg/mL of CNT (in 1.0% of CHIT solution) were prepared and higher responses for glucose in compared with the CNT free biosensors were observed with 0.2 mg/mL of CNT for *G. oxydans* based systems. CNTs are new materials with unique properties. It was previously reported that CNTs with chitosan created new chitosan–CNT nanomaterials with combined features [35]. CNT modified chitosan is a good alternative to construct microbial biosensors. In our previous study, *P. fluorescens* and *P. putida* based

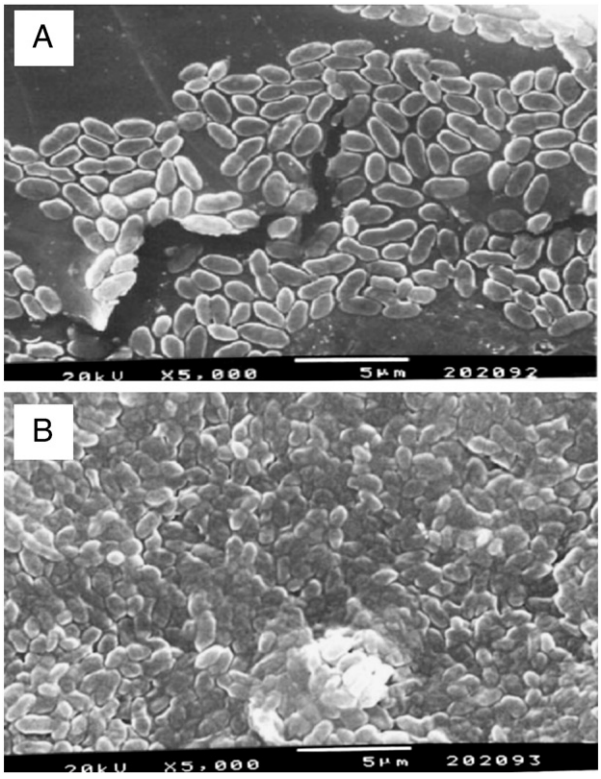

Fig. 2. SEM images of *Gluconobacter oxydans* cells on the surface of CNT-free (A) and CNT-modified (B) electrodes.

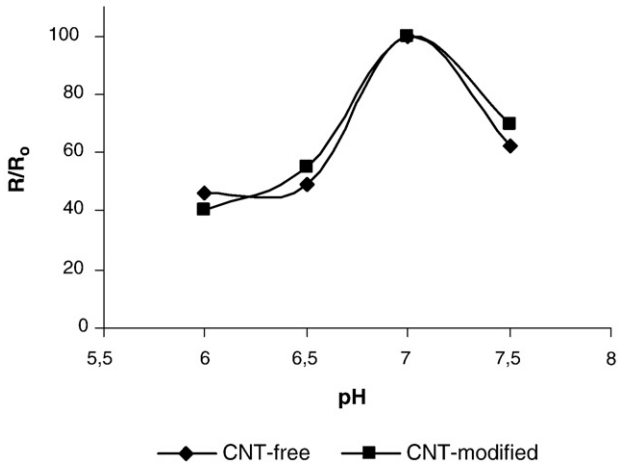

Fig. 3. Effect of pH on the response of *Gluconobacter oxydans based* biosensors (6.0–7.5: PBS (50 mM), 30 °C, −0.7 V, CNT-free (♦), CNT-modified (■); for the biosensor *x* and *y* shows pH and biosensor response %, *R*: current and R_o: maximum current, respectively).

microbial biosensors were prepared by using CNT–chitosan matrices and it is observed that CNT modification caused to provide more efficient immobilization platform to keep higher cell amount on the electrode surface [41].

SEM technique is used to detect the surface characteristics of the matrices and to show the interaction between biological materials and immobilization platforms. In this part, morphologies of both modified and unmodified matrices for *G. oxydans* biosensors were imaged by SEM (Fig. 2(A) and (B)). As can be seen from the micrographs, CNT modification provided more compact structure compared to the other one so that higher cell amount could be loaded and kept on to the surface (Fig. 2(B)) which might cause higher sensor responses.

3.1.2. Effect of pH

The effect of pH on the sensor response was tested using PBS (50 mM) between 6.0 and 7.5. Relative current signals versus pH were drawn and shown in Fig. 3. Optimum pH of the bacterial biosensor was obtained as 7.0. As a result, pH 7.0, which is much closer to the pH of the growth medium, was chosen as optimum and used for further studies.

3.1.3. Effect of temperature

Since the metabolic activity of microorganisms is dependent on the temperature, electrode response was measured at different temperatures ranging from 20 to 40 °C using unmodified *G. oxydans* biosensors (Fig. 4). Increase in temperature caused higher signals until 30 °C. At higher temperatures a drop in the response was obtained. Hence, 30 °C was conducted for all measurements. These data were in good accord with the general knowledge of the physiology of *Gluconobacter* sp. [4].

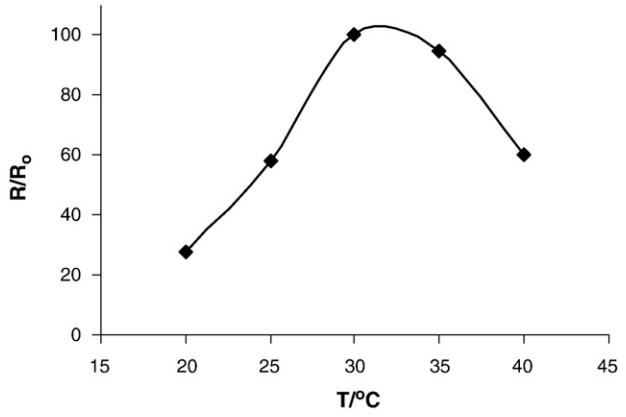

Fig. 4. Effect of temperature on the microbial sensor response (in PBS, 50 mM, pH 7.0, −0.7 V; for the biosensor *x* and *y* shows temperature, °C and biosensor response %, *R*: current and R_o: maximum current, respectively).

3.1.4. Effect of applied potential

The hydrodynamic voltammograms of CNT-free and modified *G. oxydans* biosensors between −0.55 and −0.85 V (increments of 0.05 V) for 2.0 mM glucose were shown in Fig. 5. Higher current values were observed at −0.7 V for both microbial biosensors. As can be seen from the figures CNT modification was not effective on working potential in order to follow oxygen consumption at this potential.

3.2. Analytical characteristics

The biosensors (modified with CNT and unmodified) were demonstrated for the quantification of glucose in the range of 0.05–1.0 mM at 30 °C and pH 7.0 with the 40 s of response time. At higher concentrations, standard curves for proposed systems showed a deviation from linearity. For both types of *G. oxydans* biosensors, linear graphs were obtained by the equation of $y = 1.160x + 0.151$ ($R^2 = 0.990$) and $y = 1.261x + 0.197$ ($R^2 = 0.982$), respectively (*y* shows current density as μA/cm^2 and *x* shows glucose concentration in mM). According to data, it can be said that CNT modification didn't affect the linear ranges but slightly higher current responses were registered. This is probably due to the surface properties of CNT modified system in which higher cell amount could be loaded. In our previous works, higher responses were obtained by the mediated microbial biosensing system when CNTs were incorporated to the osmium redox polymer which was used as immobilization matrix [43].

Repeatability was also examined. According to the results of 10 replicates of trials for 0.4 mM glucose, standard deviations (S.D) and variation coefficients (c.v) were calculated as ±0.013 mM and 2.9% and ±0.010 mM and 2.3% for CNT-free and modified electrodes, respectively.

When operational stability was concerned, microbial sensors were kept in the reaction cell containing working buffer with the adjusted temperature to 30 °C. And, 24% and 15% decrease of the response was observed up to 5th hour for CNT-free and modified electrodes, respectively.

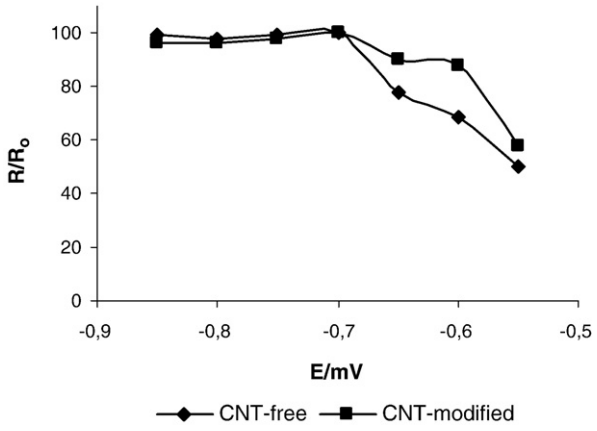

Fig. 5. Hydrodynamic voltammograms of CNT modified and unmodified microbial biosensors (in 50 mM PBS, pH 7.0, 30 °C, 1.0 mM glucose, CNT-free (♦), CNT-modified (■); for the biosensor *x* and *y* shows potential, V (versus the Ag|AgCl reference electrode) and biosensor response %, *R*: current and R_o: maximum current, respectively).

Table 2
Effects of various substrates to electrode response in the presence of glucose

Substrate	CNT-free		CNT-modified	
	Current density (μA/cm^2)	Recovery %	Current density (μA/cm^2)	Recovery %
Glc (0.2 mM)	0.405	–	0.491	–
EtOH (0.2 mM)	0.260	–	0.353	–
Glc+EtOH	0.627±0.030	94	0.917±0.068	96
Gal (0.2 mM)	–	–	–	–
Glc+Gal	0.407±0.006	100	0.496±0.011	101
Man (0.2 mM)	–	–	–	–
Glc+Man	0.411±0.012	101	0.508±0.014	103
Fru (0.2 mM)	–	–	–	–
Glc+Fru	0.416±0.015	103	0.497±0.013	101
Xyl (0.2 mM)	–	–	–	–
Glc+Xyl	0.412±0.012	102	0.517±0.015	105
GlcNAc (0.2 mM)	–	–	–	–
Glc+GlcNAc	0.401±0.0017	99	0.511±0.013	104
Lac (0.2 mM)	–	–	–	–
Glc+Lac	0.4±0.011	99	0.515±0.013	105
Sac (0.2 mM)	–	–	–	–
Glc+Sac	0.412±0.005	102	0.508±0.008	103
Mal (0.2 mM)	–	–	–	–
Glc+Mal	0.408±0.007	101	0.501±0.008	102
Cel (0.2 mM)	–	–	–	–
Glc+Cel	0.404±0.008	100	0.509±0.012	104

Glc: Glucose, EtOH: Ethanol, Gal: Galactose, Man: Mannose, Fru: Fructose, Xyl: Xylose, GlcNAc: N-acetyl D-glucosamine, Lac: Lactose, Sac: Saccarose, Mal: Maltose, Cel: Cellobiose.
*All measurements were repeated three times and reported as average±standard deviation.

3.2.1. Substrate specificity

Gluconobacter sp. contains several PQQ-dependent dehydrogenases: alcohol-, aldehyde-, aldose-, gluconatefructose-, and glycerol dehydrogenase. This diversity of enzymatic activities essentially determines the list of analytes detectable by *Gluconobacter* or *Acetobacter* cells [42]. So, the substrate specificities of the biosensor to various sugars and alcohols were tested by using both type of biosensors and results were given in Table 2. As can be seen, no response was observed for galactose, mannose, fructose, xylose, N-acetyl D-glucosamine, lactose, saccarose, maltose, cellobiose and methanol in our working conditions. *G. oxydans* biosensor exhibited response only for ethanol. Calibration graph for ethanol was also drawn in the range of 0.2–1.0 mM with equation of $y=0.918x+0.151$, ($R^2=0.990$) and $y=0.930x+0.058$ ($R^2=0.997$), (y shows current density (μA/cm^2) and x shows concentration (mM)) and a response time of 80 s for CNT-free and modified electrodes, respectively.

The effect of some compounds in the medium on glucose determination was also investigated and results were summarized in Table 2. For this aim, 0.2 mM of the compounds was added to the reaction cell containing of glucose (0.2 mM) in working buffer solution. Results showed that the presence of these compounds didn't interfere the biosensor response to glucose.

4. Conclusion

G. oxydans cells were immobilized together with CHIT matrix onto the surface of graphite electrodes. CHIT–CNT membrane was also prepared to search the nanoparticle effect on the biosensor response. The proposed system could be prepared without requiring any complex immobilization procedures and showed a good linearity and repeatability with a high operational stability. Furthermore, combining the properties of CNTs and the versatility and biocompatibility of CHIT produced chitosan surface-decorated carbon nanotubes. On the other hand, different substrates were applied to the system and only ethanol and glucose gave the signal. It was also observed that the presence of other compounds didn't affect the response to glucose substrate.

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