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Characteristics of third-generation glucose biosensors based on *Corynascus thermophilus* cellobiose dehydrogenase immobilized on commercially available screen-printed electrodes working under physiological conditions

Muhammad Nadeem Zafar [a,*], Gulnara Safina [b], Roland Ludwig [a,c], Lo Gorton [a]

[a] Department of Biochemistry and Structural Biology, Lund University, SE-22100 Lund, Sweden
[b] Department of Chemistry and Molecular Biology, University of Gothenburg, SE-41296 Gothenburg, Sweden
[c] Department of Food Sciences and Technology, Food Biotechnology Laboratory, BOKU–University of Natural Resources and Life Sciences, A-1190 Vienna, Austria

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ABSTRACT

In this article, we describe a third-generation amperometric glucose biosensor working under physiological conditions. This glucose biosensor consists of a recently discovered cellobiose dehydrogenase from the ascomycete *Corynascus thermophilus* (*Ct*CDH) immobilized on different commercially available screen-printed electrodes made of carbon (SPCEs), carboxyl-functionalized single-walled carbon nanotubes (SPCE–SWCNTs), or multiwalled carbon nanotubes (SPCE–MWCNTs) by simple physical adsorption or a combination of adsorption followed by cross-linking using poly(ethyleneglycol) (400) diglycidyl ether (PEGDGE) or glutaraldehyde (GA). The *Ct*CDH-based third-generation glucose biosensor has a linear range between 0.025 and 30 mM and a detection limit of 10 μM glucose. Biosensors based on SWCNTs showed a higher sensitivity and catalytic response than the ones functionalized with MWCNTs and the SPCEs. A drastic increase in response was observed for all three electrodes when the adsorbed enzyme was cross-linked with PEGDGE or GA. The operational stability of the biosensor was tested for 7 h by repeated injections of 50 mM glucose, and only a slight decrease in the electrochemical response was found. The selectivity of the *Ct*CDH-based biosensor was tested on other potentially interfering carbohydrates such as mannose, galactose, sucrose, and fucose that might be present in blood. No significant analytical response from any of these compounds was observed.

Despite the numerous existing analytical devices and sensors for the detection of blood glucose level, the problem of the development of simple yet robust tools capable of detecting glucose with high reliability still remains as one of the most significant topics in clinical analysis. Most of the currently commercially available glucose biosensors for the detection of blood glucose level are electrochemical [1]. Glucose oxidase (GOx),[1] due to its high substrate specificity and stability in combination with its easy availability, is still the most frequently employed enzyme for the development of electrochemical glucose biosensors [1–3]. However, because GOx belongs to the class of intrinsic redox enzymes with the catalytic center buried deep inside the protein globule [4,5], direct electron transfer (DET) between the enzyme molecule and the electrode surface is highly restricted. However, by using deglycosylated forms of GOx [6,7] or through special GOx-containing compressed carbon nanotube (CNT)–enzyme electrodes [8], DET between the enzyme molecule and electrode surface has been proposed recently. On the one hand, the electron transfer communication with the electrode can be solved and electron transfer can be facilitated if a redox mediator is included in the biosensor construction (second-generation biosensors) [1]. On the other hand, some of those redox mediators used in glucose biosensors can be highly toxic at certain oxidation states (e.g., $Os^{2+/3+}$-based mediators). There are also further drawbacks with GOx (e.g., its restricted turnover rate for glucose; its high turnover rate with O_2, from which it follows that there is always a risk for production of H_2O_2).

Because of the obvious drawbacks with GOx, there has been a continuous search for alternative glucose-oxidizing enzymes to be used in glucose biosensors (e.g., nicotinamide adenine dinucleotide [NAD]-dependent glucose dehydrogenase [9,10], pyrroloquinoline quinone [PQQ]-dependent glucose dehydrogenase [11–14], flavin adenine dinucleotide [FAD]-dependent glucose dehydrogenase [15,16]). However, these all fail in the ability for DET communication

* Corresponding author.
E-mail address: znadeempk@yahoo.com (M.N. Zafar).

[1] *Abbreviations used:* GOx, glucose oxidase; DET, direct electron transfer; CNT, carbon nanotube; FAD, flavin adenine dinucleotide; CDH, cellobiose dehydrogenase; IET, interdomain electron transfer; *Ct*CDH, CDH from *Corynascus thermophilus*; SWCNT, single-walled carbon nanotube; SPCE, screen-printed electrode made of carbon; MWCNT, multiwalled carbon nanotube; PBS, phosphate-buffered saline; PEGDGE, poly(ethyleneglycol) (400) diglycidyl ether; GA, glutaraldehyde; AA, ascorbic acid; UA, uric acid; AP, acetaminophen.

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with electrodes. Finding a glucose-oxidizing enzyme that has the ability for DET would be an excellent alternative to GOx because the simple construction of a third-generation glucose biosensor contains no potentially toxic redox mediators. One such enzyme that is able to transfer electrons gained by the substrate oxidation directly to the electrode surface is cellobiose dehydrogenase (CDH) [17].

CDH (EC 1.1.99.18; cellobiose: [acceptor] 1-oxidoreductase) is an extracellular flavocytochrome secreted by both basidiomycete and ascomycete fungi involved in wood degradation [18]. CDH consists of a larger flavin-associated (dehydrogenase) domain, DH_{CDH}, and a smaller haem-containing (cytochrome) domain, CYT_{CDH}. The cofactors in the DH_{CDH} and CYT_{CDH} domains are FAD and haem *b*, respectively. The surface-exposed haem *b* of the CYT_{CDH} makes it possible to deliver the electrons from the DH_{CDH} to the CYT_{CDH} and from the CYT_{CDH} to a polarized electrode surface via interdomain electron transfer (IET) and subsequently DET [17,19]. The mechanism involves oxidation of the substrate in a 2 e^-, 2 H^+ reaction at the DH_{CDH} via formation of the product and the fully reduced FAD. Then, the DH_{CDH} sequentially delivers the electrons to the CYT_{CDH} via IET, followed by shuttling the electrons to the electrode (DET). In general, the IET from the DH_{CDH} to the CYT_{CDH} is strongly pH dependent [17,18,20–23].

Both basidiomycete and ascomycete fungi produce CDHs [18] and today are classified into three different groups [23]. The basidiomycete ones form class I CDHs, and the ascomycete ones form class II and III CDHs. Class I CDHs oxidize only cellodextrins and lactose, whereas class II and III CDHs may also oxidize mono-, di-, and oligosaccharides other than lactose and cellodextrins to their corresponding lactones at the DH_{CDH} domain [24–26]. Previously, it was reported that in CDHs produced by basidiomycetes, the electron transfer from DH_{CDH} to the CYT_{CDH} is more efficient at acidic pH (pHs 4.0–4.5) [20,24,26,27] and strongly restricted at neutral pH, probably due to repulsive electrostatic forces that hinder domain association and, thus, result in less efficient DET at neutral pH. In contrast to CDHs from basidiomycete fungi, CDHs from some ascomycete fungi catalyze the oxidation of mono- and disaccharides like glucose and maltose with high turnover rates at neutral and slightly alkaline pHs and, thus, allow DET at physiological pH [25,28].

It has been recently reported that the newly discovered CDH from the ascomycete *Corynascus thermophilus* (*Ct*CDH), which oxidizes glucose at neutral pH and exhibits most efficient IET [23], shows great promises and perspectives to be exploited for construction of a third-generation mediator-free glucose biosensor [22]. *Ct*CDH was adsorbed on the surface of both bare carbon electrodes and carbon electrodes modified with oxidatively shortened single-walled carbon nanotubes (SWCNTs) [29]. The possibility of DET via IET was studied both in the presence and in the absence of substrate. It was assumed that the presence of CNTs facilitates DET between the immobilized enzyme and the electrode. Moreover, the analytical response obtained by the electrode modified with SWCNTs was approximately one-third higher than the one obtained using just bare carbon electrode; however, the stability was insufficient.

In this work, we have continued the exploration of *Ct*CDH to make a third-generation glucose biosensor with improved working performance and operational stability based on *Ct*CDH. Commercially available screen-printed electrodes made of carbon (SPCEs) modified with single-walled carbon nanotubes (SPCE–SWCNTs) and multiwalled carbon nanotubes (SPCE–MWCNTs) were used instead of the previously used graphite rod electrodes [29]. This allowed miniaturization of the glucose assay format and made it more convenient and suitable for on-line mode use. The effect of cross-linking on the activity of the immobilized *Ct*CDH was also studied because it was previously noted for another type of CDH when immobilized on screen-printed electrodes that cross-linking not only increased the stability but also increased the current density [20]. We believe that this biosensor provides a simple and robust assay format for fast and reliable screening and quantification of glucose at physiological conditions.

Materials and methods

Chemicals and materials

*Ct*CDH (volumetric activity with cytochrome *c* at pH 7.5 = 53 U ml^{-1}, protein concentration = 8.4 mg ml^{-1}) was used for electrode modification. *Ct*CDH was purified from the culture supernatant of the ascomycete *C. thermophilus* (CBS 405.69) obtained from the Centralbureau voor Schimmelcultures (Baarn, The Netherlands). Cultivation and purification of the enzyme were similar to previously reported protocols for *Myriococcum thermophilum* CDH [24]. The homogeneous preparation of the enzyme was stored in a 50 mM acetate buffer (pH 5.5 at −80 °C).

All salts for making phosphate-buffered saline (PBS) and phosphate buffers used in this work were purchased from Sigma–Aldrich (Stockholm, Sweden). Sodium hydroxide solution obtained from Merck/VWR International (Stockholm, Sweden) and hydrochloric acid obtained from Sigma–Aldrich were used to adjust the pH. D(+)-Glucose, D(+)-mannose, D(+)-galactose, D(+)-xylitol, D(−)-fructose, fucose, and D(+)-trehalose were of analytical grade and obtained from Sigma–Aldrich. D(+)-xylose and rhamnose were obtained from ICN Biomedicals (Columbus, OH, USA). Sucrose was obtained from Chemicon (Malmö, Sweden). Poly(ethyleneglycol) (400) diglycidyl ether (PEGDGE) was purchased from Polysciences (Warrington, PA, USA), and glutaraldehyde (GA) was purchased from Sigma–Aldrich. Both PEGDGE and GA were used to cross-link *Ct*CDH on the electrode surface. All solutions were prepared using Milli-Q water (18.2 MΩ cm, Millipore, Bedford, MA, USA). The buffers and substrate solutions were prepared with the addition of 0.1 M potassium chloride (Sigma–Aldrich) to stabilize the pseudo-reference electrode of the screen-printed electrodes used and degassed before use to avoid the appearance of air bubbles in the flow system.

The electrochemical measurements were carried out using different carbon-based electrodes, both solid graphite rods and commercial screen-printed electrodes. Graphite rod electrodes (RW001, 3.05 mm diameter, 13% porosity) were purchased from Ringsdorff Werke (Bonn, Germany). The surface area of the graphite rods was 0.071 cm^2 with a roughness factor of approximately 5 [30]. The graphite rods were only used for investigating the variation of the response of immobilized *Ct*CDH with pH, and all other experiments were performed with the screen-printed electrodes. Three types of screen-printed electrode were purchased from DropSens (Oviedo, Spain) and used for the preparation of the biosensor. The screen-printed electrodes used in this work consisted of a working electrode made of carbon (DRP-110, denoted SPCEs), carboxyl-functionalized SWCNTs (DRP-110SWCNT, denoted SPCE–SWCNTs), or MWCNTs (DRP-110CNT, denoted SPCE–MWCNTs), a silver pseudo-reference electrode, and a carbon counter electrode deposited by printing technology on the ceramic support. These screen-printed electrodes are low-cost disposable devices specially designed to work with micro sample volumes. The general dimensions of these electrodes are 3.4 × 1.0 × 0.05 cm, and the working electrode has a diameter of 4 mm. They are robust electrodes that can be used in flow injection systems [20,31,32].

Electrochemical measurements

The screen-printed electrodes modified with *Ct*CDH were placed into a commercial methacrylate wall-jet flow-through electrochemical cell (DropSens, Oviedo, Spain) for measurements. The

graphite rod electrodes were fitted into a Teflon holder, inserted into a homemade wall-jet flow-through amperometric cell [33], and positioned at a constant distance (1–2 mm) from the inlet nozzle. The enzyme-modified graphite electrode was used as the working electrode, an $Ag|AgCl_{0.1\ M\ KCl}$ electrode served as the reference electrode, and a platinum wire was used as the auxiliary electrode.

All types of electrodes used were connected to a three-electrode potentiostat (Zäta Elektronik, Höör, Sweden). The electrochemical cells were connected to a flow injection system consisting of a peristaltic pump (Gilson, Villier-le-Bel, France) and a six-port valve electrical injector (Rheodyne, Cotati, CA, USA). A 50-μl aliquot of the substrate was applied by a loop mounted onto the injector. The resulting electrical current was recorded on a strip chart recorder (Kipp & Zonen, Utrecht, The Netherlands). All measurements were performed at room temperature.

Electrode preparation

To modify graphite rod electrodes, 5 μl of *Ct*CDH solution (8.4 mg ml^{-1}) was spread evenly on the top of the polished electrode. After the enzyme solution was dried completely, 1 μl of a freshly prepared PEGDGE solution (10 mg ml^{-1}) was placed on the top of the enzyme layer. All modified electrodes were kept overnight at 4 °C under constant humidity to complete the process of cross-linking [29].

To modify the screen-printed electrodes, 5 μl of the same *Ct*CDH solution as above was deposited on the surface of the working electrode, allowing the enzyme to adsorb. *Ct*CDH was carefully spread over the whole surface of the working area and was allowed to dry. To stabilize the enzyme layer, cross-linking of *Ct*CDH with PEGDGE or GA was carried out. Here, 1 μl of a PEGDGE solution (10 mg ml^{-1}) or 1 μl of a GA solution (1%) was deposited separately on the top of the adsorbed and dried enzyme layer, and then they were kept overnight at 4 °C under constant humidity to complete the process of cross-linking.

Results and discussion

Influence of pH

After applying a potential to the *Ct*CDH-modified electrodes, a stable baseline was attained within a few minutes. After that, a sugar sample was injected into the flow system. On oxidation of the substrate, the corresponding lactone and fully reduced DH_{CDH} are formed. In the reoxidation reaction of the reduced DH_{CDH} domain, the two electrons are then sequentially transferred through an interdomain electron transfer reaction via the CYT_{CDH} domain to the polarized graphite electrode acting as an electron acceptor, and the response is registered as a flow injection peak. The optimal applied potential was chosen, where the response was as high as possible and at the same time long-term stable (results not shown), and was found to be at +100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$. Similar to previously studied CDHs [17,24,34], for *Ct*CDH too high an applied potential (>300 mV vs. Ag|AgCl) irreversibly deactivated the ability of the enzyme to transfer the electrons directly to the electrode.

Most CDHs have an acid pH optimum for efficient interdomain electron transfer between the catalytic DH_{CDH} and the smaller haem-containing CYT_{CDH} domain [17,18]. However, *Ct*CDH is one of the hitherto discovered CDHs with both a pH optimum close to 7.4 and a reasonably high turnover rate for glucose [23], making this CDH an interesting enzyme for possible glucose monitoring. Therefore, the pH of the running buffer of the sensing system was varied to find the pH where the highest analytical response signal can be found. Results with three different substrates (1 mM cellobiose, 1 mM lactose, and 10 mM glucose) showed that the response of *Ct*CDH-modified graphite electrodes when the pH was varied from 6.0 to 9.0 attained a plateau at pH 7.5 and remained almost constant (Fig. 1A) in the slightly alkaline region up to pH 9.0.

The catalytic response of *Ct*CDH-modified SPCEs was also investigated at acidic and slightly alkaline pH values. For this purpose, 20 mM of each substrate (cellobiose, lactose, or glucose) was used. The results are presented in Fig. 1B. The catalytic response of each sugar was approximately 1.7 times higher at pH 7.4 compared with the catalytic response at pH 5.5. Thus, it can be concluded that the proposed biosensor prefers a slightly alkaline milieu and makes it possible to carry out simple analysis of glucose under human physiological conditions (pH 7.4).

Analytical characteristics of biosensor based on CtCDH immobilized on screen-printed electrodes

The working properties of the biosensors based on *Ct*CDH immobilized onto different commercially available screen-printed electrodes were studied in detail. All measurements reported on below were carried out in PBS buffer (pH 7.4) containing 0.1 M KCl at a flow rate of 0.5 ml min^{-1}. The potential applied was +100 mV versus pseudo-$Ag|AgCl_{0.1\ M\ KCl}$.

Comparison of different linking agents

To compare the effect of two different cross-linkers on the activity of *Ct*CDH, PEGDGE and GA were investigated. To accomplish cross-linking, 1 μl of PEGDGE (10 mg ml^{-1}) or 1 μl of GA (1%) [20] was used to modify the SPCEs along with *Ct*CDH. The analytical responses obtained from the electrodes modified with *Ct*CDH in both the presence and absence of PEGDGE or GA as cross-linkers were compared (Fig. 2). From Fig. 2, one can observe that *Ct*CDH/SPCEs modified with cross-linkers gave a higher analytical signal in comparison with *Ct*CDH/SPCEs modified without any cross-linker. *Ct*CDH modified with GA showed a 1.5 to 2 times higher response, whereas *Ct*CDH modified with PEGDGE showed a 3 to 5 times higher response. These results are similar to previous results when cross-linking a class I CDH on the surface of such screen-printed electrodes [20].

The responses of SPCEs, SPCE–SWCNTs, or SPCE–MWCNTs modified with *Ct*CDH or with *Ct*CDH together with the cross-linker PEGDGE were investigated and compared. PEGDGE (1 μl of 10 mg ml^{-1}) was applied on the surfaces of the enzyme-modified screen-printed electrodes. The analytical responses obtained from the electrodes modified with *Ct*CDH in both the presence and absence of PEGDGE as a cross-linker were compared (Fig. 3). It was also observed that the enzyme-modified SPCE–SWCNTs and SPCE–MWCNTs showed higher analytical response when also modified with PEGDGE cross-linker in comparison with SPCEs. The reason why cross-linkers are responsible for the increase in the response current is unknown. One possible explanation might be that the added PEGDGE can be connected to amino or carboxylic groups at either the DH_{CDH} or CYT_{CDH} domain and may increase the interdomain electron transfer rate, which is the rate-limiting step. It can also be speculated that PEGDGE has linked a larger amount of the enzyme molecules and enhanced their direct contact with the electrode surface. Another possible explanation is that PEGDGE (or GA) dissolves some of the components of the screen-printed ink and, thus, will expose a larger conducting surface area to the immobilized enzyme molecules.

Effect of choice of CNT-based screen-printed electrodes on response of biosensor

For quite some time now, CNTs have been used in electrochemistry and as electrode modifier [35–37]. In Fig. 4, it can be seen that

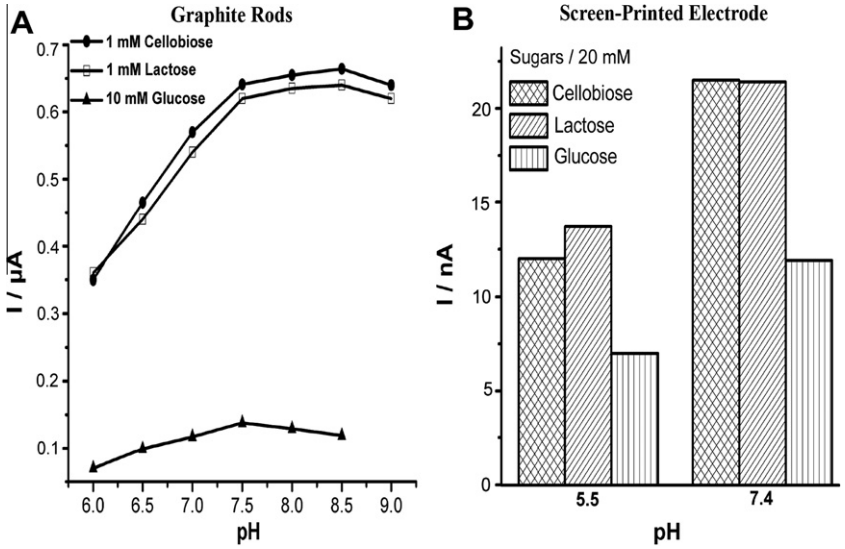

Fig.1. (A) pH profile of response current of *Ct*CDH-modified spectrographic graphite electrodes for samples of 1 mM cellobiose, 1 mM lactose, and 10 mM glucose. Experiments were performed in PBS with a flow rate of 0.5 ml min^{-1} and a working potential of 100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$. (B) Comparison of current response of *Ct*CDH-modified screen-printed electrodes (SPCEs) at pHs 5.5 and 7.4 for samples of cellobiose, lactose, and glucose (all 20 mM).

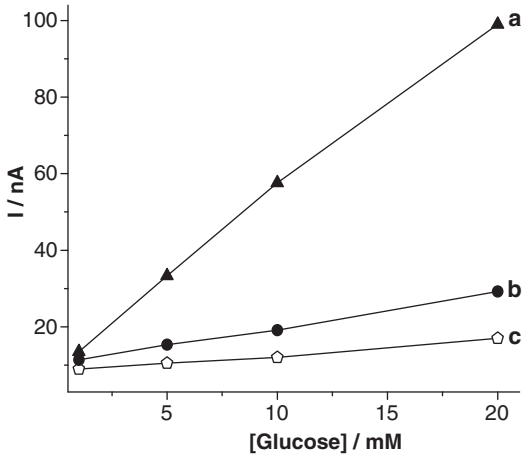

Fig.2. Comparison of *Ct*CDH-modified SPCEs in the absence and presence of PEGDGE and GA cross-linkers: (a) SPCEs modified with *Ct*CDH and 1 µl of PEGDGE; (b) SPCEs modified with *Ct*CDH and 1 µl of GA; (c) SPCEs modified with only *Ct*CDH. Experiments were performed in PBS with a flow rate of 0.5 ml min^{-1} and a working potential of 100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$.

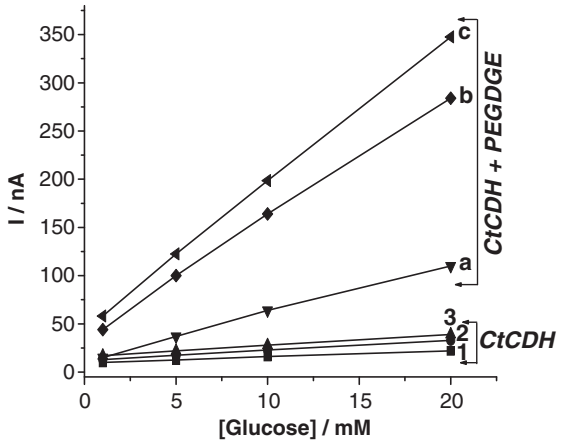

Fig.3. Effect of CNTs and PEGDGE cross-linker on the catalytic response of *Ct*CDH-modified screen-printed electrodes without cross-linker [SPCEs (1), SPCE–MWCNTs (2), and SPCE–SWCNTs (3)] and with cross-linker [SPCEs (a), SPCE–MWCNTs (b), and SPCE–SWCNTs (c)] using glucose as substrate. Experiments were performed in PBS with a flow rate of 0.5 ml min^{-1} and a working potential of 100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$.

the electrochemistry of $Fe(CN)_6^{4-/3-}$ becomes more reversible on the SPCE–MWCNTs and SPCE–SWCNTs compared with when using SPCEs. However, especially on the SPCE–SWCNTs, one can also see that the area under the waves is larger than those on the SPCE–MWCNTs and SPCEs. Thus, the nanostructured electrode surfaces of the CNT-modified electrodes not only increase the rate of the heterogeneous electron transfer reaction but also increase the real electrode surface area.

SPCEs, SPCE–SWCNTs, and SPCE–MWCNTs modified with *Ct*CDH or with *Ct*CDH together with PEGDGE were investigated. Three independent sets of each type of *Ct*CDH-modified electrode were prepared and investigated. Glucose solutions of different concentrations prepared with PBS buffer (pH 7.4) were used as analyte solution. The analytical responses obtained from the SPCEs, SPCE–SWCNTs, and SPCE–MWCNTs were compared. Fig. 3 shows that the analytical response is higher for SPCE–SWCNTs, followed by SPCE–MWCNTs and SPCEs, in both the absence and presence of PEGDGE. The results show that when using the modified SPCE–SWCNTs, a 3 to 4.5 times higher analytical response was obtained in comparison with when using the SPCEs, and also *Ct*CDH-modified SPCE–MWCNTs showed a 2.5 to 3.5 times higher response than *Ct*CDH-modified SPCEs. Thus, the *Ct*CDH biosensors based on both types of CNT-modified SPCE showed higher analytical response than those based on SPCEs. A probable explanation for this effect is that the presence of CNTs increased the active surface area of the electrode, resulting in adsorption of higher quantities of the enzyme. In addition, it can be anticipated that the CNTs will increase the percentage of adsorbed enzyme molecules oriented in a position on the conducting surface favoring DET contact with the electrode [20]. In a previous work, when adsorbing hemoglobin on such screen-printed electrodes, it was also shown that the DET electrochemistry of the immobilized hemoglobin became much clearer on the CNT-modified electrodes [38]. SPCE–SWCNTs showed better performance in comparison with SPCE–MWCNTs because SWCNTs

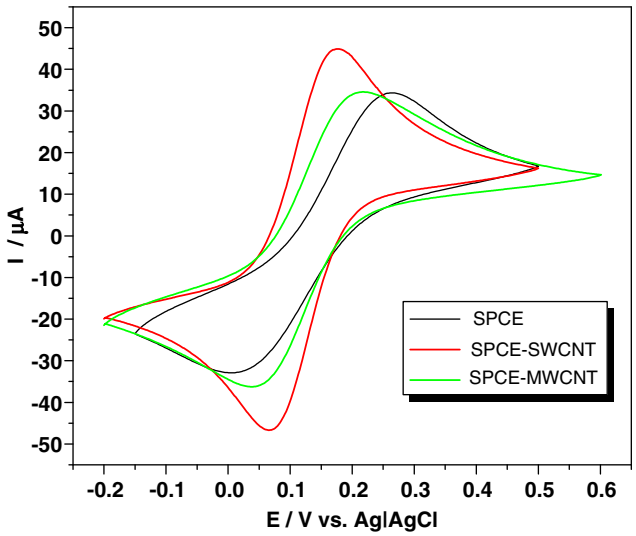

Fig.4. Cyclic voltammograms of $Fe(CN)_6^{4-/3-}$ on different screen-printed electrodes.

have a large surface area to volume ratio and better electrical, mechanical, and chemical properties than MWCNTs [39,40].

Analytical and working characteristics of glucose biosensor based on CtCDH and screen-printed electrodes

Glucose biosensors based on *Ct*CDH (with PEGDGE as a cross-linker) immobilized on the surface of SPCEs, SPCE–SWCNTs, and SPCE–MWCNTs were tested using the electrochemical flow-through cell. The biosensors were placed in the cell, and various concentrations of glucose dissolved in PBS buffer (pH 7.4) were injected into the flow system. Fig. 5A shows calibration curves obtained using the biosensors based on *Ct*CDH immobilized on SPCEs, SPCE–MWCNTs, and SPCE–SWCNTs. All three types of enzyme-modified electrode showed the same linear range (0.025–30 mM glucose). Previously, we reported that a similar linear response range (0.1–30 mM glucose) was obtained for solid graphite rod electrodes modified with *Ct*CDH in the presence and absence of SWCNTs [29], albeit with a higher detection limit. Fig. 5B shows the linear ranges of the calibration curves for all three types of the enzyme-modified electrode. The detection limit for glucose was as low as 0.01 mM and, thus, was 5 times lower compared with our previous results [29]. The glucose concentration in the blood of a healthy person is usually between 4 and 8 mM, and in the blood of a diabetes patient it can be up to 20 to 25 mM [1]. Hence, the linear range of the developed third-generation glucose biosensor based on *Ct*CDH and screen-printed electrodes should, in principle, cover the human physiologically relevant concentrations.

All developed biosensors showed good reproducibility of the analytical response. The sensor-to-sensor reproducibility was calculated using the analytical responses obtained from three equivalently prepared biosensors and had a relative standard deviation (RSD) of 3.3% to 3.9%.

Stability and selectivity of novel glucose biosensor

The operational stability of a biosensor response may vary considerably depending on the sensor geometry, method of preparation, biological recognition reactions, and the like [41], and therefore it was evaluated. Fig. 6 shows the results of the operational stability test of *Ct*CDH-modified SPCE–SWCNTs. For this experiment, aliquots of 50 mM glucose solution were successively

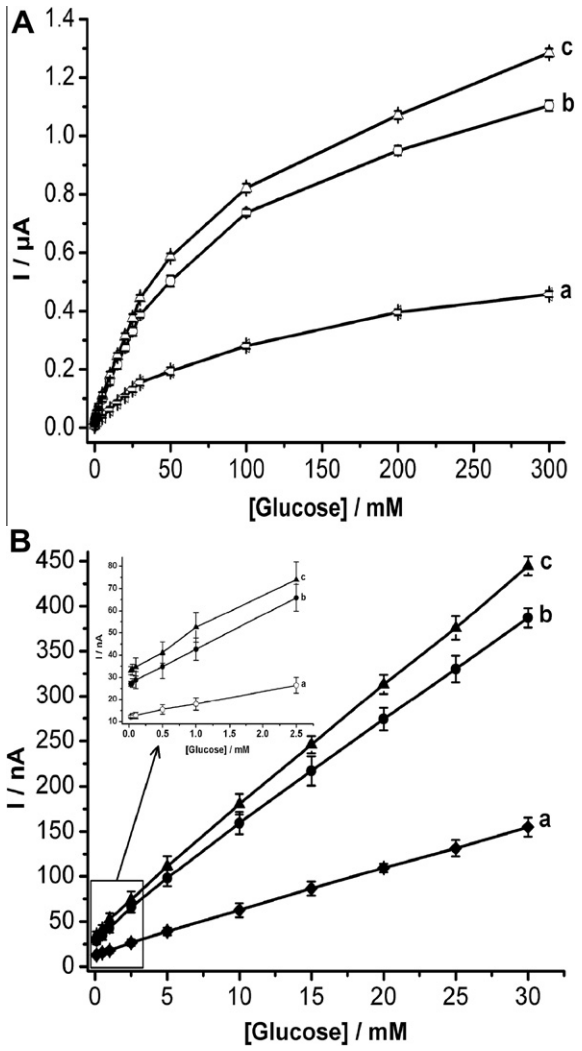

Fig.5. (A) Calibration graphs (catalytic current) of *Ct*CDH/PEGDGE cross-linker-modified screen-printed electrodes with glucose as substrate [SPCEs (a), SPCE–MWCNTs (b), and SPCE–SWCNTs (c)]. (B) Linear range of glucose calibration curves of *Ct*CDH/PEGDGE cross-linker modified screen-printed electrodes [SPCEs (a), SPCE–MWCNTs (b), and SPCE–SWCNTs (c)]. Experiments were performed in PBS with a flow rate of 0.5 ml min^{-1} and a working potential of 100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$.

injected into the electrochemical cell for nearly 7 h. The glucose biosensor exhibits excellent stability given that a decrease of only approximately 10% in the analytical response was observed with time. This is a great improvement compared with our previous results [29] using a solely physically adsorbed enzyme and an already denaturing potential (390 mV vs. NHE [normal hydrogen electrode]).

The response of the enzyme-based amperometric biosensors for the detection of glucose in real samples (e.g., in human blood) may be affected by interference from other electrochemically active species such as ascorbic acid (AA), uric acid (UA), and acetaminophen (AP). These interfering substances normally appear in very low concentrations (<1 mM) in human blood [42]. The normal ranges of AA, UA, and AP in human blood are 34–80, 178–416, and 130–150 µM, respectively [43], and the current responses of these species are very low compared with that of glucose [43,44]. Those substances can be directly electrochemically oxidized at sufficiently high working potentials and interfere with the major analytical response from the analyte of interest [45]. The necessary applied potentials to oxidize AA, UA, and AP are approximately 115, 415, and 215 mV versus $Ag|AgCl_{0.1\ M\ KCl}$ [43]. Lowering the

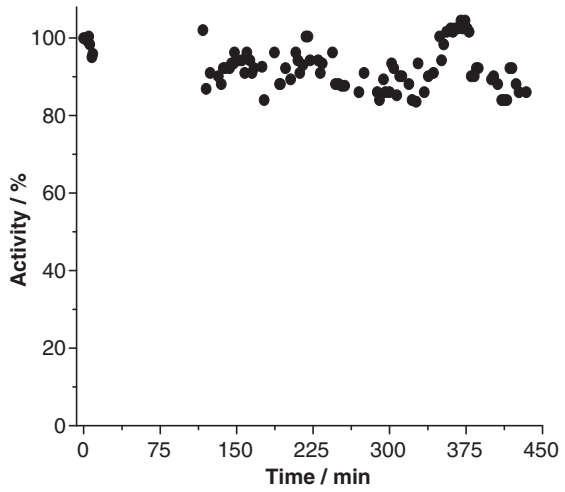

Fig.6. Variation of response for glucose with time obtained for a *Ct*CDH-modified screen-printed electrode (SPCE–SWCNT) with 50 mM glucose as substrate. Experiments were performed in PBS with a flow rate of 0.5 ml min^{-1} and a working potential of 100 mV versus $Ag|AgCl_{0.1\ M\ KCl}$.

working potential partially can minimize the contribution of such interferences. This explains the reason why a relatively low working potential in this type of research is preferred. Moreover, other carbohydrates (e.g., galactose, mannose) that could possibly be substrates for *Ct*CDH and, thus, might interfere with the analytical response of the enzyme-based biosensor may be present in blood samples. To investigate the effects of these sugars on the biosensor's response, *Ct*CDH-modified SPCE–SWCNT electrodes (with PEGDGE as a cross-linker) were used. No analytical signal was observed using galactose, xylose, fucose, rhamnose, sucrose, and xylitol (5 mM each sugar). A 10% signal in comparison with that for 5 mM glucose was observed with 5 mM mannose as substrate. However, in comparison with the glucose concentration, the concentration of mannose in blood is approximately 100 times lower and, thus, will interfere with the glucose measurement only at very low glucose concentrations (<50 μM). Such a low glucose concentration is unlikely to be found in blood samples.

Conclusion

A third-generation glucose biosensor has been developed by simple modification based on a combination of adsorption and cross-linking of *Ct*CDH onto commercially available SPCE, SPCE–MWCNT, and SPCE–SWCNT electrodes. The electrochemical characteristics of the biosensors were examined and compared with each other. The linear range and detection limit of the proposed *Ct*CDH glucose biosensor under physiological conditions were 0.025–30 and 0.01 mM, respectively. The biosensor revealed good selectivity and excellent operational stability. Furthermore, modification of the electrodes with a cross-linker improves both stability and sensitivity of these analytical devices. Taking into consideration its wide linear concentration range, low detection limit, and high selectivity and stability, the third-generation glucose biosensor has good potential to be further successfully applied for glucose monitoring in patients or blood samples. In another application of *Ct*CDH, it was shown that this enzyme exhibits long-term stability when exposed to both plasma and whole blood [46].

Acknowledgments

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