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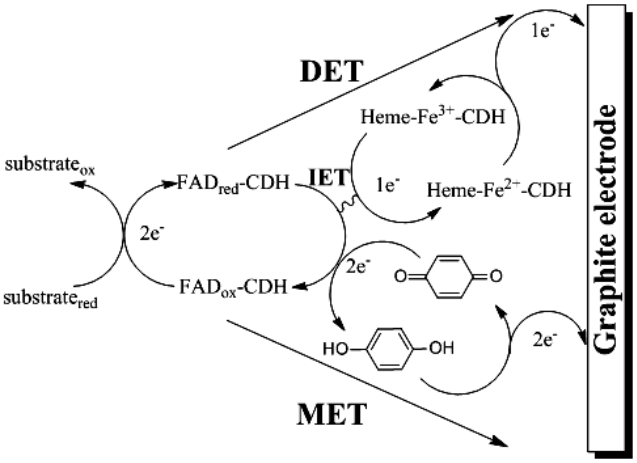
PhD thesis summary

Amperometric biosensors for detection of analytes of biotechnological interest



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Instead of introduction

How could I start this thesis about biosensors used for detection of analytes with biotechnological interest? This was the first question I had to answer. And, as usual in this type of papers, I found the typical sentence what is used in reviews and historical summaries: “Even the ancient Greeks used also…” “biosensors” for detection of poisonous analytes from food, wine and other type of beverages (Figure 1.).

Figure 1. Using a food tester to prevent poisoning.

The history of this type of “biosensors” (it also could be mentioned the using of canaries for detection of methane in the mines) could be interesting, but the size of this thesis is limited and I also need to limit the content of this work for more scientific facts, therefore I need to jump over thousands of years, until the early 1960’s…

I. Literature overview

1. About biosensors in general

The history of biosensors started in the year 1962 with the development of enzyme electrodes by the scientist Leland C. Clark [1]. Since then, research communities from various fields such as Physics, Chemistry, and Material Science have come together to develop more sophisticated, reliable and mature biosensing devices for applications in the fields of medicine, agriculture, biotechnology, as well as in the military for bioterrorism detection and prevention [2].

Various definitions and terminologies are used depending on the field of application. Biosensors are known as: *immunosensors*, *optrodes*, *chemical canaries*, *resonant mirrors*, *glucometers*, *biochips*, *biocomputers*, and so on. A commonly cited definition is the one accepted by IUPAC: "an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element" [3].

Biosensors can have a variety of biotechnological applications. The major application is so far, in blood glucose sensing because of its abundant market potential. However, biosensors have tremendous potential for commercialization in other fields of application as well. In spite of this potential, the commercial adoption has been slow because of several technological challenges, such as complexity of the real samples, miniaturization and simplification of the technology, selectivity and stability of the recognizing element [4-6].

2. Methods for biosensors characterization

During the last half century, various forms of biosensors have been developed, as well as many other sensing technologies and biosensing devices. This section attempts to describe briefly the operating principles and characteristics of the electrochemical systems used in this thesis.

The basic electrochemical process at the electrode is the redox reaction of a couple (Ox and Red), as follows:

$$\mathbf{Ox + ne^- \leftrightarrow Red} \qquad \text{(Equation 1)},$$

where Ox and Red are the oxidized and reduced species and n represents the number of electrons involved in the reaction. The potential of the electrode is related to the standard potential of the redox couple and the activities of the species involved in the conversion and is given by the Nerst equation [7]:

$$\mathbf{E = E^{0'} + (RT/nF)*ln(a_{Ox}/a_{Red})} \qquad \text{(Equation 2)},$$

where $E^{0'}$ is the formal potential of the redox reaction, **R** is the universal gas constant (8.314 J/mol*K), **T** is the absolute temperature (in Kelvin), **n** is the number of the electrons involved in the reaction, **F** is the Faraday constant (96485 C/mol), $\mathbf{a_{Ox}}$ and $\mathbf{a_{Red}}$ are the chemical activities for the oxidant and reduced species (the product between concentration and activity coefficient, which is close to unity at low concentration values).

In the next paragraphs, the methods used more frequently during my PhD studies (amperometry with flow injection analysis and (cyclic) voltammetry) will be described briefly.

Voltammetry belongs to a category of electro-analytical methods, through which information about an analyte is obtained by varying a potential and measuring the resulting current. There are many ways to vary a potential, there are also many forms of voltammetry, such as: polarography [8,9], linear sweep, differential staircase, normal pulse, reverse pulse, differential pulse etc. [10,11].

Cyclic voltammetry (CV) is one of the most widely used forms of voltammetric techniques and it is useful to obtain information about the redox potential and

electrochemical reactions (*e.g.* the rate constant) of analyte solutions. In CV, the potential of the working electrode is changed constantly in time with a defined rate (named scan rate), forward and backward (cyclic) between the starting and switching potential and the resulting current is registered and analyzed. A typical representation of the cyclic voltammetry is shown below, where plots of potential (a) and current (b) *vs.* time as well as a typical voltammogram are visible (Figure 2) [12].

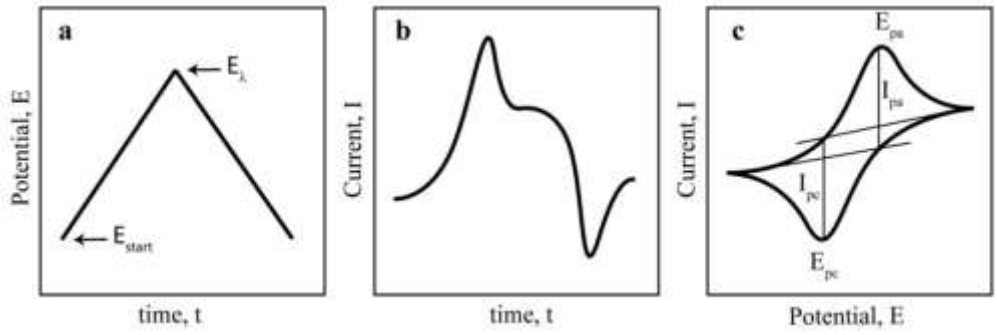

Figure 2. The excitation signal (a), response – current in time (b) and current *vs.* potential (c) of a CV.

CV is also one of the most commonly used electrochemical techniques throughout the works published about the interaction of enzymes and thiol modified gold electrodes. The direct redox conversion of the heme-cofactor and the effect of the scan rate on the peak current of the membrane electrodes have been proved that these electrodes are working as thin-layer electrodes[13]. The same technique was used in evaluation of the origin of the redox currents obtained with intact CDH trapped under a permselective membrane at a gold electrode surface (modified with cysteamine). It was concluded that the peaks observed are due to the direct communication between the heme-domain of the enzyme and the electrode, because the other subunit of the enzyme (FAD) did not gives any peaks in the CVs[14].

Cyclic voltammetry was used in this work for the electrochemical investigation of CDH from *Neurospora crassa* on gold electrodes and its interaction with different types of thiols-SAM modifications in presence of lactose and in absence of substrate, in different buffers and pHs.

The most common and simple electrochemical detection method is the measurement of the current at a constant potential, known as **amperometry**. Keeping the potential at a fixed value has the advantage of avoiding effects of changes of the electrochemical double-layer charging at the interface between the working electrode and the solution causing non-faradaic currents. Amperometry is used widely both in batch and flow systems. For a

controlled mass transport of bulk solution to the electrode surfaces either a flow-through or a rotating disc electrode are used commonly.

Electrochemical detection methods have been used in **flow analysis** since its early years of development[15-17]. Flow analysis is considered as analytical technique where the analytical signal is based on introducing a sample by aspiration or injection, on-line sample processing, and detection of analyte in the flowing medium. The main attributes of flow analysis are on-line sample processing carried out to provide/enhance selectivity of detector response, and detection carried out during the flow of the analyte (in non-derivatized or chemically derivatized form) through the detector.

A typical flow injection analysis system (FIA) is based on a peristaltic pump, an injection valve, an electrochemical cell, potentiostat, a signal processor and a recorder (Figure 3a). The electrochemical cell used for amperometry consists usually of a three-electrode set-up: working electrode (WE), reference electrode (RE) and a counter electrode (CE) (Figure 3b). During the experiments carried out for this thesis, the potential of the working electrode (spectrographic graphite rod) was maintained at a constant value versus a reference electrode, in our case Ag | AgCl in 0.1 M KCl, which has a potential of + 0.197 V *vs.* NHE at 25 ^{0}C [18]. In order to minimize changes of the potential at the reference electrode, the current was collected by a counter electrode (a Pt wire).

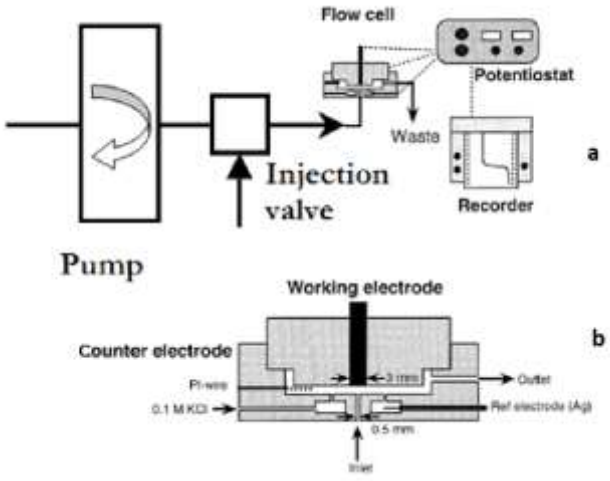

Figure 3. A FIA system (a) and the electrochemical cell used for amperometry (b) [19].

FIA has been one of the most commonly used electrochemical techniques throughout the experimental part of this thesis, especially when investigating the electrochemistry of cellobiose dehyrdrogenase (CDH) from *Neurospora crassa* using both direct and mediated electron transfer for a large number of substrates at different pH values.

3. Cellobiose dehydrogenase (CDH)

Cellobiose dehydrogenase (CDH) (EC 1.1.99.18) is an extracellular enzyme produced by a variety of different fungi. More than 25 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 and plant pathogen basidiomycete [20,21]. Recently, as more CDH sequences from genome researches became available, a third class of hypothetical CDHs found in the ascomycetes was introduced [21] by Zamocky *et.* al. without experimental confirmation of their electrocatalytic properties [22].

Typical class I basidiomycete CDHs shows a higher selectivity toward cellobiose and cello-oligosaccharides, these being oxidized at the anomeric carbon atom, while glucose and other monosaccharides are very poor substrates. Class II CDHs, produced by ascomycetes, also prefer cellobiose as substrate, but the monosaccharides, such as glucose and maltodextrines, are less discriminated compared to their basidiomyceteuos counterparts [21]. The class I CDHs have their pH optimum around 3.5-4, but usually no more than 5.5. The class II CDHs, depending on their origin, are working also at neutral or alkaline conditions, showing an effective DET at higher pH's. This can be ascribed to the fungus adaptation to its natural habitat and a polymer degradation mechanism[20,23].

The activity of CDH was first discovered in 1974 as a cellobiose-dependent reduction of quinones in white-rot fungi by Westermark and Eriksson[24,25].

CDH is an interesting enzyme in the field of bioelectrochemistry mostly recognized for its multi-faceted applications (Table 1.) and flexibility in regard to its electron-transfer mechanism. This fungal enzyme has been applied in biosensors for detection of cellobiose and other cellodextrines [26,27], maltose [28], lactose [29], diphenolic compounds [30] as well in biofuel cell anodes fuelled by glucose, lactose or cellobiose [31].

The catalytic domain of CDH is composed of a flavin containing dehydrogenase domain belonging to the glucose-methanol-choline oxidoreductase superfamily with flavin adenine dinucleotide (FAD) as prosthetic group which is connected by a flexible linker to a cytochrome domain carrying a haem domain [Figure 4]. Molecular masses are usually 90 to

100 kDa. The linker between the two domains can be cleaved by proteases present in the culture supernatant with a still catalytically active (DH_{CDH}).[23]

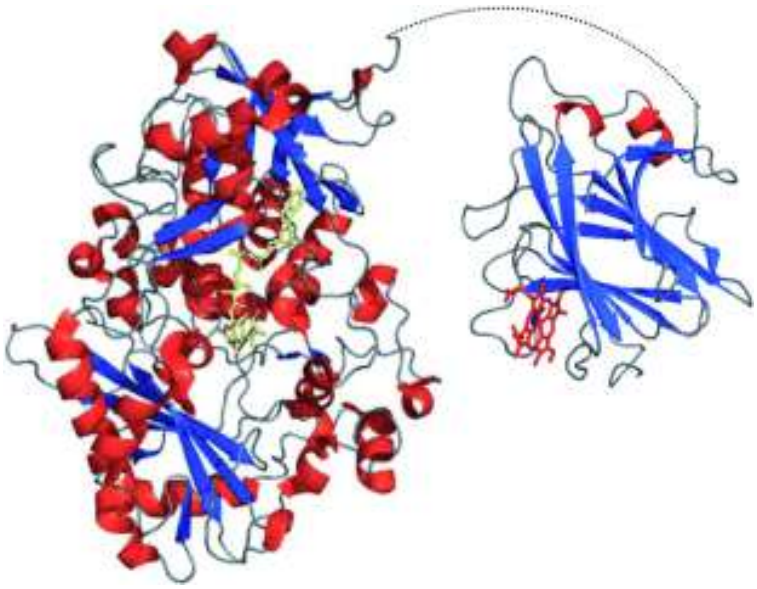

Figure 4. Dehydrogenase domain (left, PDB entry 1KDG) and cytochrome domain (right, PDB entry 1D7C) of *P. chrysosporium* CDH. The position of the interdomain linker is indicated by dots. Both domains are turned from their face-to-face position by 45° towards the observer to give a better view of FAD (yellow) and the haem *b* (red) [23].

There are two possible ways, DET and MET, to study the electrocatalytical behavior of the CDH enzymes. These electron transfer pathways are represented in Figure 5.

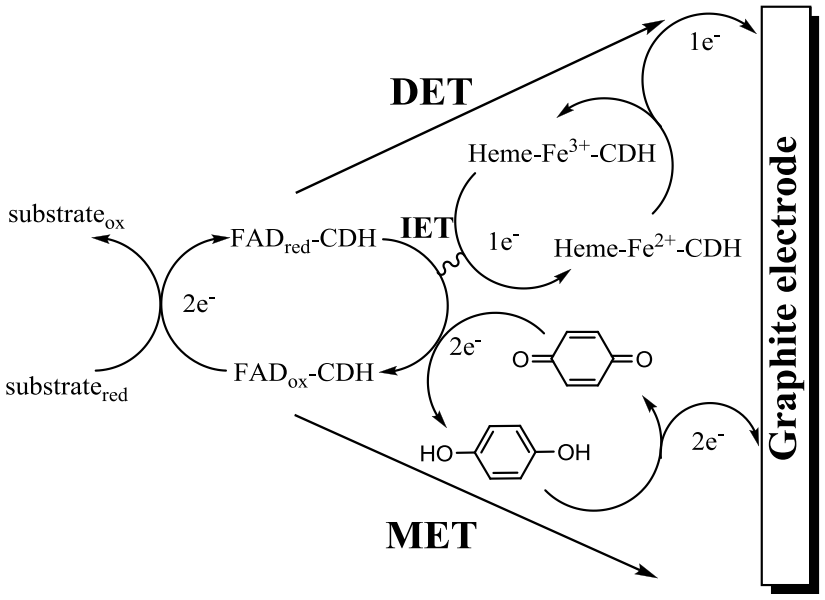

Figure 5: Schematic representation of DET and MET between CDH and electrode.

These electronic pathways can be studied using amperometric flow injection analysis with the enzyme physically adsorbed on the surface of the graphite electrode. By injection of

cellobiose in the carrier buffer, an oxidation peak current is obtained due to the DET, whilst by the addition of cellobiose into the carrier buffer a steady-state DET current results as a plateau. Using benzoquinone mixed with the solution of the substrate generates a much higher current, due to the recycling of quinone/benzoquinone molecules between FAD domain and the electrode. This current will be referred to as mediated electron transfer (MET).

Due to its special ability to show efficient DET reactions with electrodes through the cytochrome domain and its long term stability, CDH has shown already a great potential for practical use in biosensors and for biofuel-cell anodes. The possible future applications for bioelectrosynthesis are challenging as well.

As has been shown above, CDH enzymes can be divided in class I and II, as they differ in selectivity toward their substrates and also in pH optimum for DET as well. Class I CDHs (from Basidiomycota) are strongly selective for cellodextrins and lactose, therefore they are better suited for making biosensors for lactose [29,32]. On the other side, CDHs from class II show a broader selectivity profile toward different mono-, di- and oligosaccharides, such as glucose, xylose, arabinose, mannose, maltose and other maltodextrines. These CDHs are showing efficient DET in the neutral pH range, with a possibility for the future applications in glucose and lactose sensors [23].

Another approach in the application of CDH is the field of constructing biofuel-cells (BFCs). These are devices that use biocatalysts (enzymes or living cells) in order to convert the energy of the substrates/fuels into electrical energy. The typical substrates which have been used in the investigation for "feeding" BFCs are glucose [33,34], methanol [35] and ethanol [36] but a wider variety of fuels can be used, because the living organisms used in the construction of BFCs can oxidize a large variety of substrates including many alcohols, carbohydrates and fatty acids. A large number of reviews written in recent years reflect the great interest in this field [37-40]. Some CDHs have been used in BFC prototypes, using both DET [33,41] and MET [42,43] approaches with promising results.

II. Original contributions

1. Aim of the thesis

(i) The studies presented below have been performed with the intention to characterize a new CDH from ascomycete fungus *Neurospora crassa* on graphite and gold electrodes, taking into account some experimental factors, such as concentration of its main substrate and other saccharides, presence or absence of a redox mediator, the effect of different buffers and pHs. The section dedicated to the original contributions is split in two parts: in the first part are shown the results obtained regarding to the characterization of this CDH immobilized by simple adsorption on graphite electrode, using FI measurements, the second part is summarizing the results about the behavior of CDH adsorbed on self-assembled monolayer (SAM)-modified gold electrodes.

(ii) A larger variety of thiols was used to construct **SAMs on gold** electrodes aiming to improve the electrochemistry of the immobilized CDH. The enzyme exhibits DET and catalytic currents at SAM-modified Au electrodes, but the electron communication was toughly influenced by the length of the spacer and by the head functionality of the thiol used for the preparation, suggesting the importance of the relation between the CDH structure and the SAM nature.

(iii) Referring to the possible analytical applications, the electrochemistry of CDH modified graphite electrodes was studied for a large number of substrates such as cellodextrins, lactose and glucose, analytes having a great importance in the biotechnology. The electrode had shown good operational stability under the working conditions for a long period of time with promising results in future applications, such as amperometric biosensors and for biofuel cells as well.

2. Graphite Electrodes Modified with *Nc*CDH[44]

2.1. Influence of the Applied Potential

The G/CDH modified electrode, prepared using the protocol described in [27], was press-fitted into a Teflon holder and it was inserted in a flow-through wall jet amperometric cell. Maintaining the carrier flow rate at a constant rate of 0.5 mL min^{-1}, the applied potential was gradually varied from -100 to +600 mV *vs.* RE, in steps of 50 mV. For each value of applied potential, the current response of the G/CDH electrode was monitored during the injection of 5 mM lactose solution (50 µL).

The results are illustrated in Figure 6.

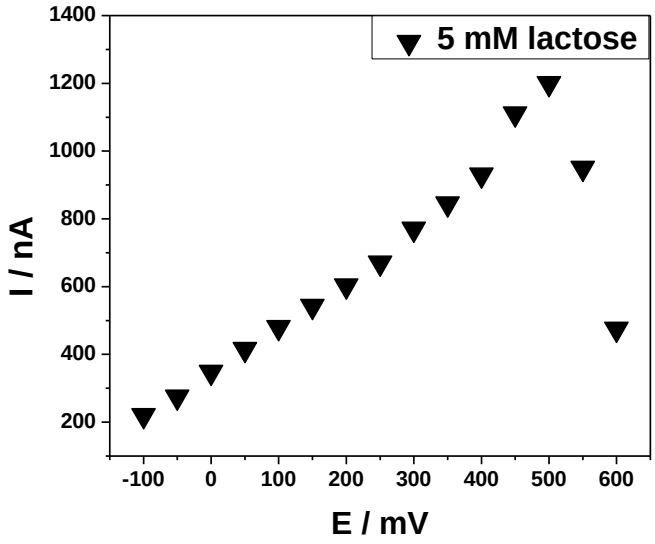

Figure 6: Dependence of the peak current on the applied potential (vs.Ag|AgCl 0.1 M KCl) for injections of 5 mM lactose (DET) in 50 mM phosphate buffer, pH 7.0. The flow rate was 0.5 mL/min .

As can be seen from Figure 6, an increase in the applied potential results in an increase in the G/CDH amperometric response, until a maximum at +500 mV *vs.* RE is reached. For higher applied potentials the bioelectrode response decreases significantly, probably due to the irreversible denaturation of the enzyme. This supposition was checked and confirmed by recording the affected electrode response at applied potentials under the critical value (i.e. +600 mV *vs.* RE) when the observed currents were significantly lower than those initially recorded at the same applied potentials (data not shown). It is worth mentioning that the irreversible deactivation of the DET occurring at high applied potentials was already

reported for other types of CDHs isolated from *Phanerochaete chrysosporium* [45], *Sclerotium rolfsii* [46] or *Myriococcum thermophilum* [27].

Previous studies [19,47,48] have shown that the formal potential (E°') of the Cyt_{CDH} from different CDHs, involved in the DET process between CDH and the electrode surface, is situated below 0 mV vs. Ag|AgCl, 0.1 M KCl, in the pH range between 5 and 7, however, varying with around 30 mV per pH unit of the solution. However, in our case, for all further measurements performed with G/CDH modified electrode under DET operation mode, the electrode potential was poised at +300 mV vs. RE, which is a potential range sufficiently more anodic than the E°' of the *Nc*CYT_{CDH}. This value is in agreement with previously reported applied potentials for CDH modified electrodes.

For the MET operation mode, the G/CDH modified electrode was investigated in a similar mode as was used previously for another class II CDH [27] making use of a 2 e-, 2 H+ acceptor redox mediator, i.e., 1,4-benzoquinone (BQ), in order to directly re-oxidize the reduced *Nc*$_{DH}$CDH (enzyme bound-FADH2 to FAD, Figure 5). In order to facilitate the straightforward comparisons between the operational parameter characteristics for DET and MET, the value of the applied potential used for MET was chosen identical to that selected for DET (i.e. +300 mV vs. RE). In the same context, aiming at estimating the optimal concentration of the mediator, the BQ concentration was increased stepwise in the injected sample containing 5 mM lactose (pH 7.0), and the amperometric response of G/CDH modified electrode was recorded under FI conditions. The maximum response of the bioelectrode was reached for ~50 µM BQ. Considering that an increase in BQ concentration by a factor of two is not justified by a 13.3% increase in response, furthermore all experiments under MET operation mode were performed with a BQ concentration of 25 µM..

2.2. pH influence

The amperometric response of the G/CDH modified electrode, operated both in DET and MET modes, was recorded under flow conditions for injections of 5 mM lactose or cellobiose dissolved in 50 mM buffer solution; the buffer without enzymatic substrates was used at the same time as flow carrier. The investigated pH range was from 4.0 to 8.5 and it was covered using two buffer solutions: 50 mM acetate buffer for pH 4.0 to 6.5 and 50 mM phosphate buffer for pH 6.0 to 8.5.

As can be seen from Figure 7A, for both investigated substrates, the optimum pH range for DET is situated between 4.5 and 5.5. This result is in agreement with previously published data on NcCDH in solution, when using the one-electron acceptor cytochrome c [21]. Cytochrome c is known to be exclusively reduced at the CYT_{CDH} [20,23], hence the similarity to DET behavior at electrode surfaces. In the current study, the highest currents were observed at pH ~5.2 for lactose and ~4.9 for cellobiose. These results confirm that both DET and IET processes are substrate and pH dependent. Compared to other similar class II CDHs [21,27], NcCDH exhibits a narrow optimum working acidic pH range, placed slightly closer to the neutral pH. Moreover, judged against MtCDH modified graphite electrodes [27], operated in similar DET conditions, the registered current values are several times higher for NcCDH reflecting an improved DET communication. The current responses at pH 7.0 are 43% and 48% of the maximum current registered in DET mode, for lactose and cellobiose respectively.

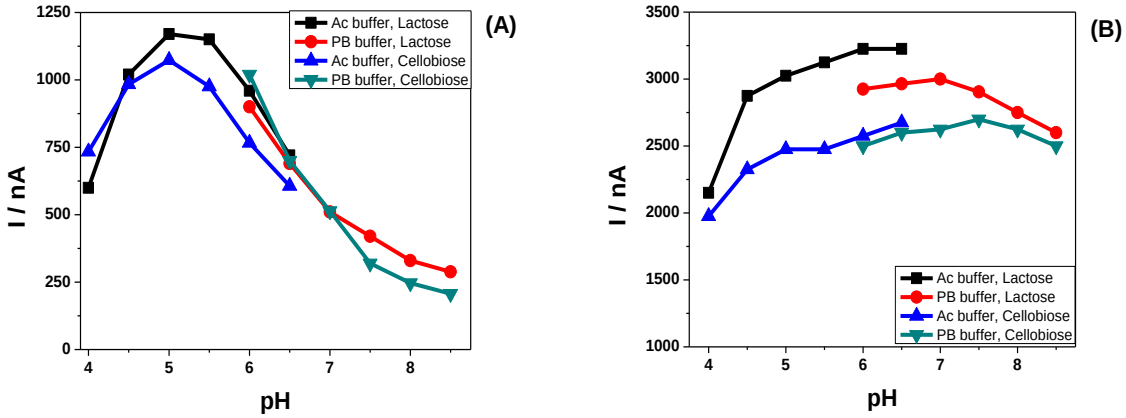

Figure 7: Dependence of the amperometric response on the pH and buffer composition in the case of DET (A) and MET using (1,4-benzoquinone) (B). The applied potential was +300 mV (*vs.* Ag | AgCl 0.1 M KCl) for injections of 5 mM lactose and cellobiose in 50 mM buffer and the flow rate of the solution was 0.5 mL/min

As expected, the currents measured under MET operation mode for the G/CDH modified electrode for lactose and cellobiose (Figure 7B), are higher than those recorded for DET mode (Figure 7A). Thus, the ratio between the maximum currents observed for MET *vs.* DET at the optimum pH value for DET was 2.75 for lactose and 2.5 for cellobiose, increasing at pH 7.0 to 5.9 and 5.1, respectively. In MET conditions, the optimum working pH for lactose and cellobiose was slightly higher (pH 6.5) than that found for DET (pH 5.2) and the working pH range is much wider (pH 5 to 8) than in the case of DET. This result is similar to that obtained in solution when using DCPIP as electron acceptor [49]. Moreover, in MET mode the bioelectrode response was more strongly influenced by the nature of the

substrate ($I_{max,lactose}/I_{max,cellobiose} \approx 1.21$ at pH 6.5) than in DET mode ($I_{max,lactose}/I_{max,cellobiose} \approx 1.09$ at pH 5.0). Finally, comparing the results from Figure 7A and B it can be stated that in DET mode the pH influence on the bioelectrode response is superior to the one in MET mode, having a strong impact on the conformational changes of the enzyme domains and linker region, which affect the IET and DET to the electrode surface, respectively.

2.3. Calibration curves for different sugars

The G/CDH modified electrode was calibrated for lactose and cellobiose both in DET and MET operation modes using the optimal experimental conditions: applied potential of +300 mV *vs.* RE and pH 5.2 (50 mM acetate buffer). Taking into consideration the potential applications of this bioelectrode in physiological (neutral) conditions, additional measurements have been carried out at pH 7.0 (50 mM phosphate buffer). The catalytic currents for each substrate concentration were calculated as the average of the amperometric responses recorded for 4 consecutive injections at two similar G/CDH electrodes using both substrates and the results are exhibited in Table 1 and Table 2.

As expected, irrespective of the pH value, the G/CDH bioelectrode gives in DET mode well shaped and similar Michaelis-Menten behavior for lactose and cellobiose (Figure 8A). As already mentioned, regardless of the substrate used, the bioelectrode response is strongly influenced by the pH value, showing the highest efficiency in acidic media. The calibration curves obtained under MET operation mode sustain the conclusions formulated in the previous section. Thus, the presence of BQ as redox mediator diminishes the differences observed in DET mode between lactose and cellobiose as well as the effect of the surrounding pH on the CDH activity (Figure 8B). This is to be expected if we consider that the mediator is directly reduced at the Nc_{DH}CDH (Figure 5) and the pH changes have a stronger effect on the linker region and on the IET process[14,50].

The kinetic and analytical parameters, estimated by fitting the calibration curves illustrated in Figure 8A and B to the Michaelis-Menten equation, are summarized in Table 1 and Table 2.

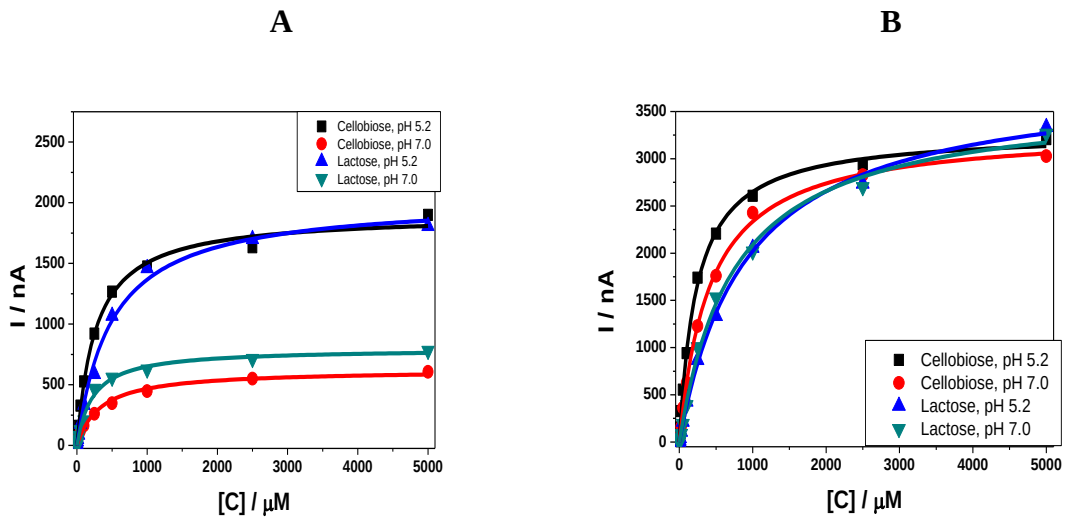

Figure 8: Dependence of the amperometric response on the pH and buffer composition in the case of DET (A) and MET (B) conditons (using the solution of the mixture of 1,4- benzoquinone, 25µM and), in different concentrations of lactose and cellobiose .The applied potential was +300 mV (vs. Ag | AgCl 0.1 M KCl), flow rate 0.5 mL/min.

The use of mediator has increased the I_{max} values (the difference caused by pH were smaller than in DET) and as well as the I_{max}/K_m ratio, which can be interpreted as the catalytic efficiency [51], for all the substrates studied in this group of saccharides. The increase of I_{max}/K_m was the biggest for cellobiose; for lactose the mediator doesn't have a significant effect toward this ratio. The pathway of the electron transfer and the pH had no clear influence toward the linear range (0.5-500 µM) and detection limit (0.5-1 µM), these values remained more or less the same within this group of substrates.

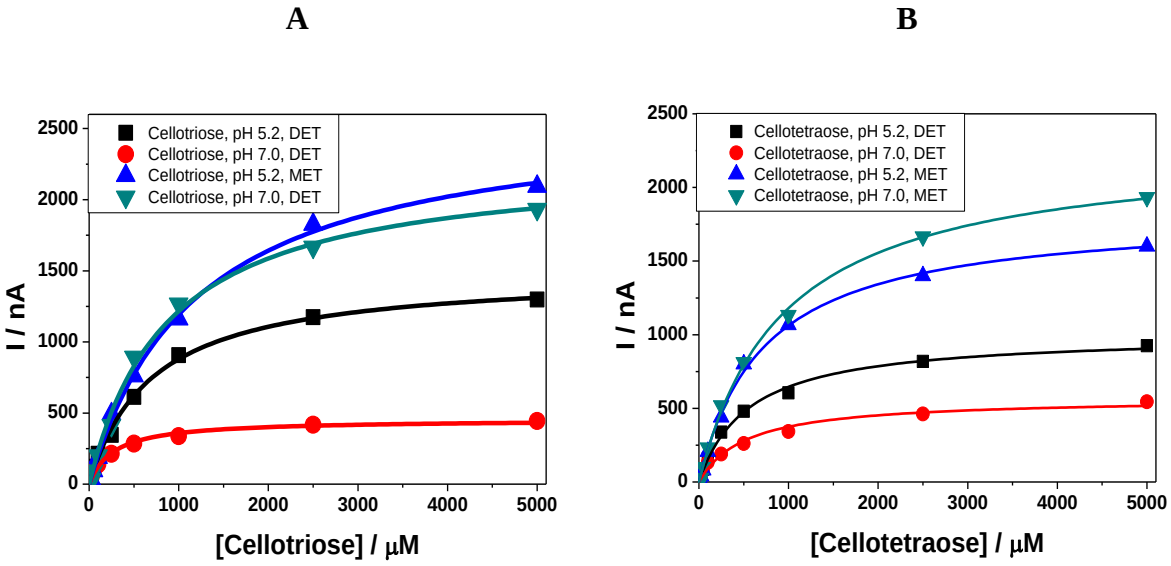

Figure 9: Dependence of the amperometric response on the pH and buffer composition in the case of DET (A) and MET (B) conditons (using the solution of the mixture of 1,4- benzoquinone, 25µM and), in different concentrations higher cellodextrines (cellotriose and cellotetraose). The applied potential was +300 mV (vs. Ag | AgCl 0.1 M KCl), flow rate 0.5 mL/min.

In the case of *Nc*CDH used under DET conditions, the K_M^{app} and Imax values for all investigated substrates (Table 1) were higher than those reported under similar experimental conditions for *Mt*CDH [27], suggesting that NcCDH has a lower substrate affinity but a higher efficiency. As expected, irrespective of the substrate type, higher I*max* values were registered in acidic media (pH 5.2) compared to those measured under neutral conditions (pH 7.0). This behavior confirms once again that *Nc*CDH belongs to the intermediate class II CDHs [21], having a clear preference for acidic conditions when operated in DET mode, but keeping a significant amount of activity under neutral pH conditions.

In MET operation mode (see Table 1 and Table 2) the kinetic parameters of adsorbed *Nc*CDH for the investigated substrates are roughly similar in acidic and neutral conditions, confirming the slight influence of the surrounding pH on the catalytic properties of the DH_{NcCDH}. Irrespective of the substrate nature, all I_{max} values were higher in MET compared to DET, the difference being more pronounced at pH 7.0. Consequently, it can be stated that the usage of the mediator increased the catalytic efficiency (estimated as the I_{max}/K_M^{app} ratio) for all the studied substrates

In both operation modes, NcCDH exhibits higher activity for the substrates with β-1,4-linkage (lactose and cellodextrins) than for the α-1,4-linkage substrates (maltodextrins). At the same time, as reported for all investigated CDHs [21], the highest affinities were observed for lactose and cellobiose. For all substrates, the detection limit and linear range were not significantly different in DET and MET modes, the values for the β-1,4-linkage substrates being situated in the μM range, while for α-1,4-linkage substrates the values are located in the mM range (Table 1 and Table 2).

The K_m values for maltodextrines were around 100 times higher than for lactose and cellodextrines; the smallest values from this group were the K_m of maltose and maltopentaose both in DET and MET. The use of mediator increased the K_m as it can be observed mostly in the case of maltotriose and maltotetraose (except for maltopentaose, where at pH 5.2, the K_m decreased with the usage of BQ). The value of I_{max} varies from 46 nA (maltose, pH 7.0) to 232 nA (maltotriose, pH 5.2). The effect of BQ could be also observed at MET, the I_{max} increases comparing to DET and the difference between the two different buffers/pHs are smaller between each other. The detection limit varies between 1-10 mM, the linear range between 1-100 mM, with a tendency of decrease to smaller molar range, when the measurement is done without mediator.

Aiming at evaluating the enzyme selectivity towards monosaccharides, calibration curves have been attempted in DET and MET modes for glucose (Figure 10), galactose, arabinose, and xylitol. In both operational modes, no notable responses were recorded for galactose, arabinose, and xylitol. For glucose, the K_M^{app} values (from 200 to 500 mM) confirmed that similarly to other class II CDHs [21,27,33,52-54], NcCDH possesses a reduced activity towards this substrate[21,49]. The kinetic parameters (K_M^{app} and I_{max}), as well as the detection limit (~10 mM), were not significantly affected by the detection mode (DET or MET).

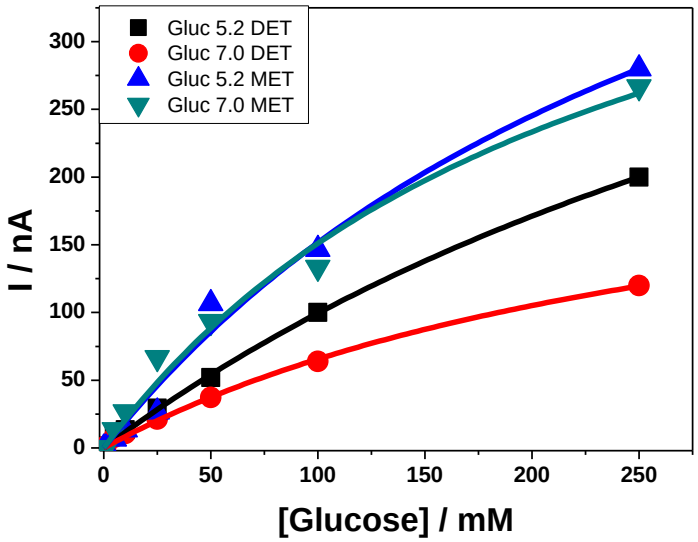

Figure 10: Dependence of the amperometric response on the pH and buffer composition in the case of DET/MET, using the solution of the mixture of 1,4- benzoquinone (25µM) and different concentrations of glucose. The applied potential was +300 mV, flow rate 0.5 mL/min

Comparing the sensitivities calculated for the two operation modes, it can be clearly stated that NcCDH is more efficiently connected to the electrode when it is exploited in presence of the redox mediator. Irrespective of the operation mode and pH it can be noticed that: (i) cellodextrins and lactose are preferred to maltodextrins; (ii) within the cellodextrins substrate group the sensitivity sequence remains practically unchanged, being cellobiose > (cellotriose ≈ cellopentose). For the maltodextrins the pH influence was stronger than that observed for the cellodextrins, and the individual sensitivities decrease roughly in the following order: maltopentaose > maltotetraose > maltotriose > maltose. For both operation modes, the NcCDH sensitivity to glucose was the lowest among the investigated substrates, being close to that observed for maltose and similar to that already reported for MtCDH [27].

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Table 1. **Apparent kinetic parameters (calculated using the Michaelis-Menten kinetics) and analytical parameters (calculated from the calibration curves) estimated for different substrates with** G/CDH **modified electrodes operated in DET mode.**

Substrate	**pH**	**K_M^{app} (mM)**	**I_{max} (μA)**	**Linear range (mM)**	**DL* (mM)**	**R^2 / N**
Cellobiose	5.2	0.265 ± 0.019	1.90 ± 0.04	0.005 – 0.25	0.001	0.9963 / 11
	7.0	0.334 ± 0.040	0.62 ± 0.02	0.01 – 0.25	0.005	0.9902 / 11
Cellotriose	5.2	0.698 ± 0.042	1.49 ± 0.03	0.01 – 0.25	0.004	0.9978 / 11
	7.0	0.289 ± 0.024	0.46 ± 0.01	0.01 – 0.25	0.006	0.9940 / 11
Cellopentaose	5.2	0.556 ± 0.036	1.01 ± 0.32	0.001 – 0.25	0.005	0.9972 / 11
	7.0	0.522 ± 0.084	0.57 ± 0.03	0.025 – 0.25	0.008	0.9818 / 11
Lactose	5.2	0.488 ± 0.041	2.03 ± 0.05	0.005 - 0.5	0.002	0.9953 / 11
	7.0	0.225 ± 0.021	0.80 ± 0.02	0.005 – 0.25	0.003	0.9939 / 11
Maltose	5.2	35.5 ± 3.4	0.056 ± 0.002	10 - 50	5.8	0.9947 / 6
	7.0	40.8 ± 3.4	0.047 ± 0.001	10 - 50	7.7	0.9962 / 6
Maltotriose	5.2	69.3 ± 8.4	0.23 ± 0.01	5 – 25	2.7	0.9932 / 6
	7.0	63.3 ± 9.8	0.15 ± 0.01	5 – 25	3.8	0.9881 / 6
Maltotetraose	5.2	47.8 ± 2.7	0.226 ± 0.006	5 – 25	1.9	0.9990 / 6
	7.0	53.6 ± 9.1	0.20 ± 0.02	5 – 25	2.5	0.9913 / 6
Maltopentaose	5.2	44.3 ± 6.7	0.17 ± 0.01	5 – 25	2.4	0.9921 / 6
	7.0	36.0 ± 7.0	0.11 ± 0.01	5 – 25	2.9	0.9857 / 6
Glucose	5.2	514 ± 48.4	0.61 ± 0.04	10 – 100	7.6	0.9993 / 6
	7.0	302 ± 35.6	0.26 ± 0.02	25 – 100	10.3	0.9977 / 6

***** The detection limit (DL) was estimated for a signal-to-noise ratio of 3.

Table 2. Apparent kinetic parameters (calculated using the Michaelis-Menten kinetics) and analytical parameters (calculated from the calibration curves) estimated for different substrates with G/CDH modified electrodes operated in MET mode.

Substrate	pH	K_M^{app} (mM)	I_{max} (μA)	Linear range (mM)	DL* (mM)	R^2 / N
Cellobiose	5.2	0.419 ± 0.016	3.31 ± 0.04	0.005 – 0.25	0.002	0.9991 / 6
	7.0	0.239 ± 0.010	3.28 ± 0.03	0.01 – 0.25	0.004	0.9989 / 6
Cellotriose	5.2	0.874 ± 0.070	2.28 ± 0.06	0.025 – 0.25	0.014	0.9962 / 6
	7.0	1.20 ± 0.06	2.63 ± 0.05	0.025 – 0.25	0.011	0.9987 / 6
Cellopentaose	5.2	0.936 ± 0.044	2.29 ± 0.04	0.025 – 0.25	0.012	0.9987 / 6
	7.0	0.719 ± 0.039	1.83 ± 0.03	0.025 – 0.25	0.012	0.9982 / 6
Lactose	5.2	0.743 ± 0.053	3.64 ± 0.09	0.01 – 0.25	0.007	0.9969 / 6
	7.0	0.912 ± 0.044	3.87 ± 0.07	0.01 – 0.5	0.006	0.9986 / 6
Maltose	5.2	44 ± 11.7	0.082 ± 0.008	25 - 100	16.3	0.9684 / 6
	7.0	41.1 ± 9.6	0.086 ± 0.007	25 - 100	14.3	0.9747 / 6
Maltotriose	5.2	173 ± 25.7	0.74 ± 0.06	10 – 100	7.0	0.9942 / 6
	7.0	98 ± 15.2	0.57 ± 0.04	10 – 50	5.2	0.9910 / 6
Maltotetraose	5.2	106.2 ± 5.7	0.67 ± 0.02	10 – 50	4.8	0.9989 / 6
	7.0	86 ± 10.8	0.66 ± 0.04	10 – 50	3.8	0.9933 / 6
Maltopentaose	5.2	82.5 ± 9.5	1.10 ± 0.07	5 – 25	2.3	0.9972 / 6
	7.0	36.6 ±8.7	0.88 ± 0.09	5 – 10	1.2	0.9779 / 6
Glucose	5.2	239 ± 63.9	0.51 ± 0.08	25 – 100	14.0	0.9851 / 6
	7.0	323 ± 104.3	0.64 ± 0.13	25 – 100	15.1	0.9854 / 6

***** The detection limit (DL) was estimated for a signal-to-noise ratio of 3.

2.4. Operational stability of the biosensor

Two different experimental protocols were used to assess the operational stability of the G/CDH modified electrode at room temperature: (i) repetitive injections of 5 mM lactose in a continuously flowing flow carrier (pH 7) consisting of phosphate buffer (Figure 11A); (ii) a continuous flow of 5 mM lactose solution dissolved in pH 7 phosphate buffer (Figure 11B). The FI protocol results pointed out that in the first part of the measurements (which took 11 h and corresponded to ~10% of the duration of the whole experiment) the bioelectrode response decreased linearly and reached ~86% of its initial response; in the second part of the measurements (~90% of the duration of the whole experiment) the slope of the response drop was much lower compared to the first part, reaching ~70% of the initial biosensor response after a total of 5 days. This biphasic behavior [23,27] proves that the operational stability of the investigated system is affected mainly by two factors: (i) the desorption of weakly adsorbed enzyme molecules from the surface of the graphite electrode, dominating the first part of the measurements; (ii) the loss of the enzyme activity operated under intermittent conditions, occurring during the whole experiment.

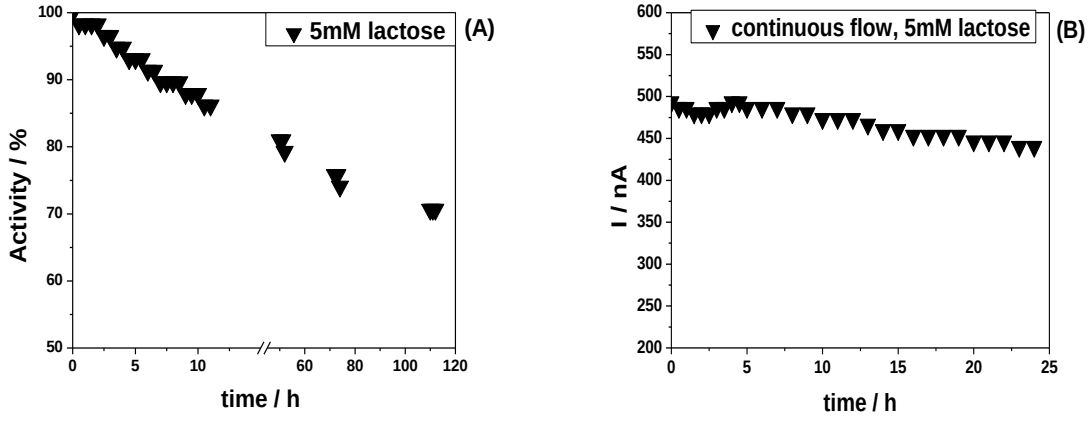

Figure 11 Stability measurements in FIA for a CDH modified graphite electrode with successive injection of 5mM lactose (A) and under continuous 5 mM lactose flow as a carrier solution (B). The applied potential was +300 mV (*vs.* Ag | AgCl 0.1 M KCl) for injections of the 5mM lactose in 50 mM buffer and the flow rate of the solution was 0.5 mL/min.

When the G/CDH modified electrode was operated under a constant flow conditions (Figure 11B) the decrease in response was lower compared to the first protocol. Thus, after 24 h of continuous running under a constant flow rate of a 5

mM lactose solution, the amperometric response of the G/CDH modified electrode showed a relative decrease of less than 11% from the initial response. The results from both experiments allow the conclusion that a relative stable bioelectrode can be obtained by simple adsorption of NcCDH on the surface of a graphite electrode, which keeps its operational activity for around one week. This stability feature can be considered promising enough to justify the manufacturing of biosensors for biotechnological applications and of biofuel cells.

2.5. Conclusions

In order to better understand the role of CDH enzymes and the mechanisms occurring at DET and MET in the presence of different substrates, the electrochemical characterization of a class II CDH, isolated from the ascomycete fungus Neurospora crassa, adsorbed on graphite, was performed under DET and MET operation modes. Aiming at finding the optimum experimental conditions, the effects of the applied potential, mediator (1,4 benzoquinone) concentration and flow carrier pH on the amperometric response of the G/CDH modified electrodes were investigated under flow conditions.

From the calibration curves, recorded at two pH values (5.2 and 7.0) for nine different sugars, the kinetic and the analytical parameters were evaluated under DET and MET operation modes. These results showed that: (i) for all nine investigated sugars the enzyme sensitivity was significantly higher for MET than for DET and for pH 5.2 compared to pH 7.0; (ii) irrespective of DET or MET operation mode, the sensitivity of the new enzyme towards the investigated sugars decreased in the following sequence: cellobiose > lactose > (cellotriose ≈ cellopentose) >> (maltotriose ≈ maltotetraose ≈ maltopentose) > (maltose ≈ glucose); (iii) for both operation modes, the NcCDH sensitivity to glucose was the lowest among the investigated substrates, being close to that observed for maltose; (iv) when NcCDH is operated in DET mode it prefers acidic media, but keeps a significant amount of activity under neutral pH conditions as well; (v) the obtained G/CDH bioelectrodes exhibit a good operational stability for around one week of exploitation under intermittent conditions.

3. Comparison between the electrochemical behavior of G/CDH and Au-S-Ph-OH-CDH modified electrodes[55]

3.1. Immobilization of CDH on different electrode materials

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 12A). Another approach 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 12 B).

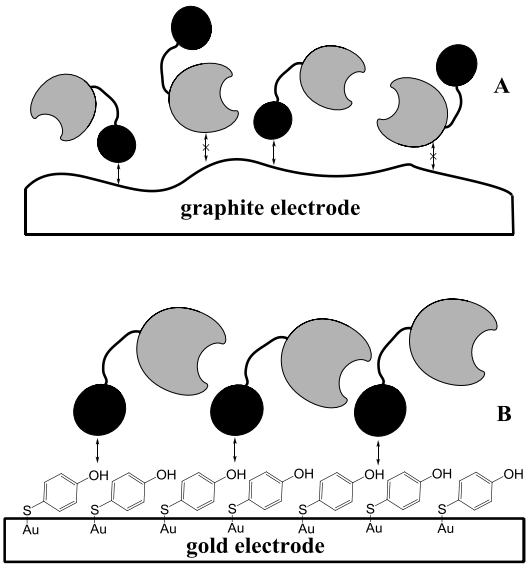

Figure 12. Schematic diagram showing the adsorption/orientation of the CDH enzyme (FAD domain – grey; heme domain – black; linker – black line) on graphite (A) and Au electrode modified with a self-assembled monolayer of 4-mercaptophenol (B).

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).

3.2. 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-S-Ph-OH/CDH) are shown in Figure 13.

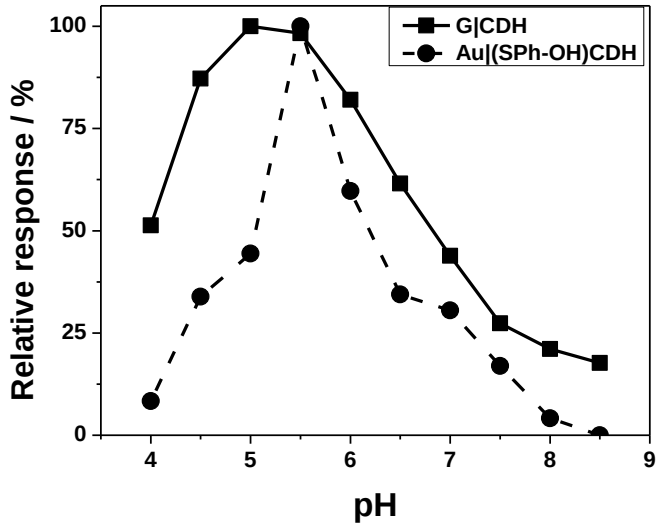

Figure 13. pH influence on the relative amperometric responses of G/CDH (■, ──) and Au-SPh-OH/CDH (●,─ ─) modified electrodes. Experimental conditions for G/CDH: flow injection mode, injections of 5 mM lactose, volume of injected sample, 50 µL; flow rate, 0.5 mL / min; applied potential, +300 mV *vs.* 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).

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-S-Ph-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-S-Ph-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-S-Ph-OH/CDH modified electrode decreases with more than 50% of its maximum value, for a pH change of 0.5 units (Figure 13).

3.3. 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 (Kmapp) 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) $\mu A*mM^{-1}cm^{-2}$.

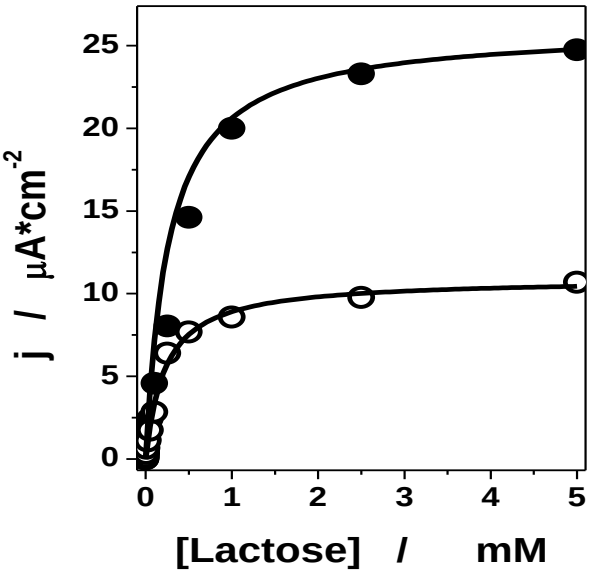

Figure 14. Calibration curves of G/CDH modified electrode towards lactose, recorded at two different pH values. Experimental conditions: applied potential, +300 mV vs. Ag|AgCl, 0.1M KCl; volume of injected sample, 50 µL; flow rate, 0.5 mL/min; flow carriers, 50 mM acetate (pH 5.5) or 50 mM phosphate 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.

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 15). 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 [56], 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-S-Ph-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.

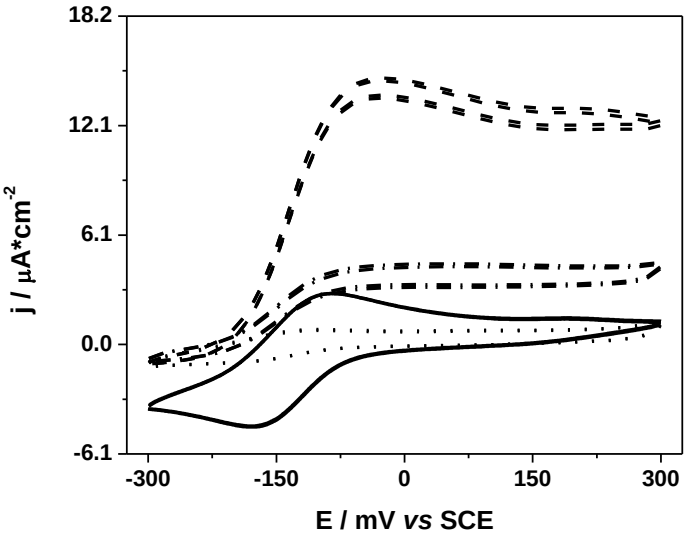

Figure 15. Voltammetric response of Au-SPh-OH/CDH modified electrode in absence [pH 5.5 (——); pH 7.0 (····)] and in presence [pH 5.5(- -); pH 7.0 (- · -)] of 5 mM lactose. Experimental conditions: starting potential, -300 mV *vs.* SCE; potential scan rate, 10 mV/s; 50 mM acetate (pH 5.5) or 50 mM phosphate buffer (pH 7.0).

The values estimating the catalytic efficiency for the two investigated electrodes are shown in Table 3**.** 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 3. 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

3.4. 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, built on the electrode surface. 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.

4. Electrocatalytic behavior of Au-SYX-CDH modified electrodes[57]

4.1. CDH immobilization on Au-SYX-CDH modified electrodes

In order to obtain an efficient DET coupling between the redox centers of the protein/enzyme and the electrode, a suitable orientation of the enzyme on the electrode surface is crucial. According to the Marcus theory [58], DET between a protein and an electrode is dependent on three major factors: i) the distance between the redox site and the electrode surface; ii) the reorganization energy, which reflects the structural rigidity of the redox site in its oxidized and reduced forms; iii) the thermodynamic driving force of the ET, which is related to a proper synchronization between the redox potential of the protein and the polarization of the electrode surface [59-61].

Among the enzymes known to exhibit DET characteristics at various electrodes, cellobiose dehydrogenase (CDH, cellobiose: acceptor 1-oxidoreductase, EC 1.1.99.18) was thoroughly studied in the recent years [20,23,62,63]. The best studied CDHs up to date are the ones from basidiomycetes (class I), while only limited information is available on class II CDHs [20,21]. The DET behavior at SAM modified Au electrodes for a number of CDHs (predominantly belonging to class I) have been studied previously using cyclic voltammetry and UV-Vis spectroelectrochemistry [32,48,64-67]. A schematic representation of the functioning principle, involving a DET process, illustrated in the case of a bioelectrode constructed by immobilizing CDH on a SAM-modified Au electrode is shown in Figure 16A. In the presence of a substrate (e.g. lactose) CDH oxidizes the sugar to the corresponding lactone at the catalytic domain (FAD) and the resulted electrons are transferred through an internal pathway (IET) to the heme binding domain. If the enzyme is properly oriented on the modified electrode surface (Figure 16B), the heme domain transfers the electrons further to the electrode surface through an efficient DET process.

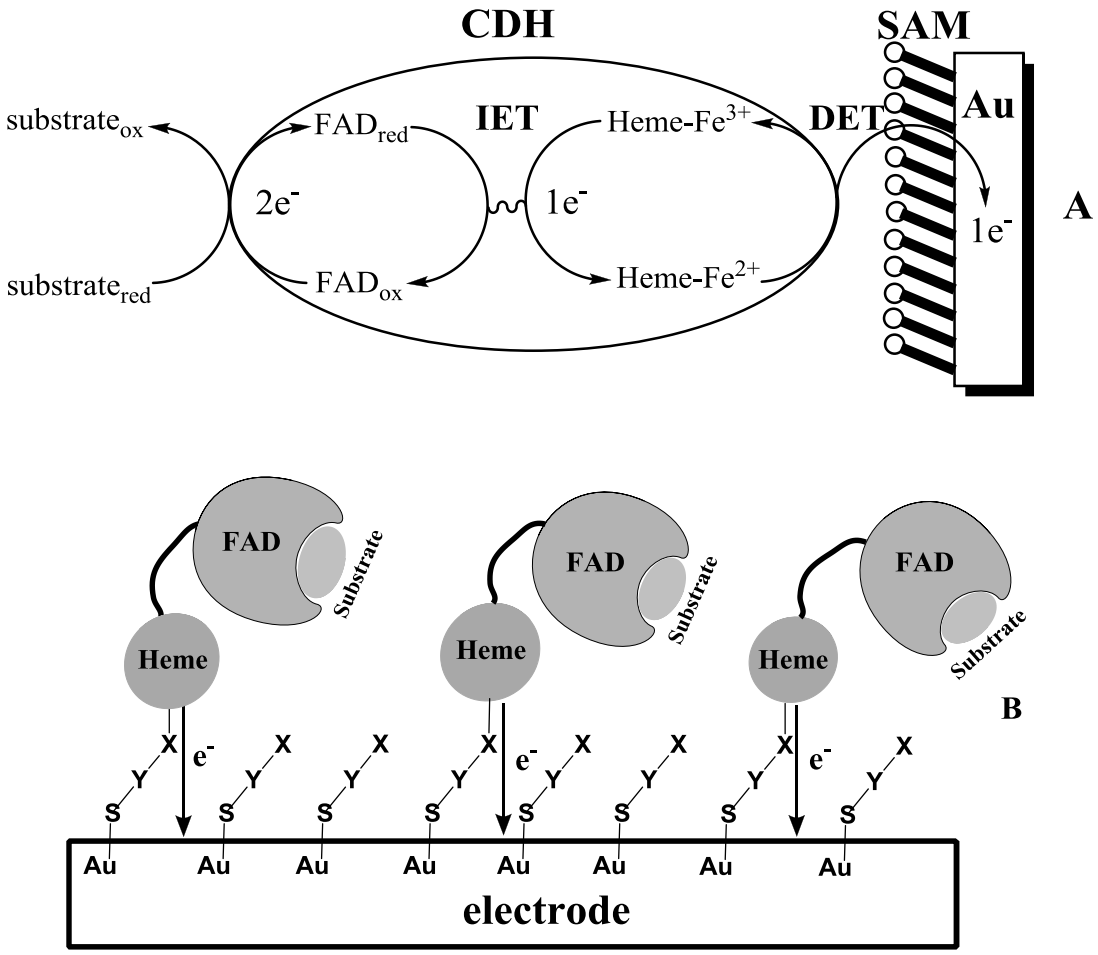

Figure 16: (A) The schematic diagram of DET (underlying the internal ET) for adsorbed CDH on a SAM-modified Au electrode; (B) the electrochemical interface structure of the bioelectrode based on CDH adsorbed at different SAM-modified Au electrodes, where Y = -$(CH_2)_2$-, -C_6H_4-, -$(CH_2)_{11}$-, and X = -OH, -NH_2, -COOH.

4.2. Voltammetric behavior of Au-SYX-CDH modified electrodes

The aim of the current section was to provide more information on the DET process in the case of *Nc*CDH. For this purpose, CDH was trapped under a permselective membrane applied onto the surface of SAM modified Au electrodes, as it was previously described in [68]. Cyclic voltammetric measurements, performed at different pH values, were used to estimate the catalytic efficiencies towards lactose. The observed behavior was explained in terms of the influence of the SAM structure on DET process occurring between CDH and the Au electrode surface. At the same time, the pH dependence of the standard formal potential of the heme group,

corroborated with data for electrocatalytic efficiency, confirms once again that CDH, deposited on thiolic SAMs, is able to sustain an efficient DET process.

As can be seen from Figure 17, three types of voltammetric behaviors were recorded for the investigated Au-SYX-CDH modified electrodes: (i) a well-shaped electrocatalytic response, observed for Au electrodes covered with HS-$(CH_2)_2$-NH_2 and HS-C_6H_4-X (X= -OH, -COOH); (ii) a mixed response, observed in the case of HS-$(CH_2)_2$-OH, HS-C_6H_4-NH_2 and HS-$(CH_2)_{11}$-COOH, which can be considered an overlapping of the electrocatalytic response with a partial direct oxidation of the substrate on the unmodified Au surface; (iii) a poor electrocatalytic response, noticed in the case of HS-$(CH_2)_{11}$-OH, being due to the direct oxidation of the substrate on the unmodified Au electrode surface [65,69].

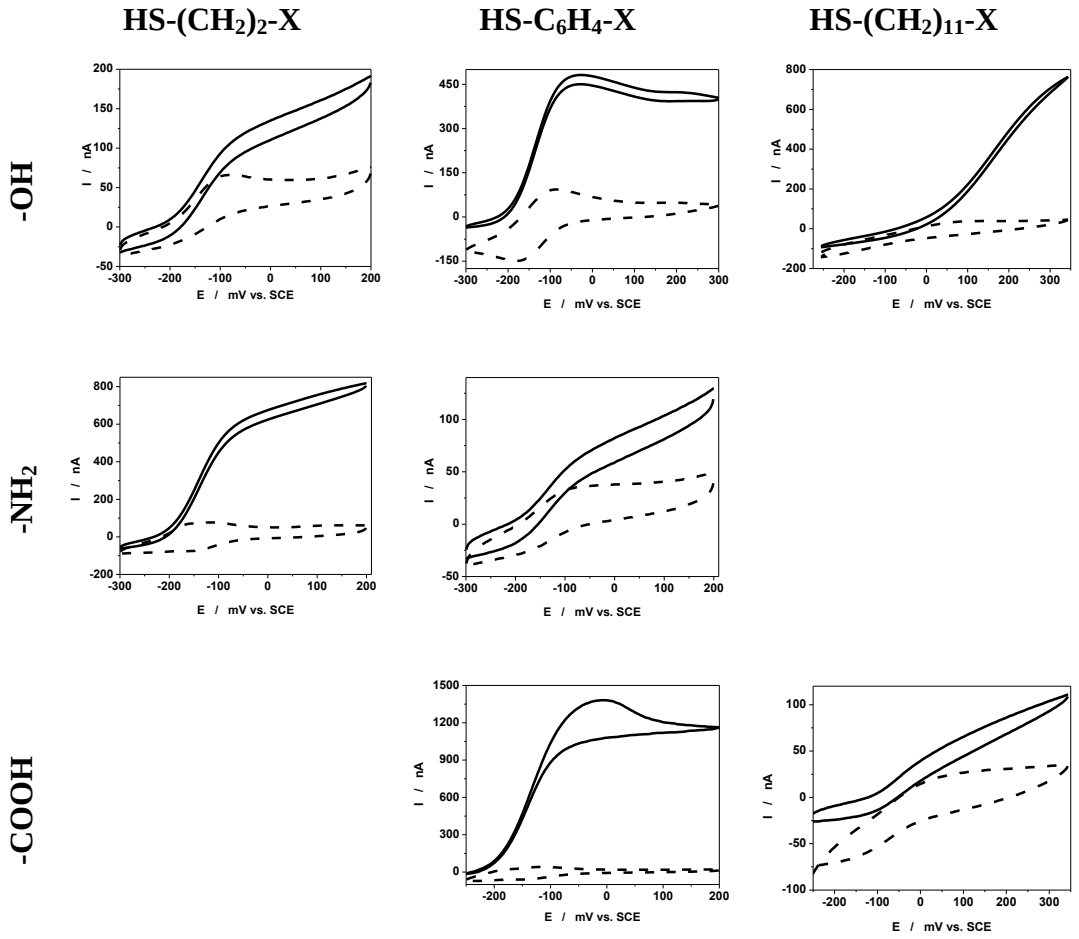

Figure 17.: Voltammetric responses of Au-SYX-CDH modified electrodes in absence (dash line) and in presence (solid line) of 5 mM lactose. Experimental conditions: scan rate, 10 mV/s; supporting electrolyte, 50 mM citrate buffer (pH 5.5).

It should be mentioned that the Au-S-$(CH_2)_2$-COOH-CDH modified electrode showed a high instability and, for this reason, all corresponding results were disregarded.

These behaviors could be understood as resulting from a complex combination of two factors: the distance between the electrode surface and the enzyme active center, which is controlled by the length of thiolic molecule; the interactions existing, for a given SAM, between the CDH molecule and the terminal functional group.

For a given SAM, the electrostatic interactions between CDH molecule and the terminal functional group depend strongly on the surrounding pH, which controls the ionization state of both terminal functional groups and enzyme surface. An example illustrating this situation is the case of HS-$(CH_2)_2$-NH_2, which at pH 5.5 is positively charged, and consequently, will develop attractive interactions with CDH molecules, promoting the DET process [70]. The apparent discrepancy observed between the electrocatalytic efficiencies observed for Au-S-C_6H_4-NH_2-CDH and Au-S-$(CH_2)_2$-NH_2-CDH modified electrodes should be due to the higher alkalinity of the aliphatic amines compared to the aromatic ones.

However, besides the electrostatic nature, other interactions such as hydrogen bonds, van der Waals and hydrophilic/hydrophobic interactions etc., should be considered in order to understand why, for example, the Au-S-C_6H_4-COOH-CDH modified electrode showed the highest catalytic activity among the investigated modified electrodes.

4.3. Electrocatalytic behavior of Au-SYX-CDH modified electrodes

In order to compare the electrocatalytic activity of the investigated modified electrodes at different pH values, the electrocatalytic efficiency was estimated as $(I_{[S]}-I_{[0]})/I_{[0]}$), where $I_{[S]}$ and $I_{[0]}$ stand for the catalytic current and the background current, respectively. For all investigated electrodes, excepting the Au-S-$(CH_2)_{11}$-COOH-CDH modified electrode, the maximum electrocatalytic efficiency was observed around pH 5 (Figure 18). Thus, the already reported preference of *Nc*CDH [21,71] for acidic media was confirmed once again. Concerning the peculiar behavior of the Au-S-$(CH_2)_{11}$-COOH-CDH modified electrode (Figure 18C), the pH increase induces the gradual ionization of the carboxyl terminal group causing a monotone decrease of the electrocatalytic efficiency. At pH values higher than 5, the

electrocatalytic activity of the electrode vanishes, probably because the unfavorable interactions between the CDH molecule and the negatively charged surface of the SAM.

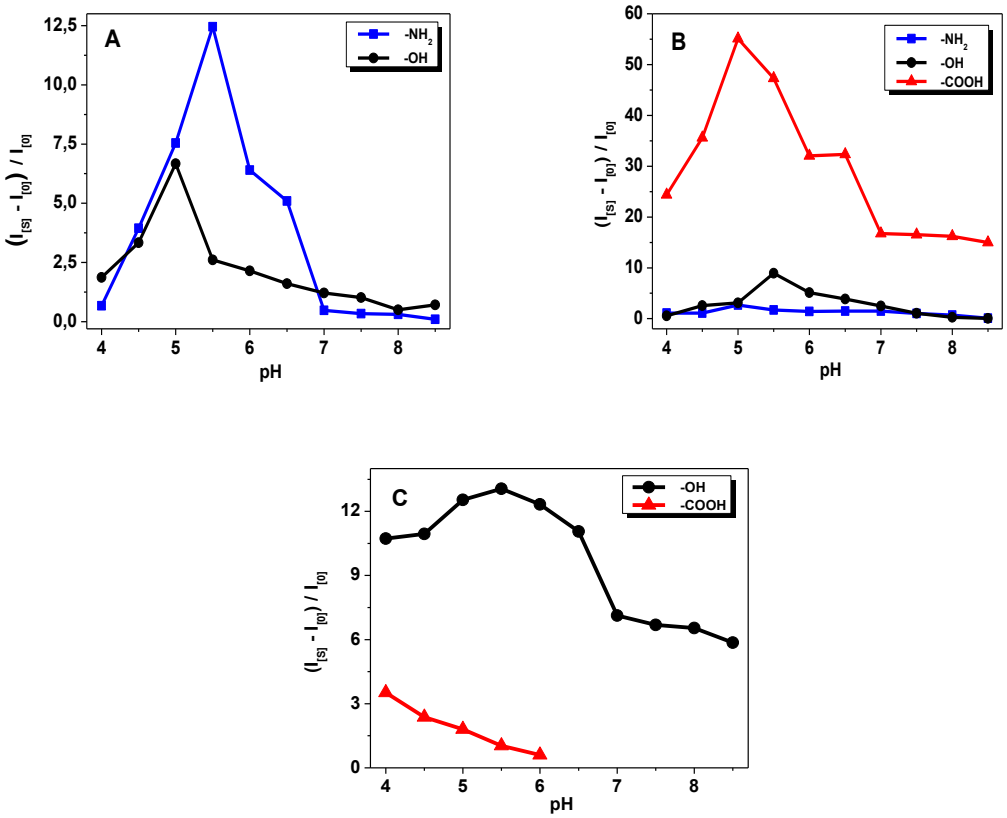

Figure 18: pH dependence of the electrocatalytic efficiencies, calculated as $(I_{[S]}-I_{[0]})/I_{[0]}$, for Au-SYX-CDH modified electrodes, where Y = $-(CH_2)_2-$ (A); $-C_6H_4-$ (B) and $-(CH_2)_{11}-$ (C). Experimental conditions: applied potential, +200 mV *vs.* SCE; substrate, 5 mM lactose.

The effect of the terminal functional group on the electrocatalytic efficiency can be better put in evidence when the maximum values of the electrocatalytic efficiencies, estimated for all Au-SYX-CDH modified electrodes, are plotted grouped for the same -Y- unit (Figure 19). Thus, within the limits of the experimental errors, it can be stated that: (i) when the –COOH group is connected to the thiol group (HS-) via a conducting unit ($-C_6H_4-$) it will clearly enhance the electrocatalytic activity; (ii) the higher alkalinity of the aliphatic amines, compared to the aromatic ones, will favor the attractive interactions between CDH and the modified electrode surface, resulting in an increase of the electrode electrocatalytic efficiency.

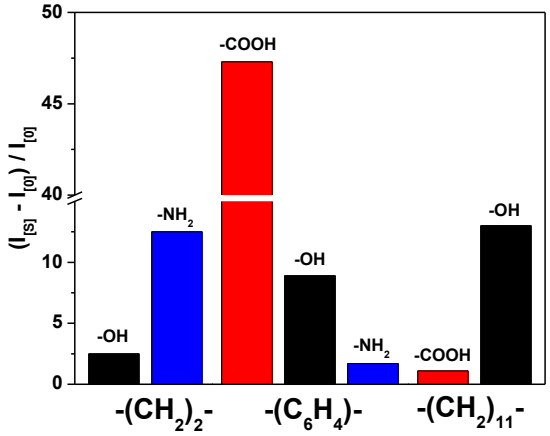

Figure 19. Influence of the SAM type on the electrocatalytic efficiencies for Au-SYX-CDH modified electrodes. Experimental conditions: applied potential, +200 mV *vs.* SCE; pH 5.5; substrate, 5 mM lactose.

4.4. pH influence on $E^{0'}$ of the heme redox couple from CDH

The voltammetric responses corresponding to the heme redox couple, hosted by the cytochrome domain [14,32,48,64,65], were recorded at different pH values for the Au-SYX-CDH modified electrodes. Their characteristic electrochemical parameters were estimated at pH 5.5 and are summarized in Table 4.

Table 4. The electrochemical parameters of the heme voltammetric response observed at different Au-SYX modified electrodes (pH 5.5).

Thiolic compound	X	ΔE_p (mV)	$E^{0'}$ (mV)
HS-$(CH_2)_2$-X	**-OH**	50	-135
	-NH_2	40	-130
HS-C_6H_4-X	**-OH**	70	-135
	-COOH	30	-135
	-NH_2	70	-135

It can be seen that the formal standard potential ($E^{0'}$) remains practically unchanged when the SAM nature changes and the peak-to-peak separation (ΔE_p) points out to a surface confined redox couple. The ΔE_p lowest value, observed for the Au-S-C_6H_4-COOH-CDH modified electrode, points out that in this case there are the

strongest interactions between CDH and the electrode surface, which corroborates well with the highest observed electrocatalytic efficiency.

4.5. Conclusions

Cyclic voltammetric measurements, performed at different pHs for Au-SYX-CDH modified electrodes (Y = -$(CH_2)_2$-, -C_6H_4- and -$(CH_2)_{11}$-; X = -OH, -COOH, -NH_2) in absence or in presence of the CDH substrate (lactose), allow estimation of the electrocatalytic efficiencies of the immobilized CDH towards lactose.

The variations observed between the different electrocatalytic efficiencies were explained in terms of the influence of the SAM structure on the direct electron transfer between CDH and the Au electrode surface. This behavior was explained as being the result of a complex combination of two factors: the distance between the electrode surface and the enzyme active center, which is controlled by the length of thiolic molecule; the interactions existing, for a given SAM, between the CDH molecule and the terminal functional group.

The pH dependence of the standard formal potential of the heme group, validates the hypothesis that the CDH extracted from *Neurospora crassa* and immobilized on Au-SYX-CDH modified electrodes is able to sustain an efficient DET process, which consists of three consecutive steps: (i) the substrate oxidation by the FAD domain; (ii) the internal electron transfer to the heme domain via a fast IET; (iii) the electrical connection between the heme domain and the modified electrode surface.

5. References

(1) Clark, L. C., Jr.; Lyons, C. ***Ann. N. Y. Acad. Sci.*** **1962,** ***102,*** **29-45.**

(2) Mohanty, S. P.; Kougianos, E. ***IEEE Potentials*** **2006,** ***25,*** **35-40.**

(3) Thevenot, D. R.; Toth, K.; Durst, R. A.; Wilson, G. S. ***Biosens. Bioelectron.*** **2001,** ***16,*** **121-131.**

(4) Mao, X.-L.; Wu, J.; Ying, Y.-B. ***Chinese Journal of Analytical Chemistry*** **2008,** ***36,*** **1749-1755.**

(5) Velasco-Garcia, M. N.; Mottram, T. ***Biosystems Engineering*** **2003,** ***84,*** **1-12.**

(6) Luong, J. H. T.; Male, K. B.; Glennon, J. D. ***Biotechnology Advances,*** ***26,*** **492-500.**

(7) Bard, A. J.; Faulkner, L. R. ***Electrochemical methods: fundamentals and applications, 2nd ed.*****; Wiley: New York, 2001.**

(8) Heyrovsky, J. ***Analyst*** **1956,** ***81,*** **189-192.**

(9) Heyrovsky, J. ***Analytica Chimica Acta*** **1948,** ***2,*** **533-541.**

(10) Speiser, B. In ***Encyclopedia of Electrochemistry*****; Bard, A. J., Stratmann, M., Eds.; Wiley-VCH: Weinheim, 2003; Vol. 3 Instrumentation and Electroanalytical Chemistry; pp 81-104.**

(11) Laviron, E. In ***Electroanalytical Chemistry: A series of advances*****; Marcel Dekker: New York, 1982; Vol. 12; pp 53-157.**

(12) Coman, V. In ***Doctoral Thesis*****; Lund University, Sweden, 2009; pp 18-20.**

(13) Lindgren, A.; Gorton, L.; Ruzgas, T.; Baminger, U.; Haltrich, D.; Schülein, M. ***Journal of Electroanalytical Chemistry*** **2001,** ***496,*** **76-81.**

(14) Lindgren, A.; Larsson, T.; Ruzgas, T.; Gorton, L. ***J. Electroanal. Chem.*** **2000,** ***494,*** **105-113.**

(15) Ruzicka, J.; Hansen, E. H. ***Anal. Chim. Acta*** **1975,** ***78,*** **145-57.**

(16) Ruzicka, J.; Stewart, J. W. B. ***Anal. Chim. Acta*** **1975,** ***79,*** **79-91.**

(17) Ruzicka, J.; Hansen, E. H. ***Flow injection anlysis, 2nd Edition*****; John Wiley & Sons: New York, 1988.**

(18) Stoica, L. Dissertation, Lund University, 2005.

(19) Appelqvist, R.; Marko-Varga, G.; Gorton, L.; Torstensson, A.; Johansson, G. ***Anal. Chim. Acta*** **1985,** ***169,*** **237-47.**

(20) Zamocky, M.; Ludwig, R.; Peterbauer, C.; Hallberg, B. M.; Divne, C.; Nicholls, P.; Haltrich, D. ***Curr. Prot. Pept. Sci.*** **2006,** ***7,*** **255-280.**

(21) Harreither, W.; Sygmund, C.; Augustin, M.; Narciso, M.; Rabinovich Mikhail, L.; Gorton, L.; Haltrich, D.; Ludwig, R. ***Applied and environmental microbiology*** **2011,** ***77,*** **1804-1815.**

(22) Zamocky, M.; Schumann, C.; Sygmund, C.; amp; apos; Callaghan, J.; Dobson, A. D. W.; Ludwig, R.; Haltrich, D.; Peterbauer, C. K. ***Protein expression and purification.*** **2008,** ***59,*** **258-265.**

(23) Ludwig, R.; Harreither, W.; Tasca, F.; Gorton, L. ***ChemPhysChem*** **2010,** ***11,*** **2674-2697.**

(24) Westermark, U.; Eriksson, K. E. ***Acta Chem. Scand. B*** **1974,** ***28,*** **204-208.**

(25) Westermark, U.; Eriksson, K. E. ***Acta Chem. Scand. B*** **1974,** ***28,*** **209-214.**

(26) Larsson, T.; Elmgren, M.; Lindquist, S.-E.; Tessema, M.; Gorton, L.; Tessema, M.; Henriksson, G. ***Anal. Chim. Acta*** **1996,** ***331,*** **207-215.**

(27) Harreither, W.; Coman, V.; Ludwig, R.; Haltrich, D.; Gorton, L. ***Electroanalysis*** **2007,** ***19,*** **172-180.**

(28) Ludwig, R.; Salamon, A.; Varga, J.; Zámocky, M.; Peterbauer, C. K.; Kulbe, K. D.; Haltrich, D. ***Applied Microbiology and Biotechnology*** **2004,** ***64,*** **213-222.**
(29) Safina, G.; Ludwig, R.; Gorton, L. ***Electrochimica Acta*** **2010,** ***55,*** **7690-7695.**
(30) Rabinovich, M. L.; Vasil'chenko, L. G.; Karapetyan, K. N.; Shumakovich, G. P.; Yershevich, O. P.; Ludwig, R.; Haltrich, D.; Hadar, Y.; Kozlov, Y. P.; Yaropolov, A. I. ***Biotechnology Journal*** **2007,** ***2,*** **546-558.**
(31) Coman, V.; Vaz-Dominguez, C.; Ludwig, R.; Harreither, W.; Haltrich, D.; De Lacey, A. L.; Ruzgas, T.; Gorton, L.; Shleev, S. ***Physical Chemistry Chemical Physics*** **2008,** ***10,*** **6093-6096.**
(32) Stoica, L.; Ludwig, R.; Haltrich, D.; Gorton, L. ***Anal. Chem.*** **2006,** ***78,*** **393-398.**
(33) Coman, V.; Ludwig, R.; Harreither, W.; Haltrich, D.; Gorton, L.; Ruzgas, T.; Shleev, S. ***Fuel Cells*** **2010,** ***10,*** **9-16.**
(34) Kavanagh, P.; Boland, S.; Jenkins, P.; Leech, D. ***Fuel Cells*** **2009,** ***9,*** **79-84.**
(35) Zhang, X. C.; Ranta, A.; Halme, A. ***Biosensors and Bioelectronics*** **2006,** ***21,*** **2052-2057.**
(36) Deng, L.; Shang, L.; Wen, D.; Zhai, J.; Dong, S. ***Biosensors and Bioelectronics*** **2010,** ***26,*** **70-73.**
(37) Willner, I.; Yan, Y. M.; Willner, B.; Tel-Vered, R. ***Fuel Cells*** **2009,** ***9,*** **7-24.**
(38) Bullen, R. A.; Arnot, T. C.; Lakeman, J. B.; Walsh, F. C. ***Biosensors and Bioelectronics*** **2006,** ***21,*** **2015-2045.**
(39) Ivan, I.; Tanja, V.-K.; Kai, S. ***Energies*** **2010,** ***3,*** **803-846.**
(40) Arechederra, R. L.; Minteer, S. D. ***Analytical and Bioanalytical Chemistry*** **2011,** ***400,*** **1605-1611.**
(41) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Noll, G. ***J. Phys. Chem. C*** **2008,** ***112,*** **9956-9961.**
(42) Stoica, L.; Dimcheva, N.; Ackermann, Y.; Karnicka, K.; Guschin, D. A.; Kulesza, P. J.; Rogalski, J.; Haltrich, D.; Ludwig, R.; Gorton, L.; Schuhmann, W. ***Fuel Cells*** **2009,** ***9,*** **53-62.**
(43) Tasca, F.; Gorton, L.; Kujawa, M.; Patel, I.; Harreither, W.; Peterbauer, C. K.; Ludwig, R.; Noll, G. ***Biosensors and Bioelectronics*** **2010,** ***25,*** **1710-1716.**
(44) Kovács, G.; Ortiz, R.; Coman, V.; Harreither, W.; Popescu, I. C.; Ludwig, R.; Gorton, L. ***Bioelectrochemistry*** **2011,** ***submitted.***
(45) Lindgren, A.; Ruzgas, T.; Gorton, L.; Stoica, L.; Ciucu, A. ***Analyst*** **1999,** ***124,*** **527-532.**
(46) Stoica, L.; Lindgren-Sjoelander, A.; Ruzgas, T.; Gorton, L. ***Anal. Chem.*** **2004,** ***76,*** **4690-4696.**
(47) Larsson, T.; Lindgren, A.; Ruzgas, T.; Lindquist, S. E.; Gorton, L. ***J. Electroanal. Chem.*** **2000,** ***482,*** **1-10.**
(48) Lindgren, A.; Gorton, L.; Ruzgas, T.; Baminger, U.; Haltrich, D.; Schulein, M. ***J. Electroanal. Chem.*** **2001,** ***496,*** **76-81.**
(49) Zhang, R.; Fan, Z.; Kasuga, T. ***Protein Expression and Purification*** **2011,** ***75,*** **63-69.**
(50) Stoica, L.; Ruzgas, T.; Ludwig, R.; Haltrich, D.; Gorton, L. ***Langmuir*** **2006,** ***22,*** **10801-10806.**
(51) Nazaruk, E.; Michota, A.; Bukowska, J.; Shleev, S.; Gorton, L.; Bilewicz, R. ***Journal of Biological Inorganic Chemistry*** **2007,** ***12,*** **335-344.**

(52) Federico, T.; Wolfgang, H.; Roland, L.; John Justin, G.; Lo, G. ***Industrial & Engineering Chemistry Analytical Edition*** **2011,** ***83,*** **3042-3049.**
(53) Coman, V.; Vaz-Dominguez, C.; Ludwig, R.; Harreither, W.; Haltrich, D.; De Lacey, A. L.; Ruzgas, T.; Gorton, L.; Shleev, S. ***Phys. Chem. Chem. Phys.*** **2008,** ***10,*** **6093-6096.**
(54) Tasca, F.; Gorton, L.; Harreither, W.; Haltrich, D.; Ludwig, R.; Noll, G. ***J. Phys. Chem. C*** **2008,** ***112,*** **13668-13673.**
(55) Kovács, G.; Popescu, I. C. ***Studia Universitatis Babes-Bolyai, Seria Chemia*** **2011,** ***56.***
(56) Stoica, L.; Dimcheva, N.; Haltrich, D.; Ruzgas, T.; Gorton, L. ***Biosensors and Bioelectronics*** **2005,** ***20,*** **2010-2018.**
(57) Kovács, G.; Ortiz, R.; Coman, V.; Harreither, W.; Popescu, I. C.; Ludwig, R.; Gorton, L. ***Revue roumaine de chimie*** **2012,** ***submitted.***
(58) Marcus, R. A.; Sutin, N. ***Biochim. Biophys. Acta, Rev. Bioenerg.*** **1985,** ***811,*** **265-322.**
(59) Gorton, L.; Lindgren, A.; Larsson, T.; Munteanu, F. D.; Ruzgas, T.; Gazaryan, I. ***Anal. Chim. Acta*** **1999,** ***400,*** **91-108.**
(60) Willner, I.; Katz, E. ***Angewandte Chemie International Edition*** **2000,** ***39,*** **1180-1218.**
(61) Armstrong, F. A. ***Russian Journal of Electrochemistry*** **2002,** ***38,*** **49-62.**
(62) Henriksson, G.; Johansson, G.; Pettersson, G. ***J. Biotechnol.*** **2000,** ***58,*** **93-113.**
(63) Cameron, M. D.; Aust, S. D. ***Enzyme Microb. Technol.*** **2001,** ***28,*** **129-138.**
(64) Larsson, T.; Lindgren, A.; Ruzgas, T. ***Bioelectrochemistry*** **2001,** ***53,*** **243-249.**
(65) Stoica, L.; Dimcheva, N.; Haltrich, D.; Ruzgas, T.; Gorton, L. ***Biosens. Bioelectron.*** **2005,** ***20,*** **2010-2018.**
(66) Coman, V.; Harreither, W.; Ludwig, R.; Haltrich, D.; Gorton; Lo; Instytut Chemii Fizycznej: Warszawa, POLOGNE, 2007; Vol. 52.
(67) Sarauli, D.; Ludwig, R.; Haltrich, D.; Gorton, L.; Lisdat, F. ***Bioelectrochemistry.***
(68) Haladjian, J.; Bianco, P.; Nunzi, F.; Bruschi, M. ***Analytica Chimica Acta*** **1994,** ***289,*** **15-20.**
(69) Vidaković‐Koch, T.; Ivanov, I.; Falk, M.; Shleev, S.; Ruzgas, T.; Sundmacher, K. ***Electroanalysis*** **2011,** ***23,*** **927-930.**
(70) Lindgren, A.; Larsson, T.; Ruzgas, T.; Gorton, L. ***Journal of Electroanalytical Chemistry*** **2000,** ***494,*** **105-113.**
(71) Kovács, G.; Ortiz, R.; Coman, V.; Harreither, W.; Popescu, I. C.; Ludwig, R.; Gorton, L. ***Bioelectrochemistry*** **2012,** ***submitted.***

III. List of publications

Papers

1. **Gábor Kovács**, Ionel Cătălin Popescu, *Electrochemical behavior of cellobiose dehydrogenase from Neurospora crassa immobilized on graphite and Au-4-mercaptophenol modified electrodes*, Studia Univ. Babeş-Bolyai, Chemia, *56*(4), **2011**.

2. **Gábor Kovács,** Roberto Ortiz, Vasile Coman, Wolfgang Harreither, Ionel Cătălin Popescu, Roland Ludwig, Lo Gorton, *Graphite electrodes modified with Neurospora crassa cellobiose dehydrogenase: comparative electrochemical characterization under direct and mediated electron transfer*, Bioelectrochemistry, *submitted*, **2011**.

3. **Gábor Kovács**, Vasile Coman, Ionel Cătălin Popescu, Lo Gorton, *Influence of SAM structure on direct electron transfer at Au electrodes modified with cellobiose dehydrogenase from Neurospora crassa*, Revue Roumaine de Chimie, *submitted*, **2012**.

4. **Gábor Kovács,** Laura Mureşan, Graziella Liana Turdean, Csaba Bolla, Ionel Cătălin Popescu, *Módosított elektródok a glükóz borból történő kimutatására / Modified electrodes for detection of glucose from wine*, Proceedings of 14th International Conference of Chemistry, Ed. Hungarian Technical Society of Transylvania, Cluj-Napoca, **2008**, 70-76.

Communications

1. **Gábor Kovács**, Csaba Bolla, Ionel Cătălin Popescu, Lo Gorton, *Új CDH (Neurospora crassa-ból előállított) enzimmel módosított harmadik generációs bioszenzorok/Third generation biosensor modified with a new CDH from Neurospora Crassa,* 16th International Conference of Chemistry, Cluj-Napoca, Romania, 11-14 November **2010**, *oral communication*.

2. **Gábor Kovács**, Laura Mureşan, Graziella Turdean, Csaba Bolla, Ionel Cătălin Popescu, *Bioszenzorok a glükóz kimutatására / Biosensors for Detection of Glucose*, 15th International Conference of Chemistry, Cluj-Napoca, Romania, 12-15 November **2009**, *oral communication*.

3. Laura Mureşan, **Gábor Kovács**, Graziella Turdean, Ionel Cătălin Popescu, *Détection du Glucose dans le Vin a l'aide d'un Biocapteur a Glucose Oxydase*, Journees d'Electrochimie, Sinaia, Romania, 6-10 July **2009**, *poster*.

4. **Gábor Kovács,** Laura Mureşan**,** Graziella Turdean, Ionel Cătălin Popescu, Csaba Bolla, *Módosított elektródok a glükóz borból történő kimutatására / Modified electrodes for detection of glucose from wine*, 14th International Conference of Chemistry, Cluj-Napoca, Romania, 13-15 November **2008**, *oral communication*.

5. Laura Mureşan, **Gábor Kovács**, Graziella Turdean, Ionel Cătălin Popescu, B*iocapteur Amperometrique Bienzymatique Pour la Detection de la Glucose Dans des Vins,* Le 2ème Congrès des Sciences Analytiques, Casablanca, Morocco, 29-31 October **2008**, *poster*.

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I will switch back a bit to English… I may have forgotten some people from this long list but that was just because for the moment Your name has slipped away from my mind and as always, I'm in lack of time☺, I have to give away for printing this thesis as soon as possible. So, if your name is missing above, these big Thanks! are for You!