STUDIA UBB CHEMIA, LVI, 4, 2011 (p. 199 - 210)

# ELECTROCHEMICAL BEHAVIOR OF CELLOBIOSE DEHYDROGENASE FROM *NEUROSPORA CRASSA* IMMOBILIZED ON GRAPHITE AND Au-4-MERCAPTOPHENOL MODIFIED ELECTRODES

**GÁBOR KOVÁCS[a], IONEL CĂTĂLIN POPESCU[a,*]**

**ABSTRACT.** The amperometric responses of G/CDH and Au-4-mercaptophenol/CDH modified electrodes to lactose were comparatively recorded under flow injection and cyclic voltammetry operating modes, respectively. The differences noticed between the characteristic parameters of the two investigated bioelectrodes were explained in terms of the influences exerted by the given experimental conditions on direct electron transfer process, existing between cellobiose dehydrogenase (CDH) and the investigated electrode materials.

***Keywords:*** *amperometric biosensors, cellobiose dehydrogenase, direct electron transfer, self-assembled monolayer, Au and graphite modified electrodes.*

## INTRODUCTION

Modification of different electrode surfaces with enzymes is the main idea of amperometric biosensor construction. The basic requirements for amperometric biosensors are: (i) an enzyme which reacts on its substrate, reducing or oxidizing it at the surface of a suitable electrode; (ii) a method for immobilizing the enzyme in close proximity to the electrode, which retains the activity of the enzyme and is offering a suitable electron transfer pathway toward the electrode; (iii) an electronic system capable of controlling (and registering) the potential of the electrode and measuring the current produced by the redox process [1].

The enzyme used for our research was cellobiose dehydrogenase (CDH) from *Neurospora crassa.* CDH (EC 1.1.99.18) is an extracellular enzyme produced by a variety of different fungi. Around 30 species of fungi have shown to produce CDH. All CDHs belong to two related subgroups: class I, produced only by basidiomycetes (filamentous fungi) and class II, with longer and more complex structure, produced by ascomycetes (sac fungi). The most common and well-known CDH's are those produced by wood-degrading

[a] Universitatea Babeş-Bolyai, Facultatea de Chimie şi Inginerie Chimică, Str. Kogălniceanu, Nr. 1, RO-400084 Cluj-Napoca, Romania

* *cpopescu@chem.ubbcluj.ro*

and plant pathogen fungi [2-4]. The interest related to CDH is due to its ability to show efficient direct electron transfer (DET) properties at various electrode materials (different type of graphite [5-10], carbon nanotubes [11] and gold [12-14]), towards various substrates (e.g. cellobiose [15, 16], glucose [17, 18], lactose [9, 19]). Thus, CDH is considered very promising for applications in the field of biosensors and biofuel cells.

The enzyme immobilization and the construction variant allowing the DET between its active center and the electrode surface can be made by various methods. One easy way is the immobilization of the enzyme through simple chemo-physical adsorption on the surface of the electrode (Figure 1). This method is used to modify different graphite electrode surfaces (e.g. spectroscopic [8, 20], screen printed [21-24]), due to good reproducibility rates, high porosity of the electrode materials (thus they can adsorb the enzymes with high efficiency), and the low cost of the electrode material.

Another method used for assuring the electrical connection for efficient DET consists in the modification of the electrode surface with self-assembled monolayers (SAMs) [25-27]. In the case of metal electrodes, especially Au, the modification of the electrode surface with thiols, having various end-group functionalities, represents a good solution to overcome the difficulties met with DET [12, 14, 28, 29]. A SAM of thiols can provide a well-ordered structure onto electrode surface and hence, the enzyme maintains a precise distance from the electrode surface and a particular orientation of the enzyme, which favors an efficient DET, and assures high currents for less enzyme consumption.

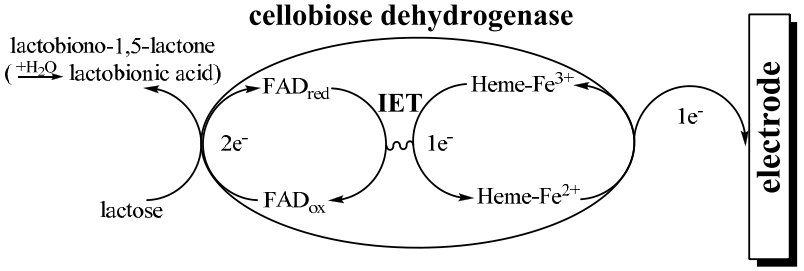


**Figure 1.** Schematic diagram of the process of direct electron transfer between the adsorbed CDH enzyme and the graphite or Au electrode modified with a self-assembled monolayer of 4-mercaptophenol.

When CDH is immobilized properly on the electrode surface (either on graphite or Au), in presence of substrate (e.g. lactose) the enzyme will oxidize it by the FAD cofactor to lactone and the electrons produced from this reaction will be transferred one-by-one at the electrode surface *via* the heme domain: the process is called internal electron transfer (IET) (Figure 1).

In the present study, in order to obtain more information about the DET efficiency for immobilized CDH, its electrochemical behavior was comparatively investigated by amperometry, performed either by flow injection measurements at a CDH-modified graphite electrode or by cyclic voltammetry at a CDH-modified Au electrode, which was previously covered with a SAM made of 4-mercaptophenol (SPh-OH). For this purpose, the responses to lactose, under DET operation mode, for both above-mentioned modified electrodes were monitored in different experimental conditions (pH and substrate concentration). The collected data served to establish the optimal constructive variant of the bioelectrodes incorporating CDH.

## RESULTS AND DISCUSSION

### Immobilization of CDH on different electrode materials

In the work presented here, spectroscopic graphite and gold electrodes modified with SAM were used as supporting materials for immobilization by simple adsorption of CDH isolated from *Neurospora crassa*. From the previous works [14] it is known that the favorable orientation of the enzyme, which is determinant for obtaining an efficient DET, is strongly affected by both the electrode material and the method used for enzyme immobilization.

Immobilization of CDH on graphite electrode involves a simple chemo-physical adsorption onto the surface of the polished graphite rod. Consequently, the optimal enzyme orientation on the surface of the electrode is not guaranteed, as the enzymes molecules are adsorbed randomly. Some adsorbed molecules will be able to participate in catalysis and electron transfer, but some other are immobilized in such a way that either the heme domain is not oriented in order to assures the transfer of the electrons produced during the catalytic process at FAD domain, or the orientation of catalytic center is unable to load the substrate from solution (Figure 2A). Often, a different construction is used for the electron transfer pathway. This requires the modification of the electrode surface (gold) with thiols, assembling a monolayer, and attaching the enzyme in an ordered layer. Theoretically, this approach supposes that all the adsorbed enzyme molecules are involved in catalysis and electron transfer (Figure 2B).

In this context, it was interesting to compare the electrocatalytic efficiency of the two construction variants described above. For this purpose, two different CDH-modified electrodes (G/CDH and Au-SPh-OH/CDH) were prepared and their electrocatalytic behavior was investigated towards the same substrate (lactose).

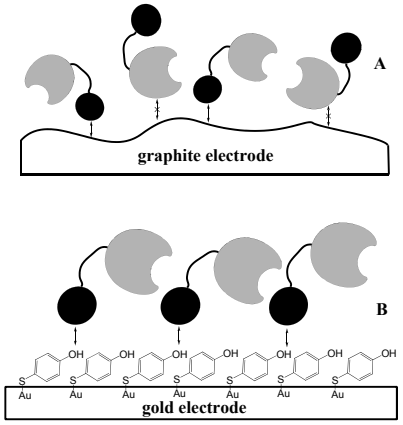


**Figure 2.** Schematic diagram showing the adsorption/orientation of the CDH enzyme (FAD domain – grey; heme domain – black; linker – black line) on graphite (A) and Au electrode modified with a self-assembled monolayer of 4-mercaptophenol (B).

## pH influence

The relative amperometric responses of G/CDH and Au-SPh-OH/CDH electrodes to 5 mM lactose at different pH values, recorded under flow conditions (G/CDH) or in cyclic voltammetry (Au-SPh-OH/CDH) are shown in Figure 3. As can be seen, for both electrodes, the optimum working pH is placed around 5.5. The difference between the pH profiles, observed in the case of investigated electrodes, should be explained in terms of the interaction between the pH induced conformation changes of the immobilized CDH molecule and their effect on the electron transfer process, occurring at different electrode surfaces. At the same time, the more organized structure, characteristic to Au-SPh-OH/CDH modified electrode, should be considered, too. Thus, it can be supposed that, within certain limits, the surface properties of the graphite and Au-SPh-OH electrodes are not significantly affected by the pH variation. Contrarily, the DET and IET processes, involved in the electron transfer between the CDH molecules and the electrode surfaces, are strongly influenced by the conformational changes of the enzyme molecules, which are induced by the variation of the distance between the two functional domains occurring when the pH changes. Concluding, the sharp maximum noticed on the pH profile of Au-SPh-OH/CDH electrode response certainly reflects the high sensitivity of an ordered structure for small conformational changes occurring around the optimal pH value. In this context, it is worth to mention that the catalytic activity observed at Au-SPh-OH/CDH modified electrode decreases with more than 50% of its maximum value, for a pH change of 0.5 units (Figure 3).

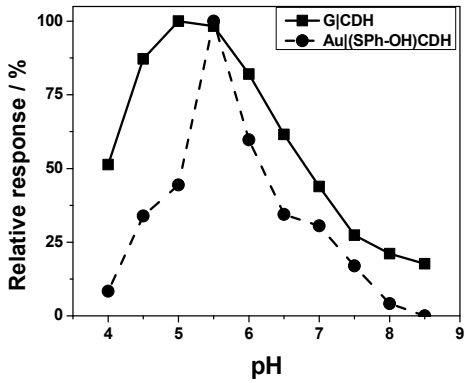


**Figure 3.** pH influence on the relative amperometric responses of **G/CDH** (■, ——) and **Au-SPh-OH/CDH** (●,— —) modified electrodes. Experimental conditions for **G/CDH**: flow injection mode, injections of 5 mM lactose, volume of injected sample, 50 µL; flow rate, 0.5 mL / min; applied potential, +300 mV *vs.* Ag|AgCl, 0.1M KCl; for **Au-SPh-OH/CDH**, cyclic voltammetry mode, starting potential, -300 mV *vs.* SCE, v=10 mV/s; supporting electrolyte 50 mM acetate buffer (pH 4 to 6) and 50 mM phosphate buffer (pH 6.5 to 8.5).

## Electrocatalytic efficiency

The amperometric responses of the modified electrodes were recorded at two different pH values: the optimum value (pH 5.5) and a value of practical interest for biotechnological applications (pH 7.0).

The calibration curves obtained for G/CDH modified electrode against lactose are shown in Figure 4. As expected, the bioelectrode gives a well-shaped Michaelis-Menten behavior at both pH values. The highest efficiency was observed in slightly acidic media. The value of the apparent Michaelis-Menten constant ($K_m^{app}$) decreases to its half at neutral pH compared to the value estimated for optimum pH: from 488 µM to 225 µM lactose. The maximum current ($I_{max}$) shows a similar behavior, decreasing from 2.03 µA (pH 5.5) to 0.8 µA (pH 7.0). Consequently, the bioelectrode sensitivity is slightly affected by the pH changes, decreasing with less than 15%, from 57.5 (pH 5.5) to 49.1 (pH 7.0) $\mu A*mM^{-1}cm^{-2}$. It should be mentioned that both values are slightly higher than those recently reported for a similar system [23].

The electrochemistry of CDH and its voltammetric response at Au-SPh-OH/CDH modified electrode was studied in absence and in presence of lactose, at pH 5.5 and at pH 7.0. In absence of the substrate, the response due to the redox-couple $Fe^{2+/3+}$ from heme domain was observed. At pH 5.5 the formal standard potential ($E^{0'}$) was found +150 mV *vs*. SCE, while at pH 7.0 $E^{0'}$ was +160 mV *vs*. SCE (Figure 5). Irrespective of the surrounding pH, in presence of the substrate (lactose) a clear catalytic current was observed. As it was suggested for a similar CDH [14], the thiols with alcohol end-group immobilized on the Au surface, induce the enzyme molecule orientation in a

favorable position at the surface of the modified electrodes. Thus, the biocatalytic process is enhanced, resulting in an active and selective bioelectrode. The current decrease noticed at neutral pH can be attributed to the decrease of DET efficiency, due to weaker (Au-SPh-OH) - CDH interactions, followed either by the decrease of the CDH adsorption rate or by unfavorable conformational changes occurring within the enzyme molecule. Consequently, the electron transfer becomes less efficient and the bioelectrode response decreases.

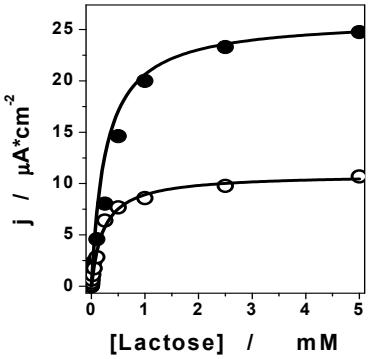


**Figure 4**. Calibration curves of G/CDH modified electrode towards lactose, recorded at two different pH values. Experimental conditions: applied potential, +300 mV vs. Ag|AgCl, 0.1M KCl; volume of injected sample, 50 µL; flow rate, 0.5 mL/min; flow carriers, 50 mM acetate (pH 5.5) or 50 mM phosphate buffer (pH 7.0); filled symbols were used for pH 5.5 and open symbols for pH 7.0. The solid lines correspond to Michaelis-Menten non-linear fittings.

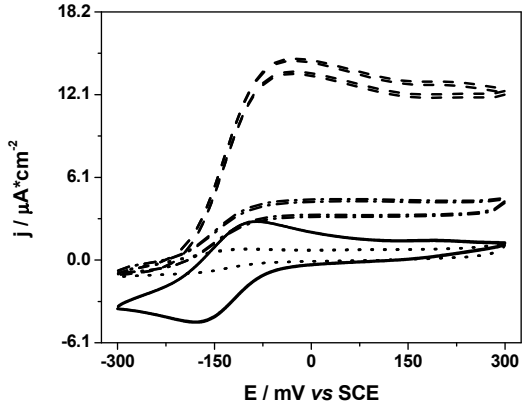


**Figure 5.** Voltammetric response of **Au-SPh-OH/CDH** modified electrode in absence [pH 5.5 (——); pH 7.0 (····)] and in presence [pH 5.5(- -); pH 7.0 (**- · -**)] of 5 mM lactose. Experimental conditions: starting potential, -300 mV *vs.* SCE; potential scan rate, 10 mV/s; 50 mM acetate (pH 5.5) or 50 mM phosphate buffer (pH 7.0).

The values estimating the catalytic efficiency for the two investigated electrodes are shown Table 1. The $I_0$ value is referring to the current measured in absence of the substrate, while the $I_{peak, S}$ value stands for the peak current measured in presence of the substrate. It can be noticed that the values of efficiency corresponding to the G/CDH modified electrode are much higher than those estimated for the Au-SPh-OH/CDH modified electrode. This behavior is obviously due to a higher enzyme loading in the case of the first bioelectrode. Indeed, a surface characterized by a high roughness factor (graphite) and allowing an unconstrained distribution of the CDH molecules will exhibit a higher enzyme activity than a surface with a lower roughness factor (Au-SPh-OH) and exerting size constraints for CDH molecules.

**Table 1.** Electrocatalytic efficiency of the CDH modified electrodes for 5 mM lactose (for experimental conditions see Figure 5 for Au-SPh-OH/CDH and Figure 4 for G/CDH).

| Electrode | Electrocatalytic efficiency ($I_{peak, S}/I_0$) | |
|---|---|---|
| | pH 5.5 | pH 7.0 |
| G/CDH | 72 | 30 |
| Au-SPh-OH/CDH | 9.76 | 3.84 |

Additionally, the sensitivity (expressed as the ratio between of the electrocatalytic efficiencies estimated at pH 5.5 and pH 7.0) of the second bioelectrode to pH changes (2.54) is slightly higher than that estimated for the first one (2.40). This behavior supports the results reported above, confirming once again that an ordered structure is more sensitive to small conformational changes of the enzyme molecules, occurring around the optimal pH value.

## CONCLUSIONS

During the half century of biosensors history various electrode materials and electron transfer pathways were investigated aiming at possible applications in medicine and biotechnology. In the presented work the similarities and differences of DET at two different CDH modified graphite electrodes were investigated.

Both approaches have their advantages and drawbacks. The CDH immobilization by “simple adsorption” on the graphite surface provides a rapid and cost effective way towards future applications for biosensors and/or biofuel cells construction. The weakness of this method consists in a random adsorption of the enzyme, resulting in a smaller reproducibility of the prepared bioelectrodes. The “SAM” approach, illustrated by Au electrodes modified with 4-mercaptophenol, offers the advantage of a huge versatility, due to the high number of thiocompounds which can be involved in this approach. Another “pro” for the “SAM” approach is the presence of a quasi-ordered structure,

including the enzyme, built on the electrode surface, which facilitates a reproducible preparation of the bioelectrodes. Its main disadvantage refers to the low enzymatic activity of the electrode surface coupled with the relative instability of the monolayer.

Besides these, the present work points out that in the case of CDH, an enzyme able to sustain DET at different electrode materials, the “SAM” approach exhibits a higher vulnerability to pH changes than the “simple adsorption” one. This behavior was explained taking into consideration the conformational changes of the CDH molecules, which, in the case of a better organized interface, exert a stronger influence on the bioelectrode activity.

## EXPERIMENTAL SECTION

### Reagents

All chemicals used were of analytical grade. Citric acid-1 hydrate, β-lactose and 4-mercaptophenol (SPh-OH) were purchased from Sigma-Aldrich (Sigma-Aldrich Chemicals, Steinheim, Germany). Sodium hydrogen phosphate monohydrate and sodium hydroxide were obtained from VWR (VWR International, Darmstadt, Germany), while hydrochloric acid was purchased from Fluka (Fluka, Buchs, Switzerland).

Cellobiose dehydrogenase from Neurospora crassa (CDH) was a kind gift received from Dr. Roland Ludwig, Department of Food Sciences and Technology, BOKU-University of Natural Resources and Applied Life Sciences, Vienna. It was obtained and purified as previously described [30]. The protein concentration was estimated using Bradford assay as 13.9 mg/mL. The enzyme activity, at pH 4 and 30°C, was found to be 118.73 U/ml and 63.6 U/ml by using DCIP assay and Cyt C assay respectively.

The buffer solutions used in all experiments were prepared using either a 50 mM citric acid solution (for pHs ranging from 4.0 to 6.5) or a 50 mM sodium hydrogen phosphate (for pHs placed in the 6.0 to 8.5 interval). The desired pH value was adjusted with 4 M NaOH or 5 M HCl. Before use, the buffer and the substrate solutions were carefully degassed.

All solutions were prepared using deionized water (18 MΩ) purified with a Milli-Q system (Millipore, Bedford, MA, USA). All measurements were performed at room temperature (22 °C).

### Flow injection electrochemical setup

A flow-through wall jet cell was used as amperometric detector [31], connected on-line to a single line flow injection (FI) system (Figure 6). The carrier flow was maintained at a constant flow rate of 0.5 mL/min by using a peristaltic pump (Gilson, Villier-le-Bel, France). The injector was an electrically controlled six-port valve (Rheodyne, Cotati, CA, USA), provided with an injection loop of 50 µL volume.

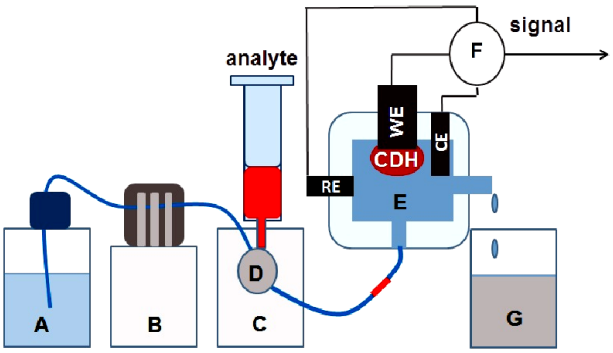


**Figure 6.** The experimental FI setup with carrier/buffer solution (A), peristaltic pump (B), injector (C), valve (D), flow-through cell (E), potentiostat (F) and waste collector (G).

The electrochemical cell consisted of a conventional three electrode system, where the working electrode (WE) was the CDH-modified graphite, the reference (RE) was a Ag|AgCl, 0.1 M KCl electrode and the counter electrode (CE) was a Pt wire. The CDH modified electrode was press-fitted into a Teflon holder, inserted into the wall-jet cell and kept at a constant distance (~1 mm) from the inlet nozzle.

The electrochemical cell was connected to a low current potentiostat (Zäta Elektronik, Lund, Sweden) and the response currents were recorded on a strip chart recorder (Kipp&Zonen, Delft, The Netherlands).

## Cyclic voltammetry measurements

Cyclic voltammetry (CV) experiments were performed under anaerobic conditions (assured by a previous degassing of the solutions and by using a flow of pure argon gas over the working solution) with a BAS 100W Electrochemical Analyzer (Bioanalytical Systems, West Lafayette, IN, USA). A three electrode cell was used with the working electrode, a saturated calomel electrode (SCE) as the reference electrode and a Pt wire as the auxiliary electrode. The scan rate of 300 mV/s was used for the electrochemical cleaning procedure or 10 mV/s for the regular measurements.

## Preparation of CDH-modified graphite electrodes

The graphite electrodes were prepared using spectroscopic graphite rods (OD 3.05 mm; Ringsdorff-Werke GmbH, Bonn, Germany). A rod of adequate length was cut and polished on wet emery paper (Tufbak, Durite, P1200); afterwards, it was carefully rinsed with deionized water and dried. The CDH was immobilized through simple chemo-physical adsorption onto the surface of the polished graphite rod by using the following procedure: 5 µL

of CDH solution was spread onto the entire active surface of the electrode (0.0731 $cm^2$). The CDH modified graphite electrode (G/CDH) was dried at room temperature for approximately 20 minutes and then stored overnight at 4ºC. Before use, the G/CDH electrode was thoroughly rinsed with Milli-Q water in order to remove any weakly adsorbed enzyme. Afterwards, the G/CDH electrode was placed into the wall jet cell filled with the buffer solution, the required potential was applied and the output current was recorded. Before performing any substrate injection into the flow system, the carrier buffer solution was continuously pumped until a stable background current was obtained.

**Preparation of Au-SPh-OH/CDH modified electrodes**

The cleaning of the disc Au electrode (CH-Instruments, Cordova, TN, USA, Ø 2 mm, area of 0.033 $cm^2$) started by dipping the Au electrode in "piranha" solution (3:1 v/v $H_2SO_4$:$H_2O_2$) for 5 min. Then, the electrode was mirror-like polished with aqueous alumina FF slurry (1 and 0.1 μm, Stuers, Denmark) deposited on Microcloth (Buehler). Furthermore, the electrodes were carefully rinsed with water, ultrasonicated for 5 min in Milli-Q water, and electrochemically cleaned in 0.5 M $H_2SO_4$, by performing 20 cycles with a scan rate of 300 mV/s between −100 and 1700 mV *vs.* SCE. Finally, they were rinsed again with Milli-Q water.

The preparation of CDH-thiol-modified electrodes started by the immersion of the clean Au electrodes in a 1mM solution of thiol dissolved in ethanol for 60 min. This treatment results in the formation of the self-assembled monolayer of thiol on the electrode surface. Before the exposure to CDH, they were carefully rinsed with ethanol in order to remove the weakly absorbed thiols and dried with Ar. CDH deposition on the Au-SAM modified electrodes was made by spreading of 2 μL of enzyme solution onto the thiol-modified Au surface. The enzyme drop was allowed to gently dry in order to avoid the spread of the enzyme drop outside of the electrode area. A dialysis membrane (molecular weight cut off 6000–8000), pre-soaked in the buffer solution, was applied onto the electrode and fitted tightly to the electrode surface with a rubber O-ring. For three equivalently prepared electrodes the enzymatic activities was found reproducible in the limits of 5%. Between measurements, the Au-SPh-OH/CDH modified electrodes were stored at 4 $^0$C in a water saturated atmosphere.

**ACKNOWLEDGMENTS**

The authors thank to Dr. Vasile Coman for fruitful discussions. GK acknowledges the financial support received from "Doctoral Studies: through Science towards Society" (contract no. POSDRU/6/1.5/S/3), project co-financed by the Sectorial Operational Program for Human Resources Development 2007 – 2013 and professor Lo Gorton (Lund University) for offering the opportunity of a research stay in his group.

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