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Poly(glycidyl methacrylate-co-3-thienylmethylmethacrylate) as an immobilization matrix for microbial glycerol biosensing based on *Gluconobacter oxydans*

Tülay Ergön-Can [a,*], Elif Erhan [b], Ömer Faruk Algur [a]

[a] Department of Biology, Faculty of Science, Atatürk University, 25240 Erzurum, Turkey
[b] Department of Environmental Engineering, Gebze Technical University, 41400 Gebze, Kocaeli, Turkey

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ABSTRACT

A commercial strain of *Gluconobacter oxydans* together with a new co-polymer Poly(glycidyl methacrylate-co-3-thienylmethylmethacrylate) (Poly(GMA-co-MTM)), which provides effective immobilization in the continuous flow system, was used in the sensor design. By taking the advantages of the nano-technology, carbon nanotubes (CNTs) were also added to the cell film and the sensitivity of the sensor was increased about 15 times. During the glycerol analysis in the continuous system, it was shown that composite film was not removed from the electrode surface and film elements were not washed out from the system. Glycerol analyses were performed by using batch loaded continuously flow cell at different flow rates of 1, 2, 4, and 6 mL/min. The linear range was found as 2–100 mM with the detection limit (LOD) of 0.057 mM according to S/N = 3. The calibration graphs were obtained for Poly(GMA-co-MTM)/*G. oxydans* and Poly(GMA-co-MTM)/CNT/*G. oxydans* biofilm electrodes in FIA mode, and sensitivities were found to be 1.50 nA/mM and 19.13 nA/mM, respectively. In this study, Poly(GMA-co-MTM) was used for the first time as a microbial matrix and was shown to be an effective immobilization agent.

1. Introduction

Glycerol is a byproduct of biodiesel and bioethanol production and one of the quality parameters in food industry and vegetable oil production. In addition, it is quantified for the purpose of biochemical diagnostics in medical practices. It can be involved as a product or substrate in bioprocesses [1], while its concentration depends on the type of process to be monitored. Therefore the analysis of glycerol is required in many fields and very important in terms of industrial and commercial purposes.

The conventional methods for glycerol analysis include oxidation, esterification, ether formation, dehydration reaction, and chromatographic procedures [2]. The use of enzymatic procedures in routine laboratories is still limited due to its high cost. The establishment of a sensitive and inexpensive method for fast determination of glycerol is therefore becoming urgent for various industrial purposes [3].

On the other hand biosensors, particularly amperometric ones, are analytical tools, that can provide such analysis and they are competitors for conventional techniques using HPLC or GC for the determination of glycerol and glycerol derivatives [4]. Both enzymatic and microbial-based biosensors can be used successfully for the determination of glycerol. But microbial biosensors are cheaper and easily to be manufactured. As well as enzymes are usually more stable in their natural environment in the cell, and also coenzymes and activators are already present in the system.

Gluconobacter oxydans is an effective microorganism to determine glycerol due to its membrane bound oxidoreductase activities. Microbial biosensors based on *G. oxydans* produce an analytical signal via their pyrroloquinoline quinone (PQQ)-dependent membrane bound dehydrogenase (EC 1.1.99.22). Since the reactive sites of the related enzymes are located in the periplasmic space, there is no need to transport the substrates into the cells [5]. Therefore, *G. oxydans* can be used as a biocatalyst for many sugars and alcohols [6]. Membrane bound PQQ-dependent glycerol dehydrogenase (GDH) converts glycerol to dihydroxyacetone by oxidizing second hydroxyl group of glycerol [7] (Fig. 1).

Conducting polymers are often used as support materials in the immobilization of biomaterials for amperometric biosensors [8,9]. Various architectures of epoxy group possessing polymers have been developed in the literature [10,11]. Copolymers of glycidyl methacrylate (GMA), an epoxy group containing methacrylate monomer, with other acrylic or vinyl monomers have received great interest because of the pendant epoxide groups. GMA is able to bind enzyme covalently via its epoxy groups.

* Corresponding author.
E-mail addresses: tulayergon@hotmail.com (T. Ergön-Can), e.erhan@gtu.edu.tr (E. Erhan), ofalgur@yahoo.com (Ö.F. Algur).

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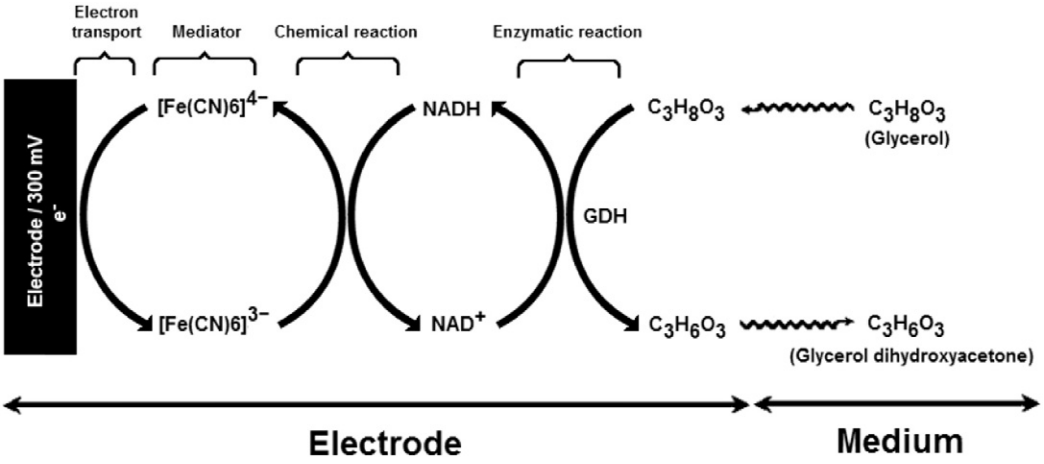

Fig. 1. Electron transfer mechanism of microbial glycerol biosensor based on *G. oxydans* on the electrode surface (GDH: glycerol dehydrogenase).

A thiophene-functionalized methacrylate monomer, 3-methylthienyl methacrylate (MTM), can be synthesized via the esterification of 3-thiophene methanol with methacryloyl chloride. Thus, the MTM monomer obtained has two polymerizable groups: the vinyl group is useful for radical polymerization while the thiophene ring, with substitution at the 3-position, can be employed in both oxidative polymerization and electropolymerization. In a previous study [12,13], horseradish peroxidase (HRP) was successfully bonded to the Poly(GMA-co-MTM) film electrode via pendant epoxy groups. Poly(GMA-co-MTM) is reported to be a promising polymeric material for the fabrication of enzyme electrode concerning its ability of forming chemical bonds with the amine groups of enzymes [12–14]. Copolymerization reaction of poly(GMA-co-MTM) can be seen in Fig. 2.

In this study, a microbial glycerol sensor based on *G. oxydans*, Poly(GMA-co-MTM)/*G. oxydans*, has been developed as an alternative to conventional chromatographic methods. Poly(GMA-co-MTM) was used as an immobilization matrix for the first time in the fabrication of a microbial biosensor. The successful use of nanoparticles as a part of the designed electrode matrix is widely reported in the current biosensor researches [15–17]. The sensing capability of the developed electrode was enhanced by benefiting superior conducting properties of nanoparticles. Poly(GMA-co-MTM)/*G. oxydans* was brought together with the advantages of nanoparticles in sensing. Carbon nanotubes (CNTs) have attracted more attention due to their high chemical stability, large surface area, high adsorption capacities, unique electronic properties and relatively high mechanical properties [18]. CNTs can donate and accept electrons in a wide range of potentials, and could therefore be used as mediators in biosensor systems [19]. The response dependences and amperometric characteristics including sensitivity, linear range, detection limit and response time of the developed Poly(GMA-co-MTM)/CNT/*G. oxydans* composite film electrode in the detection of glycerol have been investigated.

2. Experimental section

2.1. Reagents

All chemicals were of analytical grade and were used as received without any further purification, unless otherwise mentioned. Nutrient broth, agar, peptone, yeast extract, calcium chloride dihydrate, magnesium sulfate, sodium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate and ammonium chloride were purchased from Merck (Darmstadt, Germany). Glycerol and potassium ferricyanide were from Sigma-Aldrich (St. Louis, MO, USA). High purity multi-walled carbon nanotubes were purchased from Nanocs Inc. (New York, USA). Poly(GMA-co-MTM) was provided as a gift from Prof. Dr. Faruk Yilmaz (Department of Chemistry, Gebze Technical University).

2.2. Cell cultivation of G. oxydans

The strain of *G. oxydans* (ATCC 33447) was obtained from the Agricultural Research Service Culture Collection (NRRL) and inoculated into a nutrient broth. The activated *G. oxydans* strain was maintained on an agar plate containing 5 g/L peptone, 3 g/L yeast extract and 12 g/L agar, and transferred monthly. The cell biomass was prepared by aerobic cultivation at 30 °C on a rotary shaker in 250 mL flasks filled with 100 mL of growth media. A minimal medium, called M9 was used

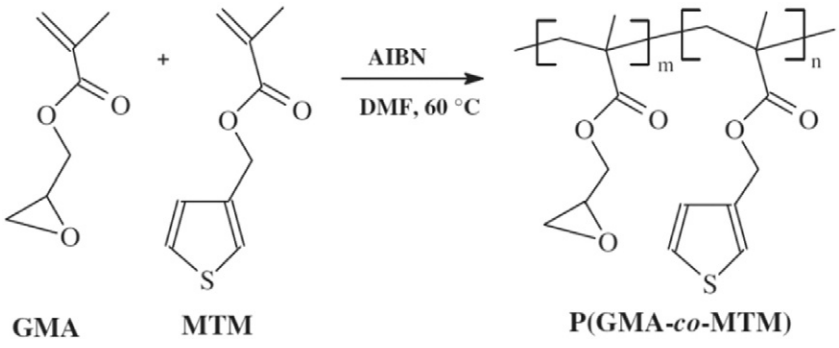

Fig. 2. The copolymerization reaction of Poly(GMA-co-MTM) (DMF: dimethylformamide, AIBN: azobisisobutyronitrile).

as growth medium for *G. oxydans*. M9 contains 5 g/L glycerol as the sole source of carbon with the following composition; 6.78 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2 \cdot 2H_2O$. The pH of the medium was adjusted to 7. The culture, inoculated from the slant agar, was incubated until reaching the late exponential phase which corresponds to the highest yield of PQQ-dependent glycerol dehydrogenase [20]. Then, the cells were collected by centrifugation (10 min, 6000 rpm), resuspended in 1 mL of cold and sterile 0.9% NaCl solution and this procedure was repeated three times. The biomass amount was calculated by weighing the cells after drying at 105 °C for approximately 2 h.

2.3. Flow injection system and electrodes

All electrochemical experiments were performed by using a CHI Model 842B electrochemical analyzer. The flow injection system (Fig. 3) was set up with a HPLC pump (Shimadzu LC-6 AD), an injection valve (Shimadzu), and a flow cell based on three electrode system (CHI 130). The HPLC pump was adjusted to deliver a running solution ranging from 1 mL/min to 6 mL/min flow rate for an accurate glycerol measurement. The running solution included minimal medium and 2 mM of potassium ferricyanide ($K_3[Fe(CN)_6]$) as a mediator at pH 7. The glycerol solution was introduced to the running solution through an injection valve with a loop volume of 0.5 mL. Glycerol concentration was quantified based on the obtained current through the electrochemical oxidation of ferrocyanide ($[Fe(CN)_6]^{4-}$) according to the scheme given in Fig. 1.

2.4. Preparation of Poly(GMA-co-MTM)/CNT/G. oxydans biocomposite film electrode

Glassy carbon electrode was polished with slurries of fine alumina powders (0.3 and 0.05 mm) on a polishing microcloth pad. The electrode was then rinsed with distilled water. The facile routine for preparation of water-soluble CNTs was a modification of the acid oxidative method developed by Zhao's group [21]. Firstly, 14 mg of multiwalled CNTs was added into 5 mL of a 9:1 concentrated H_2SO_4/H_2O_2 (30%) aqueous solution and the mixture was stirred for 30 min for CNT oxidation. After the reaction, 15 mL of the 9:1 concentrated H_2SO_4/H_2O_2 (30%) aqueous solution was added into the mixture. The mixture was placed in an ultrasonic bath (Elma 460-H) and sonicated for 5 min. Resulting CNT dispersion was diluted using 1 L of distilled water, then was filtered through a 0.45 μm cellulose membrane. Filtrate was washed with 10 mM NaOH solution and distilled water till the pH level reached 7. Then the filtrate was separated from the membrane and dispersed in distilled water (0.03 mg/L). The resulting CNT solution was sonicated for 2 min to obtain a homogeneous CNT solution.

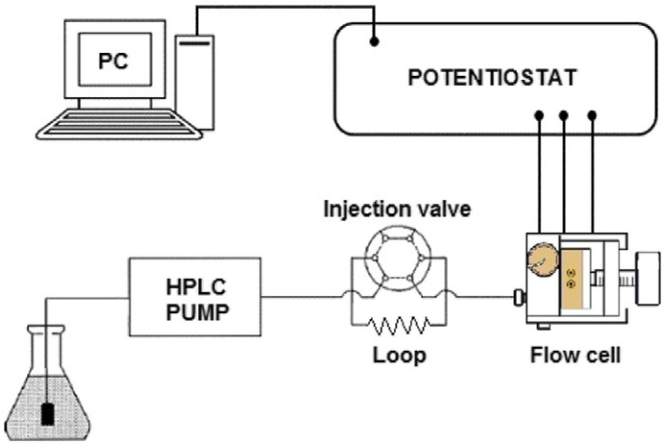

Fig. 3. Scheme of the flow injection system.

Poly(GMA-co-MTM)/CNT/*G. oxydans* biocomposite film electrode was prepared in three steps. Firstly, 5 μL of cell solution (1 g_{dw}/L) was spread onto the surface of the glassy carbon electrode and dried-up for 20 min at 30 °C. After that, 5 μL of CNT solution was dropped on the cell layer and it was dried at 30 °C for 15 min. Finally 5 μL of Poly(GMA-co-MTM) solution was spread onto the composite surface of electrode and then allowed to dry at 30 °C for 15 min.

3. Results and discussion

In this study two different configurations of the electrodes were used: Poly(GMA-co-MTM)/*G. oxydans* electrode and Poly(GMA-co-MTM)/CNT/*G. oxydans* electrode. The difference between these two configurations is the presence of CNT in the composite film. The design parameters of sensitivity, linear range, detection limit, and response time were investigated for these developed electrodes. Biosensor parameters were calculated by using the calibration curves obtained from the current peaks of flow injection experiments corresponding to the varied glycerol concentration. In addition, the effect of the flow rate on biosensor parameters was examined by operating the flow injection system with different flow rates.

3.1. Various electrode configurations and the effect of CNT in the design of electrode

Amperometric response of the electrode without CNT (Poly(GMA-co-MTM)/*G. oxydans*) at flow injection rates of 2 mL/min and 4 mL/min can be seen in Fig. 4a and b, respectively. In these experiments, the glycerol at various concentration ranges (2 to 100 mM) was loaded to the flow injection system. Apart from systematic loading in ascending concentration order for the calibration curve, the system was also loaded with different levels of glycerol concentrations to be able to test the system's performance with respect to concentration fluctuations. The results are seen to be compatible with each other. The coefficient of determination, R^2,

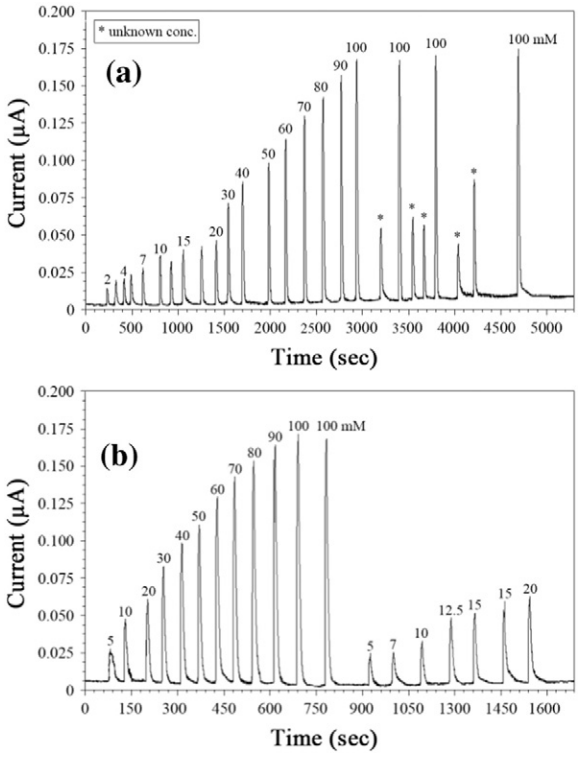

Fig. 4. Amperometric responses of Poly(GMA-co-MTM)/*G. oxydans* biocomposite film electrode to increasing concentrations of glycerol at a flow rate of (a) 2 mL/min, and (b) 4 mL/min, respectively (applied potential: 300 mV vs. Ag/AgCl, 3 M NaCl).

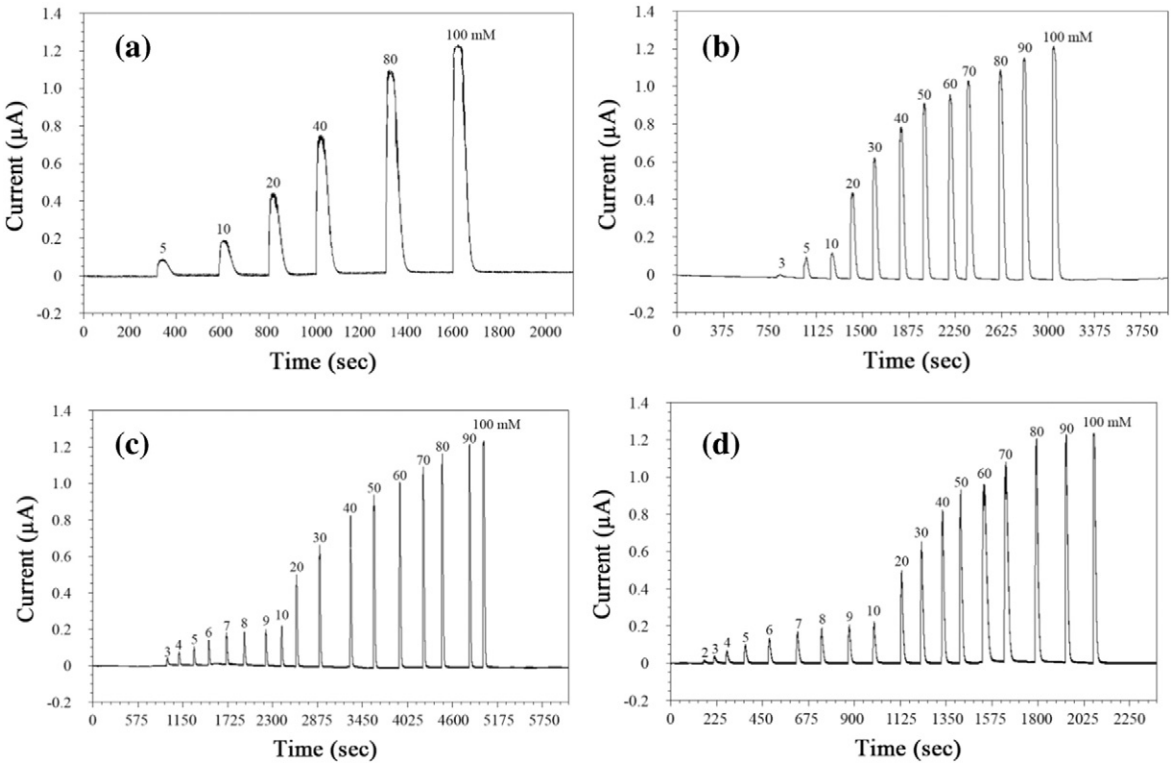

Fig. 5. Amperometric responses of Poly(GMA-co-MTM)/CNT/*G. oxydans* biocomposite film electrode to increasing concentrations of glycerol at a flow rate of (a) 1 mL/min, (b) 2 mL/min, (c) 4 mL/min, and (d) 6 mL/min, respectively (applied potential: 300 mV vs. Ag/AgCl, 3 M NaCl).

values of the calibration curve is 0.99. Response time was measured as 25 s and 40 s for operating flow rates of 4 mL/min and 2 mL/min, respectively. The sensitivity value of the electrodes were calculated as 1.39 and 1.50 nA/mM for operating flow rates of 4 mL/min and 2 mL/min, respectively.

CNT was used in order to investigate the effect of nanoparticle use in the configuration of working electrode. Amperometric response of the electrode with CNT (Poly(GMA-co-MTM)/CNT/*G. oxydans*) at flow injection rate of 1 mL/min, 2 mL/min, 4 mL/min, and 6 mL/min can be seen in Fig. 5a–d, respectively. R^2 values for the obtained calibration curves are calculated as 0.97–0.98. The sensitivity values were calculated as 12.33, 19.33, 16.54, and 17.84 nA/mM in the order of increasing flow rate.

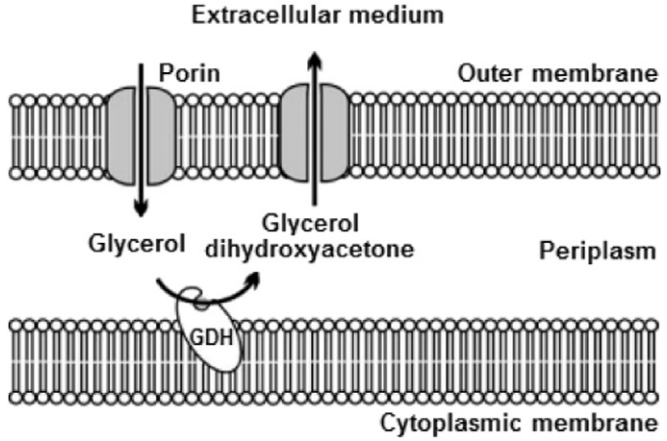

Fig. 6. Degradation mechanism of glycerol of *G. oxydans* (GDH: glycerol dehydrogenase).

3.2. Mass transfer effect: the effect of flow rates on biosensing

The working electrode has a sophisticated surface composed of polymeric material, whole cells, and nanoparticles. This complex film forms a relatively thick layer which can limit the mass transfer through the surface for biocompatibility of glycerol by *G. oxydans*. As seen in Fig. 6, the glycerol dehydrogenase is an enzyme bonded to the surface of cell membrane. The reaction of PQQ-dependent glycerol dehydrogenase enzyme, which localized in the periplasm and directly connected to the electron transport chain, with glycerol produces glycerol dihydroxyacetone. The biochemical reaction rate is directly effective on

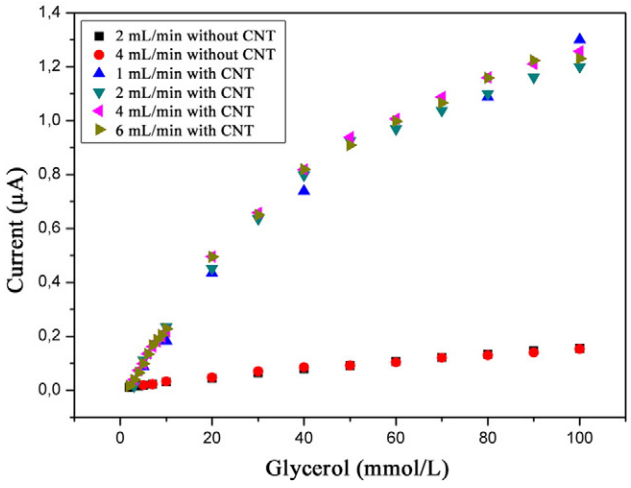

Fig. 7. Calibration curve Poly(GMA-co-MTM)/*G. oxydans* and Poly(GMA-co-MTM)/CNT/*G. oxydans* biofilm electrodes to increasing glycerol concentrations (applied potential: 300 mV vs. Ag/AgCl, 3 M NaCl).

Table 1
Biosensor parameters of working electrodes (W.E. (I): Poly(GMA-co-MTM)/*G. oxydans* and W.E. (II): Poly(GMA-co-MTM)/CNT/*G. oxydans*).

Working electrode	R^2	Sensitivity (nA/mM)	Linear range (mM)	Detection limit (mM)	Response time (s)	% RSD	% Recovery
W.E. (I) 2 mL/min	0.99	1.50	2–100	0.057	40	1.17	93.03
W.E. (I) 4 mL/min	0.99	1.39	5–100	0.103	25	2.11	95.95
W.E. (II) 1 mL/min	0.97	12.33	5–100	0.263	100	3.55	101.14
W.E. (II) 2 mL/min	0.98	19.13	3–50	0.169	85	1.14	103.64
W.E. (II) 4 mL/min	0.97	16.54	3–70	0.159	45	6.31	92.06
W.E. (II) 6 mL/min	0.97	17.84	2–60	0.145	35	4.24	91.12

biosensor parameters, especially on sensitivity. Pressure driven systems are known to be more efficient in overcoming the mass transfer limitations depending on the flow rates.

In this work, the effect of flow rate on the reaction rate was examined through operating the system at various flow rates. The results of the experiments obtained for two different electrodes at various flow rates were overlaid in Fig. 7. It can be seen from the figure that the calibration curves for both electrodes are almost entirely overlapped independent of the used flow rate. It can be said that intrinsic reaction rate is in the kinetic-controlled region not in the diffusion controlled region. Therefore, the change in flow rate shortens the response time without affecting the sensitivity of the biosensor. Even the flow rate increased to the high level of 6 mL/min, neither nanoparticles nor whole cells were washed out from the system. This proves that formed surface is extremely stable since the used matrix of Poly(GMA-co-MTM) was chemically bonded to the *G. oxydans* cells and successfully integrated with nanoparticles via forming a network on the surface. Therefore mass transferring rate is not limited by diffusional forces.

Obtained biosensor parameters based on different flow rates for each used electrode were listed in Table 1. Both this table and Fig. 7 clearly show that the electrode with CNT enhances enormously the current response 15 fold compared to the current value obtained using the electrode without CNT. Different types of nanoparticles can improve the basic characteristics of a biosensor [22]. A similar increase of sensitivity was reported for ethanol biosensor based on *G. oxydans* and CNTs [23].

Table 2 summarizes sensor parameters reported in the literature for biosensor, which sensing glycerol and its derivatives are particularly based on *G. oxydans*. Reviewing of the parameters one can see that a biosensor was developed in this work with a comparable capacity.

Table 2
Sensor parameters reported in the literature for the enzyme-based and microbial-based biosensors.

Sample	Bioreceptor	Immobilization technique	Working electrode	Detection mode	Detection limit (μM)	Linear range (mM)	Sensitivity (μA/mM)	Ref.
Glycerol	*G. oxydans*	Immobilized in a dialysis membrane	Graphite rod	Batch	20	0.02–2	0.755	[24]
Triglyceride	*G. oxydans*	Immobilized in a dialysis membrane	Graphite rod	Batch	50	0.05–12	0.058	[24]
Dihydroxy acetone	*Galactose oxidase and peroxidase*	Immobilized in a dialysis membrane	Graphite rod	Batch	0.8	0.002–0.55	4.7	[25]
Ethanol	*G. oxydans*	Immobilized in a CA membrane with ferricyanide as a mediator	Glassy carbon	Batch	0.85	0.002–0.27	3.5	[26]
1,3-Propandiol	*G. oxydans*	Immobilized in a dialysis membrane with ferricyanide as a mediator	Glassy carbon	Batch	2	0.004–0.16	0.108	[27]
1,3-Propandiol	*G. oxydans*	Immobilized in a dialysis membrane with Os-polymer as a mediator	Glassy carbon	FIA*	5.6	0.01–0.2	0.028	[27]
Ethanol	*G. oxydans*	Immobilized in a dialysis membrane	Glassy carbon	FIA	3.3	0.01–1.5	0.374	[28]
Triglyceride	*Lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish peroxidase*	Immobilized onto PVA membrane through glutaraldehyde	Platinum	Batch	210	0.56–2.25	–	[29]
Triglyceride	*Lipase, glycerol kinase, glycerol-3-phosphate oxidase and horseradish peroxidase*	Immobilized onto PVC membrane through glutaraldehyde	Platinum	Batch	110	0.56–2.25	–	[30]
Ethanol	*G. oxydans*	Immobilized in a chitosan matrix with CNT and ferricyanide as a mediator	Glassy carbon	Batch	–	–	162	[23]
Glycerol	*Glycerol kinase, creatine kinase, creatinase, sarcosine oxidase, peroxidase*	Immobilized between a chitosan sandwich	Gold	Batch	1.96	0.005–0.64	0.80	[31]
Glycerol	*Glycerol dehydrogenase*	Entrapped in a carbon paste electrode and covered with a layer of Poly(o-phenylenediamine)	Glassy carbon paste	Batch	20	0.0007–1.8	–	[32]
Glycerol	*G. oxydans*	Covalent binding on a Poly(GMA-co-MTM) copolymer	Glassy carbon	FIA	103	5–100	0.0015	This study (W.E. I)
Glycerol	*G. oxydans*	Covalent binding on a Poly(GMA-co-MTM) copolymer with entrapped CNT	Glassy carbon	FIA	145	2–60	0.0191	This study (W.E. II)

* FIA: flow injection analysis.

Although the sensitivity of the electrode with Poly(GMA-co-MTM)/CNT/*G. oxydans* is significantly higher than that of Poly(GMA-co-MTM)/*G. oxydans*, it was obtained slightly less sensitive compared with the studies reported in the literature [27,28]. In contrast, the working electrodes showed a wide linear range and high stability among the glycerol sensors [23–32]. It is thought that these results were very much dependent on the use of Poly(GMA-co-MTM) as a matrix for *G. oxydans* whole cells. Poly(GMA-co-MTM) is an inert matrix while allowing water to transfer via its porous structure. This biocompatible matrix creates a convenient microenvironment for microorganisms to sustain their bioactivity. The matrix can be in very close contact with the living cells without being toxic or adversely affecting their viability. In addition, the matrix is electroactive and can copolymerize with the conducting polymers.

4. Conclusions

Application of whole living cells opens new horizons both for basic bioelectrochemical studies and reagentless biosensing and possibly construction of more robust microbial biosensors. Whole cells represent natural environment for the enzymes and improve stability of biosensors while producing cheaper systems compared with enzyme-based biosensors.

In this study, it was shown that the use of CNT integrated into polymeric matrix enhances the biosensor's sensitivity significantly. The formed surface is extremely stable since the used matrix of Poly(GMA-co-MTM) was chemically bonded to the *G. oxydans* cells and successfully integrated with nanoparticles via forming a network on the surface. Poly(GMA-co-MTM) was used for the first time as a microbial matrix. The results obtained from the developed glycerol microbial biosensor based on *G. oxydans* showed that Poly(GMA-co-MTM) as a matrix creates highly stable biocomposites in the design of efficient working electrodes.

Conflicts of interest

The authors declare no conflict of interest.

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