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Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické chemie a kontroly léčiv

UNIVERSITY OF GOTHENBURG

Faculty of Science

Department of Chemistry and Molecular Biology

Makroporézny indium cín oxid ako potenciálna platforma pre bioanalytické použitie

Diplomová práca

Školiteľ: Prof. PharmDr. Martin Doležal, Ph.D.

Konzultant: Assist. Prof. Gulnara Safina, Ph.D.

Hradec Králové 2015

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Diploma thesis

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Abstrakt

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Názov diplomovej práce: Makroporézny indium cín oxid ako potenciálna platforma pre bioanalytické použitie

Monitorovanie hladiny glukózy v krvi je nevyhnutnou súčasťou stanovenia diagnózy diabetu. Preto sú neustále vyvíjané nové stratégie na zdokonalenie meracích prístrojov. V tejto práci bola skúmaná vhodnosť použitia elektród tvorených makroporéznym indum cín oxidom (ITO) na konštrukciu glukózových biosensorov tretej generácie. Ako biorekogničný element bola použitá celobióza dehydrogenáza z huby *Corynascus thermophilus* (CtCDH) imobilizovaná na ITO nosič. Bolo skúmaných viacero metód imobilizácie ako fyzikálna adsorpcia, elektrostatická väzba enzýmu na povrch pokrytý polyetyleniminom (PEI) a zosieťovanie glutaraldehydom (GA), s cieľom dosiahnuť primeranú citlivosť a stabilitu biosensora. Morfológia/topografia a elementárne zloženie enzymaticky modifikovaného povrchu boli skúmané rastrovacím elektrónovým mikroskopom (SEM), metódou energiovo disperznej spektroskopie (EDX) a röntgenovou fotoelektrónovou spektroskopiou (XPS). Optimálne podmienky pre elektrochemické meranie v prietokovej cele boli stanovené takto: pH 7,2, napätie 0,3 V a prietoková rýchlosť 0,5 ml/min. Prúdová odozva pri použití elektródy modifikovanej s PEI bola asi 10-krát vyššia ako pri modifikácii s GA. CDH-GA modifikovaná elektróda vykazovala zlepšenú stabilitu pri meraní v prietokovej cele po dobu 7 hodín s nástrekmi relatívne vysokej koncentrácie glukózy (10 mM). Pokles počiatočnej odpovede bol 15 % v prípade GA-CDH elektródy a 72 % v prípade PEI-CDH elektródy. Senzor ukázal linearitu v rozmedzí koncentrácii glukózy 1 – 20 mM s limitom detekcie 0,03 mM pre PEI-CDH modifikovanú elektródu a 0,37 mM pre GA-CDH modifikovanú elektródu.

Kľúčové slová: amperometrický sensor, indium cín oxid, celobióza dehydrogenáza, glukóza

Abstract

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Title of thesis: Macroporous indium tin oxide as a potential platform for bioanalytical applications

Blood glucose monitoring is an essential tool in diabetes mellitus diagnosis. Therefore new strategies have been developed to improve the performance of glucose sensing devices. In this thesis the suitability of macroporous indium tin oxide (ITO) electrodes for construction of third-generation glucose biosensors was investigated. As a biosensing part in sensor cellobiose dehydrogenase from *Corynascus thermophilus* (CtCDH) immobilized onto ITO platform was used. Several immobilization strategies based on physical adsorption, electrostatic bindings of the enzyme to the surface functionalized with polythyleneimine (PEI) and cross-linking with glutaraldehyde (GA) were studied in order to achieve reasonable sensitivity and stability of the biosensor. The morphology/topography and elemental composition of the enzyme modified surface were examined by scanning electron microscopy (SEM), energy-dispersive X-ray spectroscopy (EDX), X-ray photoelectron spectroscopy (XPS). The optimal working conditions for flow injection experiment were established as follows: pH of 7,2, working potential of 0,3 V, and flow-rate of 0,5 ml/min. The current response obtained from the electrode functionalized with PEI was about 10 times higher than the one modified with GA. CDH-GA modified electrode exhibited improved stability in flow-injection test for duration of 7 hours with relatively high concentration of glucose (10 mM). The decrease of the initial response was 15% and 72% for GA-CDH and PEI-CDH modified electrodes, respectively. The sensor showed linearity in the glucose concentration range from 1 to 20 mM with the limit of detection down to 0,03 mM for the PEI-CDH modified electrode and 0,37 mM for GA-CDH modified electrode.

Keywords: amperometric sensor, indium tin oxide, cellobiose dehydrogenase, glucose

List of abbreviations

CAT	Catalysis
CDH	Cellobiose dehydrogenase
CV	Cyclic voltammetry
CYT	Cytochrome domain
Cyt c	Cytochrome c
DET	Direct electron transfer
DH	Flavodehydrogenase domain
EDX	Energy-dispersive X-Ray Spectroscopy
ESCA	Electron Spectroscopy for Chemical Analysis
FAD	Flavin adenine dinucleotide
GA	Glutaraldehyde
GDH	Glucose dehydrogenase
GOx	Glucose oxidase
HPC	Hydroxypropyl cellulose
IET	Internal electron transfer
ITO	Indium tin oxide
LOD	Limit of detection
nano-ITOH	Ultra-small indium tin hydroxide
PBS	Phosphate buffered saline
PEI	Poly(ethyleneimine)
PMMA	Poly(methyl methacrylate)
PMSA1	Sulfonated polyaniline copolymer
PQQ-GDH	Pyrroloquinoline quinine-dependent glucose dehydrogenase
SEM	Scanning Electron Microscope
TCO	Transparent conducting oxide
XPS	X-Ray Photoelectron Spectroscopy

Content

1 The aim of the thesis

The aim of the work is to investigate the suitability of macroporous ITO as a platform for biosensor construction and compare different modification approaches:

- Physical adsorption of the enzyme onto electrode surface
- Premodification of the electrode with the polycation PEI (polyethyleneimine)
- Cross-linking with glutaraldehyde

The surface topography and elemental composition will be characterized and behaviour of the electrode is to be examined by electron microscopy, advanced spectroscopic techniques and electrochemistry.

2 Theoretical part

2.1 Biosensors

2.1.1 Definition and classification

A biosensor is an analytical device that transforms biological information into a measurable signal and provides information about chemical composition of a sample or a particular constituent concentration. The sensor consists of two main components, a biorecognition part and a transducer [1]. The biorecognition element or bioreceptor has an affinity for a target analyte and enables selective interaction and/or binding with it. It provides the sensor with a high selectivity [2]. The biorecognition process results in a change in chemical or physical properties of sensing system related to the analyte concentration. This change is registered and converted into a measurable signal by a transducer, which is also called a detector. Both components, the recognition element and transduction system are integrated in the same device [3].

Biosensors can be categorized in many ways, most often it is according to the transduction method as follows [4]:

- *Optical sensors.*

  They measure optical properties like absorbance, fluorescence, refractive index, etc.

- *Electrochemical sensors.*

  They are based on electrochemical reaction between analyte and electrode and electrical sensors, where the electrochemical reaction does not occur, for instance a conductivity sensor

- *Mass sensitive sensors.*

  They register the mass of the transducer increase when the biospecific interaction occurs on the sensor surface, e.g. piezoelectric devices and surface acoustic wave devices.

- *Magnetic sensors.*

  They are based on the change of paramagnetic properties of a gas being analysed.

- *Thermometric sensors.*

  They are based on the registering the heat effect caused by chemical reaction

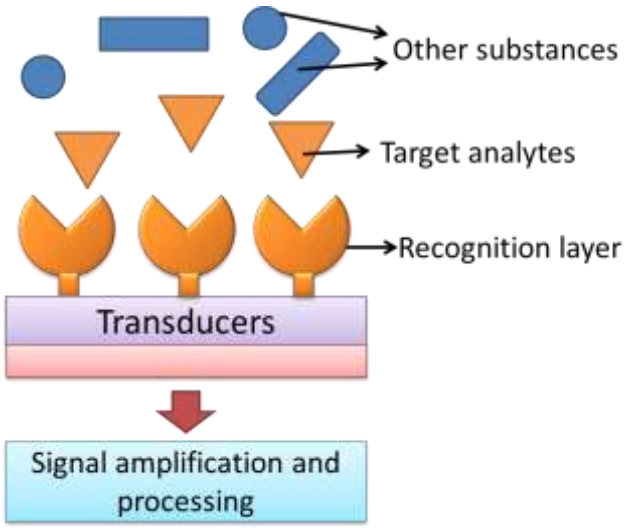


Figure 1: Schematic diagram of a biosensor. Figure was adapted from [5].

2.1.2 Application

Biosensors are sophisticated measuring devices, which are able to provide inexpensive, easy to use, sensitive and accurate analysis. They have found a wide variety of applications in different fields ranging from biomedical through agricultural to

environmental. The devices are used in both research and commercial applications (Table 1) [3, 6].

Table 1: Key biosensor application domains [7].

Domain	**Application**
Healthcare	Chronic disease control, such as glucose monitoring in diabetes patients Home pregnancy testing Cholesterol testing Bacterial infection testing Stomach ulcers: Helicobacter pylori Acute disease diagnosis, such as prostate cancer
Biotechnology/fermentation	Wine fermentation process Assay of citric acid Brewing technology Enzyme production Biopharmaceutical production
Food quality	Chemical contaminant detection, such as contamination with antibiotics Toxin detection Pathogen (bacteria) detection Hormone detection, as in milk Antibiotics content assay
Personal safety/law enforcement/employment	Alcohol testing Drug of abuse testing
Environmental monitoring	Pollution, such as testing for fecal coliforms in water Analysis of pesticides in water such as organophosphates Heavy metals assay (waters, soil) Sex hormones pollution (waters)
Security	Chemical and warfare agent detection

2.1.3 Attributes of a biosensor

A successful biosensor should meet the following requirements [8]:

- stable and specific biorecognition element
- the reaction is minimally influenced by temperature, pH and stirring
- accurate (reliable) and reproducible response
- wide linearity with minimal sample preparation
- biocompatibility, nontoxicity
- providing real-time analysis
- cheap, portable, suitable for mass production and simple to use

2.1.4 Biological recognition element

Biosensors may be classified by the type of the bioreceptor or by the transduction mode [9]. According to the biosensing mechanism, two classes of biosensors are distinguished: biocatalytic sensors and bioaffinity sensors [10].

2.1.4.1 Biocatalytic sensors

The biocatalytic sensor incorporates enzymes, whole cells or tissue slices, which catalyses a biochemical reaction of a substrate. This leads to a significant increase in the rate of a chemical reaction. Enzymes are the most commonly used bioelements thanks to their high specificity, biocatalytic activity and ability to construct a relatively inexpensive device with a simple design. Composite structure of the enzyme allows to specifically detect particular substrate in a complicated mixture, such as blood or urine, often without the need or minimal sample pretreatment. High selectivity of enzyme originates in the arrangement of enzyme's active site, where the substrate fits and is further converted to a corresponding product [9, 10].

2.1.4.2 Bioaffinity sensors

The working principle of affinity biosensors is based on strong binding of a biological receptor molecule with a desired substrate (ligand) which results in a formation of [receptor-ligand] complex. Formation of such complex results in change of physicochemical properties of system, which is usually measured as electrical signal [10]. This type of biosensors utilizes antibodies, membrane receptors or nucleic acids. The most frequently employed bioreceptors are antibodies because of their possibility to synthesize them towards particular antigen and, therefore, the high affinity in recognition of this antigen. The device incorporating antibodies in its biorecognition part is called immunosensor. It is noted for its ability to detect trace amounts of substances, therefore immunosensors have found application in food safety, environmental and clinical monitoring [11]. The recognition process in nucleic acid-based sensors is determined by complementarity of DNA/RNA chain fragments. They could be used to diagnose cancer, bacterial and viral pathogens and genetic disorders [12].

2.1.5 Electrochemical sensors

The main focus of this work will be done on electrochemical biosensors. Biosensors coupled with electrochemical transducer are simple, portable and relatively low cost devices. Potential for miniaturization opens up possibilities for construction of implantable biosensors. These properties are the reason why electrochemical transducers are the most widely used. Electrochemical biosensors are further divided into amperometric, potentiometric and conductometric [13].

2.1.5.1 Potentiometric biosensors

Petentiometry is based on measuring of the potential difference between two electrodes, reference and working, when a negligible current flows through the electrochemical cell. In potentiometric sensors a reference electrode maintains a fixed potential and an indicator electrode responds to the analyte activity or concentration.

Very frequently used working electrodes are ion-selective electrodes with various composition of a selective membrane. The change in potential is proportional to the logarithm of concentration of a sample analyte [9, 14].

2.1.5.2 Conductometric biosensors

Conductometric measurements are based on electrolyte conductivity, which is linearly dependent on ion concentration. Conductivity reflects the ability of the analyte to pass an electrical current between two electrodes which are separated by a distance or by a working medium. In enzyme sensors, the change in ionic strength, and so the conductivity, is caused by an enzymatic reaction. One of the main advantages of this type of sensor is a simplified design, as no reference electrode is required. On the other hand, the method is less sensitive and is strongly influenced by buffer capacity [9, 14].

2.1.5.3 Amperometric biosensors

An amperometric set up consists of two or three electrodes. The working electrode is typically made of inert metals or carbon. A reference electrode serves to control the potential of the working electrode. It is usually Ag/AgCl of calomel electrodes. Sometimes the third electrode that completes the current path is introduced. It is called a counter or auxiliary electrode. Amperometric sensors measure current as a function of time generated by electrochemical oxidation or reduction of an electroactive species on the electrode surface. The resulting current is proportional to the analyte concentration. Redox reaction occurs at working electrode when potential is applied. The given potential is constant with respect to a reference electrode. During this reaction, electrons are transferred between analyte and electrode. Based on the different transfer process three generations of biosensors are distinguished (Figure 2) [9, 14]:

- First generation – based on the electroactivity of the enzyme substrate or product

- Second generation – involve specific redox mediators between the reaction and the transducer
- Third generation – based on direct electron transfer (DET)

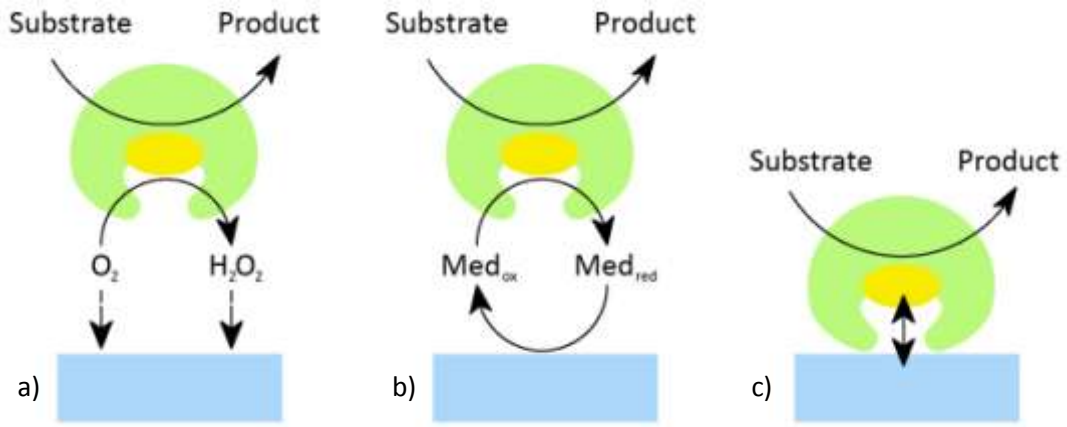


Figure 2: Electron transfer in enzyme biosensor a) first generation, b) second generation, c) third generation. Figure created with Paint.NET.

One well known example of amperometric biosensor is glucose sensor. The first electrochemical biosensor for glucose detection was invented by Clark and Lyons in 1962. The device was based on an oxygen electrode coated with glucose oxidase (GOx). This original glucose enzyme electrode gave a start to the rapidly expanding field of biosensors [15].

2.1.5.3.1 First generation amperometric biosensors

The first generation of biosensors is based on the detection of electroactive products of enzymatic reaction. Generally, there are two approaches [16]:

- measurement of oxygen consumption during cathode reduction at the potential of -0,7 V
- measurement of hydrogen peroxide anode oxidation at the potential of +0,65 V

Glucose is usually measured using GOx based biosensor. During biocatalytic reaction β-D-glucose is oxidized to β-D-gluconolacton, while reducing the flavin group in the

enzyme. Molecular oxygen functions as a cosubstrate in regeneration of the enzyme GOx(FAD). This type of biosensors has several drawbacks. One of them is oxygen dependence. Poor solubility of oxygen in aqueous solutions may lead to O_2 deficit. That is what makes it the limiting reagent, which leads to reduction in linear range and changes in sensor response. To solve this problem oxygen-enriched carbon paste, which is based on an oxygen-rich fluorocarbon (Kel-F oil) pasting liquid was designed. It can provide an internal flux of oxygen to run the enzymatic reaction [17].

Another solution to eliminate the signal dependence on the dissolved oxygen concentrations is to utilize glucose dehydrogenase (GDH) instead of GOx. Measurements based on generation of hydrogen peroxide are simpler but they require establishing a relatively high voltage, which is sufficient to oxidise H_2O_2 at the electrode surface. Under these conditions endogenous electroactive species such as ascorbic acid, uric acid, acetaminophen may interfere the measurement of glucose and therefore compromise the accuracy of the biosensor. To avoid this shortcoming, permselective membranes (e.g. Nafion) have been included to reduce the access of some interfering substances towards the surface of the electrodes. The signal from these substances can be also minimized by reducing the applied potential to the range of 0,0 to -0,2 V, although this may compromise the sensitivity of biosensor [15, 18, 19].

2.1.5.3.2 Second generation amperometric biosensors

The 2nd generation amperometric biosensors incorporates an artificial mediator, which replaces the oxygen in electron transfer process [15]. GOx is not capable of direct electron transfer because its redox centre is buried inside a molecule surrounded by a thick protein layer. Hence efficient direct electrical contact between the enzyme active site and the electrode surface cannot be established. The artificial mediator shuttles electrons from flavin adenine dinucleotide (FAD) redox centre to the electrode surface. This makes the measurement independent of oxygen partial pressure. An ideal mediator should possess these characteristics [15]:

- rapid electron transfer

- high electrochemical activity
- low solubility in aqueous medium
- chemical stability
- non-toxicity

An example of widely know mediators are ferricyanide and ferrocene derivatives commonly used in commercially available glucose meters. However, nonphysiological mediators are not suitable for in-vivo implantable devices due to possibility of leaching and toxicity. There are several possible ways how to overcome this problem [15]. First, wiring the enzyme to the electrode with a flexible redox polymer and consequently stabilizing the mediator to the surface of electrode. For example poly(vinylimidazole) or poly(vinylpyridine) covalently linked with osmium-complex electron relays were studied [20]. Furthermore, nanomaterial electrical connectors, such as gold nanoparticles and carbon nanotubes, can be used to wire the enzyme [15]. The carbon nanotubes act as the electrical connectors between the redox center of the enzyme and the electrode, and thus facilitate electron transfer [21].

2.1.5.3.3 Third generation amperometric biosensors

Biosensors based on direct electron transfer mechanism belong to the third group of amperometric enzyme biosensors. In this case, electrons are transported directly from the substrate molecule to the electrode via enzyme active site. No further electron carriers or mediators are necessary for this process, thus possible contamination of sample is avoided. However only few enzymes have been reported of being capable to DET at conventional electrodes [15, 16]. Recently, various strategies for constructing glucose biosensors have been developed. For example nanostructured metal-oxide based glucose sensors provide direct electron communication between the enzymes and conducting electrode. The sensors incorporate the enzyme with metal nanoparticles, e.g. ZnO, CeO_2, MnO_2, etc. The other approach focuses on development of non-enzymatic glucose sensors [22].

2.2 Approaches for immobilization of enzyme

The use of enzymes for technical purposes is limited, because most enzymes suffer from insufficient stability, the costs of isolation and purification are rather high, and recovery of active enzyme from the reaction mixture is technically difficult. Since the use of enzymes in solution turned out to be non-productive and economically not feasible, as an alternative, the enzymes have been attached to surfaces, i.e. immobilized. In the process of immobilization, the enzyme molecules are physically confined in a defined space, while their catalytic activity is preserved. Fixed enzymes can be used repeatedly and operated continuously. Their activity and stability may be favourably altered. Enzyme is usually incorporated into a carrier matrix and immobilized according to the chosen procedure. An ideal matrix is inert, mechanically stable, biocompatible, regenerable and inexpensive. The carrier support can be either inorganic or organic and it should be selected considering physical characteristics of the material, which have an influence on performance of the enzyme system [23, 24]. Parameters such as surface area, particle size, pore structure should be taken into account because they affect the capacity of binding of enzymes [23].

The immobilization methods can be generally divided into two categories: reversible and irreversible methods [23, 25].

2.2.1 Reversible immobilization methods

When the enzyme is reversibly bound to the support, it can be detached under mild conditions. Consequently, the carrier can be regenerated and reloaded with fresh enzyme. This approach is interesting mostly from economic point of view. There are few methods of reversible enzyme immobilization: adsorption, ionic and affinity binding, e.g. enzyme attached to antibody, chelation or metal binding, and immobilization due to formation of disulfide bonds (Figure 3) [23].

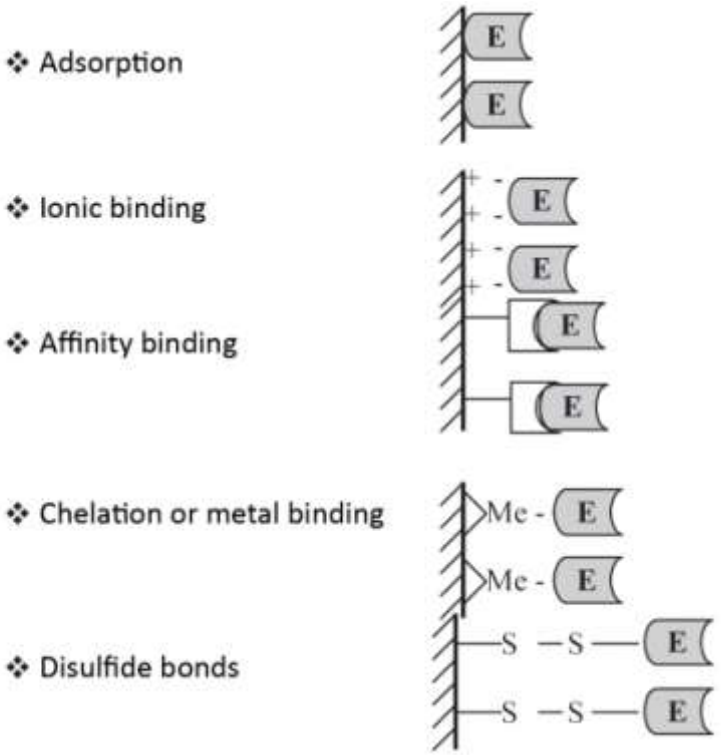


Figure 3: Reversible methods of immobilization. Figure was adapted from [23].

Adsorption is a simple method, which can be generally achieved by prolonged contact of the carrier matrix with the enzyme. It is based on noncovalent interactions, and include hydrogen bonding, van der Waals forces, hydrophobic interactions or electrostatic attraction [3, 23]. This rather weak binding usually does not change the native structure of the enzyme and enable the retention of enzyme activity [26]. On the other hand, the weak nature of these bonds may result in easy desorption of the enzyme caused by changes in pH, temperature or ionic strength [3, 23].

Another approach of reversible immobilization is based on the binding to ion exchangers. The matrix material is bearing charged groups that can capture molecules with the opposite charge. The strength of electrostatic interactions depends on ion concentration and ion charges. For example the use of cationic polymer polyethyleneimine to bind enzymes and whole cells has been reported [3, 23, 27].

2.2.2 Irreversible methods

Irreversibly immobilized enzyme cannot be detached without loss of enzyme activity or destroying the support. The most common techniques for irreversible immobilization are: covalent coupling, microencapsulation, entrapment and cross-linking (Figure 4) [23].

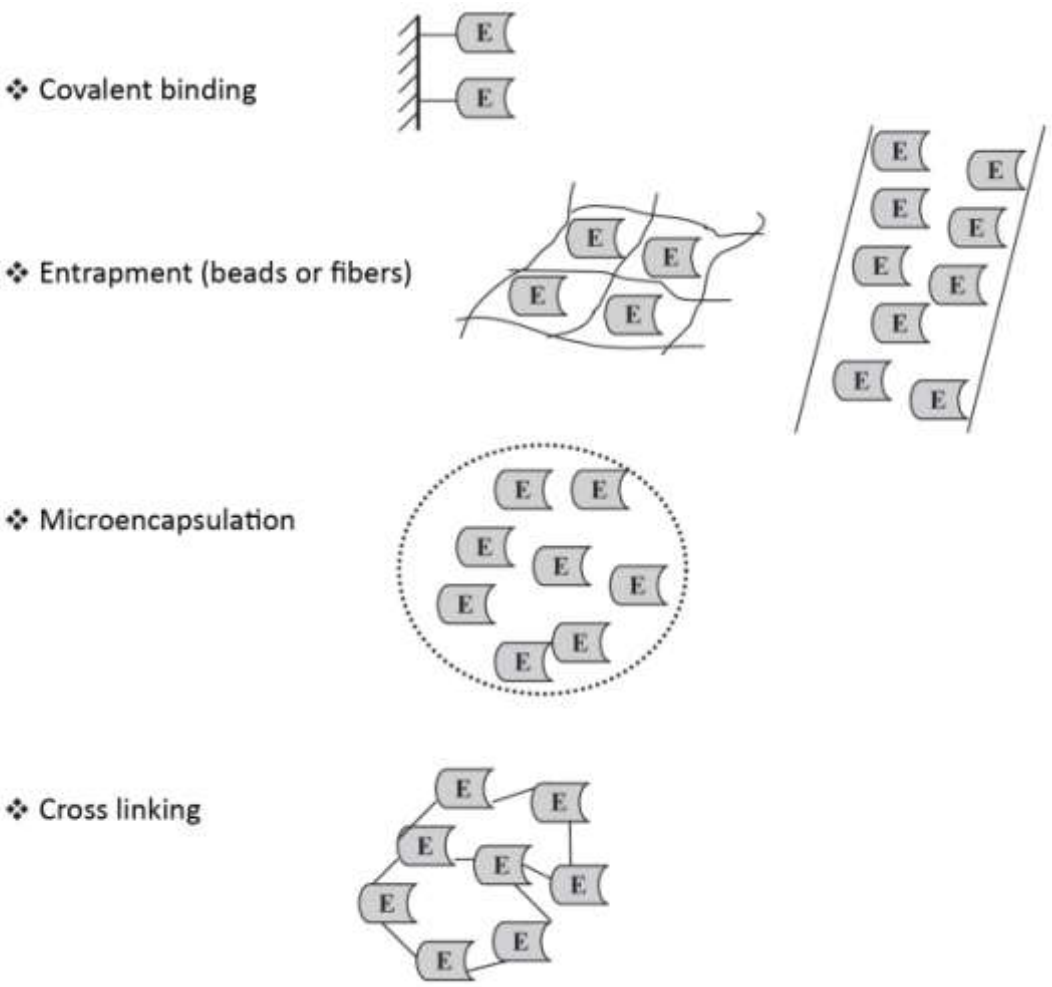


Figure 4: Irreversible methods of immobilization. Figure was adapted from [23].

- Covalent binding and cross-linking

Extensively used approach is the attachment of the enzyme to the insoluble support by the formation of covalent bonds. This type of binding is very strong; hence the enzyme is not easily released into the solution upon use. The various functional groups in the

side chains of amino acids usually take place in the formation of chemical bonds. The most frequently involved functional moieties are amino, thiol and carboxylic groups. The covalent immobilization may be accomplished either by activating the matrix with a reactive function (e.g. tresyl chloride, cyanogens bromide) or by addition of the bifunctional reagent to link together the polymer matrix and the enzyme [23, 24, 28]. The basic principle of matrix activation process is the generation of electrophilic groups on the support which react with the nucleophiles on the enzymes. In order to obtain high degree of bound activity, the amino acid residues inevitable for catalytic activity must not be involved in the covalent coupling to the support [23]. To overcome the loss in the enzymatic activity the coupling reaction may be carried out in the presence of an enzyme substrate or a competitive inhibitor [24].

Cross-linking is a type of immobilization using a bifunctional reagent such as glutaraldehyde. In this case the enzyme is not fixed to a carrier, but it acts as its own carrier. [29]. Instead the covalent bonds are formed between the enzyme and the reagent by creating a three-dimensional network [30]. The major disadvantage of this method is the possibility of the activity loss due to chemical alterations of the enzyme active site during cross-linking [31].

- Entrapment

The entrapment method is based on the occlusion of enzymes within the interstitial spaces of fibre or gel matrix or enclosing them into semipermeable membranes. The polymeric matrix allows the diffusion of substrates and products but holds the enzyme. The most popular matrices for entrapping enzymes are polyacrylamide, silicone rubber and starch gel, bovine serum albumin, gelatine and chitosan. However, there are few difficulties linked with this method like leakage of the enzyme due to heterogeneous pore size distribution, inactivation of the enzyme by the free radicals used in the polymerization process, and lowered substrate accessibility [24, 25].

- Microencapsulation

Encapsulation or membrane confinement is another effective entrapping technique. Enzyme is confined within semipermeable membrane that permits the passage of

small molecules. Thus substrate molecules can diffuse through the membrane, where are transformed to product by the entrapped biocatalyst. The products then diffuse back across the membrane to the exterior phase. In contrast, the enzyme, being larger than the membrane pore diameter, is retained in the membrane [24, 32].

2.3 Cellobiose dehydrogenase

Cellobiose dehydrogenase (EC 1.1.99.18) is an extracellular enzyme produced by several white- and brown- rot, phytopathogenic or saprotrophic fungi. It is a part of lignocellulolytic enzymes, which play an important role in wood degradation. The production of this enzyme is induced by cellulose or cellobiose present in growth medium [33, 34].

The unique properties make the CDH an interesting enzyme for bioanalytical applications. Its ability of direct electron transfer from the enzyme to the electrode is particularly attractive for design of amperometric biosensors. It was shown, that CDH can be successfully applied for the detection of carbohydrates, such as cellobiose, lactose, glucose, as well as quinones and catecholamines and also in biofuel cell anodes [33, 35, 36].

2.3.1 Classification of CDH

A number of different kinds of CDHs, which varies in their fungal producer,molecular architecture and catalytic properties, are known and studied. According to its origin enzymes are divided into three groups. Class I is formed by basidiomycete CDHs, which has a shorter sequence of amino acids. It reveals high preference towards disaccharides as substrates (e.g. cellodextrines and lactose) and discriminates monosaccharides (e.g. glucose). The efficiency of internal electron transfer (IET), thus the catalytic reaction, depends on pH conditions, for basidiomycete CDHs optimal pH is acidic (around 4 to 4,5). Class II and III comprise ascomycete CDHs. Besides cellodextrines and lactose they can also oxidise mono- and disaccharides and in

contrast to class I the IET is not limited to acidic pH, the optimum is in the neutral or alkaline pH region [33-35].

It has been reported that CDH from ascomycete Corynascus thermophilus (CtCDH) can oxidise glucose with high turnover rate at physiological pH. Thus CDH shows a potential to be used in design of novel glucose sensing devices with improved working properties [33].

2.3.2 Structure of cellobiose dehydrogenase

CDH is an extracellular flavocytochrome which belongs to the oxidoreductase family. It consists of two different domains connected by a flexible polypeptide linker. The larger flavodehydrogenase domain (DH_{CDH}) comprises a flavin adenine dinucleotide cofactor, and it possesses catalytic activity. The smaller cytochrome domain (CYT_{CDH}) has a haem b as a cofactor and it functions as a built-in mediator, which shuttles electrons between DH_{CDH} and a terminal electron acceptor. Internal electron transfer between DH and CYT becomes possible due to close proximity of these domains. Because of the existence of internal electron transfer pathway, CDH is structurally classified as extrinsic redox enzymes, that is capable of catalyzing an enzymatic reaction by means of direct electrode transfer [33-35]. This feature of CDH makes the enzyme highly suitable to be utilised for design and construction of novel third-generation biosensors.

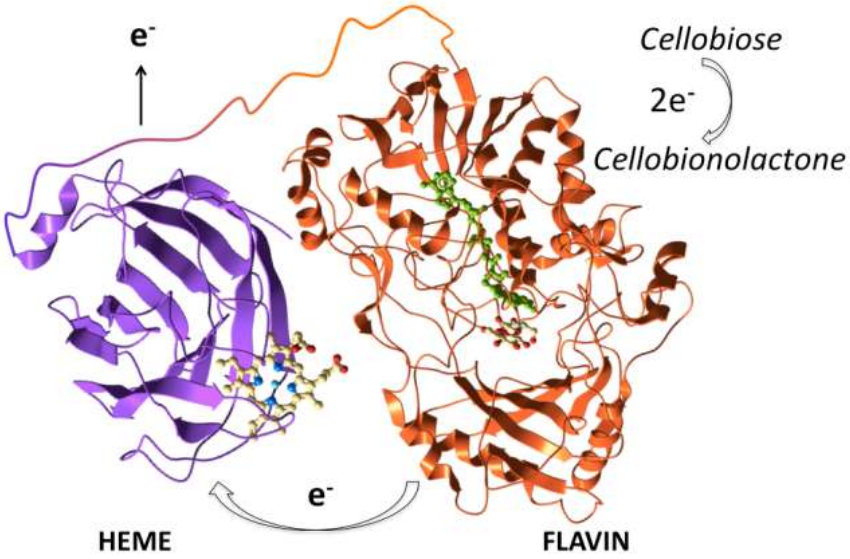


Figure 5: Schematic representation of a CDH comprising a C-terminal flavin domain with its FAD highlighted in green and an N-terminal heme domain. Oligosaccharide oxidation takes place at the flavin domain followed by electron transfer to the ferric heme group. Figure was adapted from [37]

2.3.3 Mechanism of electron transfer in cellobiose dehydrogenase

The process of CDH catalysis is very complex and still not completely understood. In general, the proposed mechanism involves electron exchange from a substrate to electrode surface via DH_{CDH} and subsequently CYT_{CDH}.

During the catalysis (CAT), the sugar substrate is oxidised into a corresponding lactone and two electrons from the substrate are delivered to the oxidised FAD cofactor (F_o) in the active site of the DH domain leading to FAD reduction (F_r). The process is described in the following reaction:

$$CDH\ (F_oH_o) + \ cellobiose \ \xrightarrow{CAT} \ CDH\ (F_rH_o) + \ cellobionolactone \qquad (1)$$

In the next step the fully reduced flavin is reoxidised and the electrons are passed in two single-electron-transfer reactions to haem cofactor. When transferring the first electron, haem is reduced and a semiquinone radical is formed.

$$CDH\ (F_rH_o) \ \xrightarrow{IET\ 1} \ CDH\ (F_r^{\cdot}H_r) + \ H^+ \qquad (2)$$

Then the reduced haem is reoxidased via a DET at the electrode in order to enable accepting of the second electrone.

$$CDH\ (F_r^{\cdot}H_r) \xrightarrow{DET\ 1} CDH\ (F_r^{\cdot}H_o) + e^-\ \text{(electrode)} \quad (3)$$

The reoxidation of haem is followed by the second IET reaction where the electron is transported from semiquinone radical to oxidised haem resulting in formation of reoxidised FAD.

$$CDH\ (F_r^{\cdot}H_o) \xrightarrow{IET\ 2} CDH\ (F_oH_r) + H^+ \quad (4)$$

Finally, the second electron is delivered from haem to the electrode surface and the redox cycle is completed.

$$CDH\ (F_oH_r) \xrightarrow{DET\ 2} CDH\ (F_oH_o) + e^-\ \text{(electrode)} \quad (5)$$

[18, 19]

2.4 Indium tin oxide glass as a platform for biosensors construction

Thanks to its favourable electrical and optical properties, indium tin oxide (ITO) has received a growing attention in the field of development of biocompatible materials and biosensing technology. ITO, also known as tin-doped indium oxide, is a solid mixture of In_2O_3 and SnO_2. It is transparent and colourless in form of a thin film while bulk material is yellowish to grey [38, 39].

ITO is the most extensively used transparent conducting oxide (TCO). TCOs are metal oxides with a wide band gap characterized by the unique combination of high electrical conductivity and optical transparency. In contrast, metals are typically great electrical conductors, but they do not transmit visible light. On the other hand, transparent materials like glass lack the electrical conductivity. TCOs are usually compound semiconductors. The opto-electrical properties of TCO can be altered by doping with a suitable dopant [39, 40].

2.4.1 Electrical properties of ITO

Electrical properties of semiconductors are related to their electronic band structure. In semiconductors there are bands of allowed energy levels, which are separated by a well-defined band gap. Valence band is located below the gap and the band above is known as the conduction band. At absolute zero temperature the valence band is entirely filled and the conduction band is empty. But completely filled or empty bands do not contribute to the electrical conductivity of the material. Only if electron gains energy equal to band gap energy it gets excited from the valence band to the conduction band. As a result electron vacancies or holes are created in the valence band [41, 42]. These holes act as positive charge carries and electrons freely moving in the conduction band are negative charge carries [43]. Conductivity of semiconductors results from the movements of electrons and holes inside the crystalline lattice [44].

The electrical properties of TCO can be modified by doping. It is the process of adding impurities to a semiconductor, which change the concentration of charge carriers. Dopants are classified as donors (n-type) and acceptors (p-type). In the n-type semiconductors there is an excess of electrons whereas p-type contains mainly free holes [40, 41, 44].

In ITO tin substitutes the indium in the In_2O_3 lattice and provides an electron to the conduction band. Thus ITO is classified as n-type semiconductor [45].

2.4.2 Application of ITO for constructing biosensors

ITO has recently found a wide range of applications. It has been used as gas sensor, transparent current spreading layers in surface light emitting diodes, in liquid crystal displays, heat-reflective mirrors, in solar cells and other opto-electrical devices. Moreover, ITO thin films have been applied in biosensors as the working electrodes for adhesion of biological substances [46, 47].

In contrast to conventional flat electrodes, TCO electrodes with a defined porous morphology offer large surface area enabling high loading of active molecules (e.g.

enzymes). Besides that, large conducting interface provides direct communication with the incorporated species. These beneficial properties of porous TCO electrodes make them attractive platforms for incorporation of biomolecules [48-50].

In recent years nanostructured electrode layers with different surface architectures have been developed. According to the IUPAC porous materials are classified by the pore size into three groups [51]:

- microporous (pore diameter less than 2nm)
- mesoporous (pore diameter between 2 and 50 nm)
- macroporous (pores greater than 50 nm)

The optimized pore size of the porous electrode film is important for incorporation of functional guests. Despite on a high surface area of microporous structure, its application in the field of biosensing is limited due to difficulty in incorporating of molecules of large size (enzymes, protein complexes). Therefore architecture with larger pores is more advantageous for this purpose. Macroporous films provide unique morphological characteristics, such as an open porous architecture allowing efficient mass transfer, an interconnected framework advantageous for charge transport, and pore sizes suitable for incorporation of bulky guests or functional layers [40, 49].

2.4.3 Synthesis of macroporous ITO thin film electrode

Latex beads are used as templates to obtain desired porosity in fabrication process of macroporous TCO layers. The bead arrays are either infiltrated or assembled directly with a suitable TCO precursor. The method of direct co-assembly appears to be simpler and less time consuming with a more efficiently controlled ratio of precursor to template [49].

The particle-based procedures for the construction of TCO films with a proper crystallinity are more advantageous than classical sol-gel route. The sol-gel techniques produces amorphous material. It requires a heat treatment to obtain adequate crystallinity, which is often difficult to control. In contrast, the usage of pre-formed

crystalline nanoparticles as building blocks can overcome this shortcoming. However, the successful assembly of a regular structure requires full dispersibility of nanoparticles in polar solvents compatible with the beads [49].

Liu and colleagues reported the fabrication of ITO electrodes with controlled morphology by direct co-assembly of ultra-small indium tin hydroxide (nano-ITOH) and poly(methyl methacrylate) (PMMA) latex beads. In a procedure, mixed dispersions of PMMA template and nano-ITOH are coated onto glass or planar ITO. Optimized PMMA/nano-ITOH ratio is important to achieve uniform porosity. Too high concentration of ITOH creates non-porous domains, while abundance of PMMA results in fragile scaffold. To improve mechanical stability and surface coverage hydroxypropyl cellulose(HPC) is added to the coating solution. The addition of HPC also increase smoothness and adhesion of the film to the substrate. After deposition, the layers are annealed in air at 400 °C. During this step, polymer template is removed and nano-ITOH particles are transformed to the cubic ITO phase [49].

The ITO films prepared by this way can be used as transparent electrode layers for spectroelectrochemistry. They feature respectable electrical conductivity of 4,0 ±0,3 Scm^{-1} and open interconnected pores with a uniform size of 300 nm [49].

3 Experimental part

3.1 Chemicals and materials

CDH (Cellobiose dehydrogenase) from Corynascus thermophilus with volumetric activity (measured with DCIP assay, pH 5.5, 30 °C) = 29 U/mL, (measured with Cyt c assay, pH 7.5, 30 °C) = 20 U/mL

Potassium hexacyanoferrate(II) trihydrate (Sigma Aldrich)

Potassium hexacyanoferrate(III) (Sigma Aldrich)

Glutaraldehyde solution 25% (Sigma Aldrich)

Poly(ethyleneimine)solution (PEI) 50% (Fluka)

Phosphate Buffer Saline (PBS) tablets (Medicago)

D-(+)-Glucose (Sigma-Aldrich)

Nafion solution 5% (Sigma-Aldrich)

L-Ascorbic Acid (Sigma-Aldrich)

Macro ITO (Flash calc. 400 °C, H2/N_2/400 °C, dip-coated 3x speed 3)

ITO coated glass slide (thickness of ITO coating 0,12-0,16 nm) (Sigma Aldrich)

All solutions and buffers were prepared using deionized water from Millipore purification system (18 MΩcm).

3.2 Electrode preparation

Electrodes were prepared by modification of macroporous ITO film with enzyme. Three different approaches were used:

1. Physical adsorption of the enzyme onto the macroporous ITO electrode. 2 µL of CtCDH solution was deposited on the surface of the ITO electrode and allowed to dry.
2. Cross-linking of CDH with bifunctional compound. The enzyme was allowed to adsorb onto the electrode surface as described above. Subsequently, 1 µL of a GA solution (1% or 0,1%) was placed on the top of the adsorbed and dried enzyme layer.
3. Adsorption of the enzyme on the macroporous ITO modified with cationic polymer PEI. 4 µL of a PEI solution (10 mg/ml) was spread over the surface of the working electrode. After the solution was dried, 2 µl of CtCDH solution was placed onto the PEI modified ITO electrode.

All modified electrodes were stored overnight at 4-8 °C under constant humidity prior to use.

3.3 Characterization methods

3.3.1 Characterization of ITO electrode by SEM and EDX

SEM measurements were performed on a Quanta 600 scanning electron microsope equipped with a field emission gun (Japan). All measurements were performed in low vacuum mode with accelerating voltage between 20 kV and 25 kV. The secondary electron images were recorded by large field detector. For obtaining back-scattered images solid state detector was used. The elemental composition of the ITO electrodes was determined by energy dispersive X-ray spectroscopy (EDX). The EDX detector was integrated with SEM and operated by INCA software (Oxford Instruments, UK).

3.3.2 Characterization of ITO electrode by XPS

X-ray photoelectron spectroscopy (XPS), also known as Electron Spectroscopy for Chemical Analysis (ESCA) is a technique used to investigate the chemical composition of surfaces. XPS spectra were acquired with a PHI 5500 instrument using a

monochromatic Al Kα X-ray source with energy of 1486,6 eV and power 350 W. The take off angle between the analyzer and the sample surface was 45°. No sample preparation procedure was required prior the measurements.

3.4 Electrochemical measurements

Electrochemical measurements were conducted using three-electrode set-up. The enzyme macroporous ITO electrode was used as a working electrode. An Ag|AgCl (0,1 M KCl) electrode was used as a reference electrode, and platinum wire was utilized as a counter electrode. All electrodes were connected to a multichannel potentiostat CHI 1030B (CH Instruments, USA).

Before using the modified ITO electrodes were rinsed with deionized water to remove loosely bound enzyme molecules. Then the electrode was inserted into a homemade flow-through amperometric cell with a rubber O-ring of 0,5 cm in inner diameter. All electrochemical measurements were held inside a home-built Faraday cage. The electrochemical cell was connected to a flow-injection system consisting of a peristaltic pump (Harvard apparatus, USA) and a six port valve manual injector Rheodyne (IDEX, Germany).

All measurements were performed at room temperature.

4 Results and discussion

4.1 Characterization of ITO electrodes by SEM and EDX

Topography and morphology of the enzyme modified and bare macroporous ITO electrodes were studied using SEM.

Figure 6 shows surface morphology of bare macroporous ITO film. The image reveals homogenous morphology with a uniform dense honeycomb like porous structure. The pores have spherical shape and are well connected to each other. The average diameter of pores is about 300 nm. The porous structure has been observed at higher magnification as shown in Figure 6c). Large pore size and open interconnected structure of macroporous ITO platform is advantageous for incorporation of bulky guest species like proteins or for deposition of functional layers.

Figure 7 shows the electrode surface coated with PEI and immobilized CDH. Figure 8 demonstrates the morphology of ITO electrode after the enzyme and GA were deposited onto the surface . It can be seen that both, PEI and GA are spread over the surface and form continues layers. When comparing the surfaces prior to and after modification, the porous architecture of ITO film are less clearly visible due to coverage layers. As the modified functionalized surface does not appear to be smooth and flat, this indicates that most likely a modifier and enzyme (molecule size ca. 18 nm) penetrates into the material's pores.

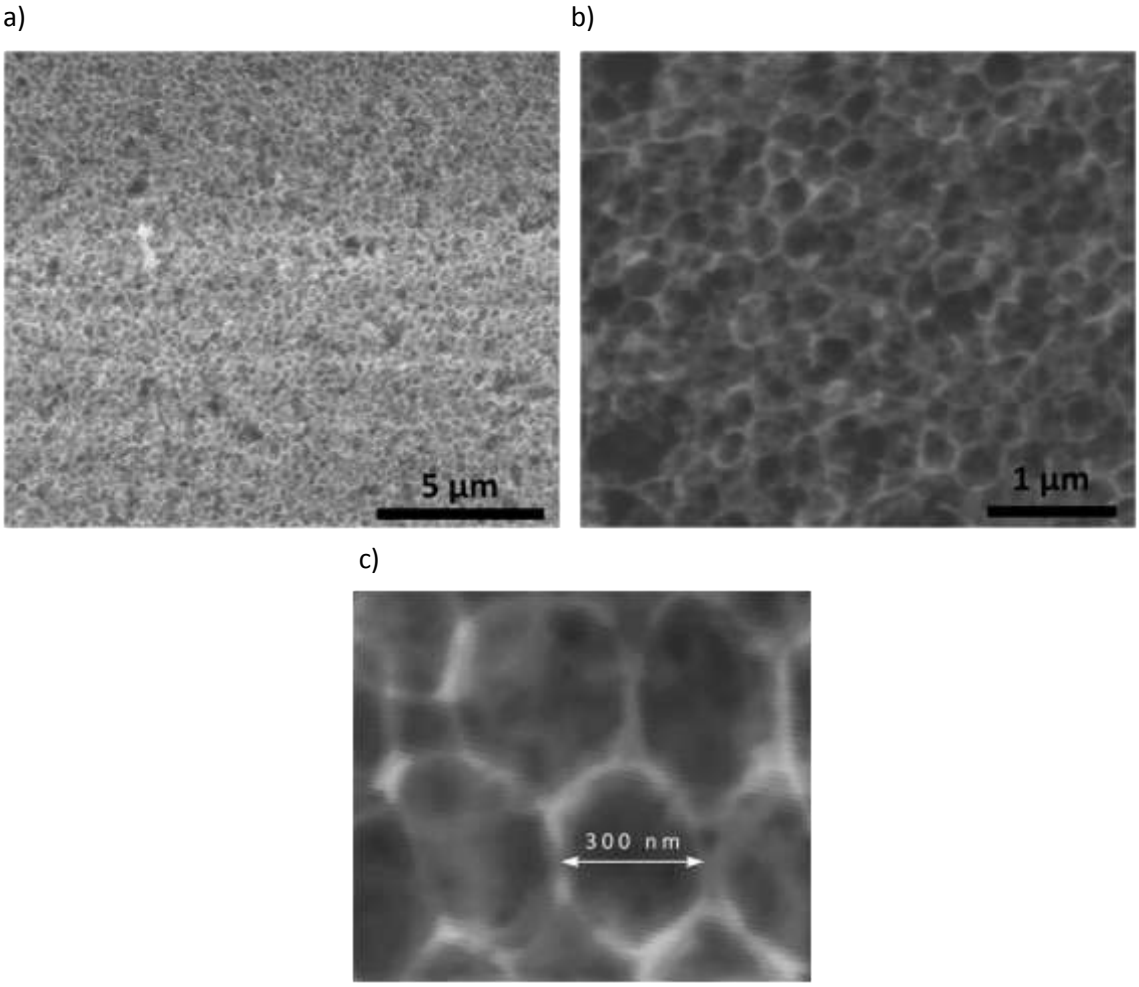


Figure 6: SEM images of bare macroporous ITO electrode at magnifcation a) 9000x b)30000x c)135000x.

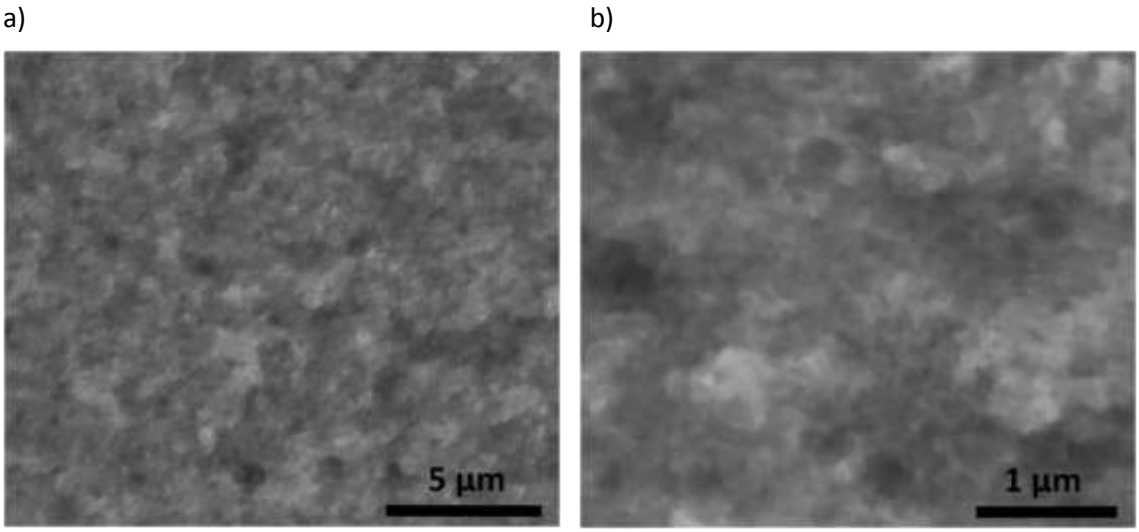


Figure 7: SEM images of PEI-CDH modified ITO electrode at magnification a) 9000x b) 30000x.

a) b)

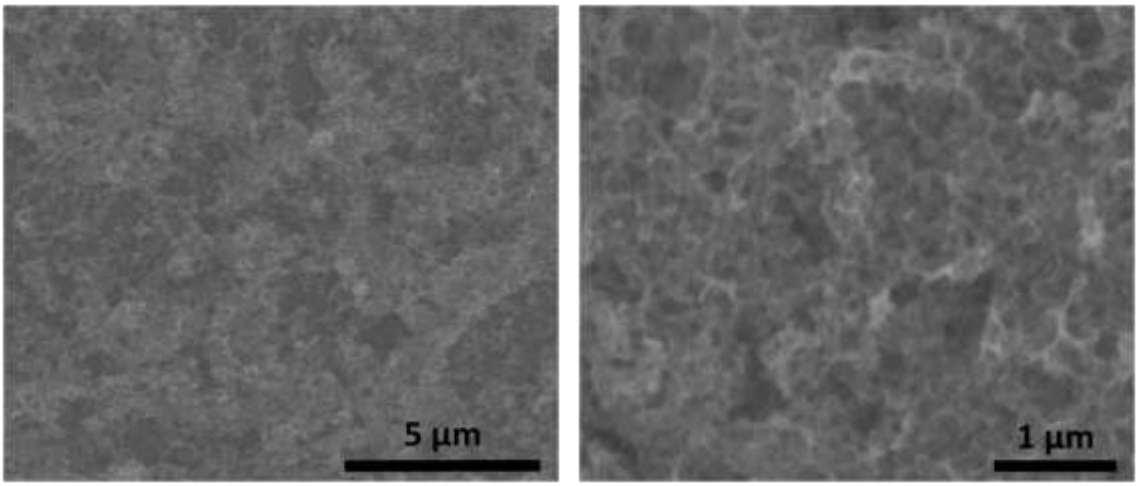


Figure 8: SEM images of CDH-GA modified ITO electrode at magnification a) 9000x b) 30000x.

The elemental composition of ITO electrodes was obtained by Energy dispersive X-ray spectroscopy. The energy of the emitted X-rays is characteristic for each element present. By measuring the energies of peaks, the corresponding elements can be identified. The peaks were identified by comparison the energy values for each element with the ones stated in the NIST database.

Figure 9 shows the EDX spectrum of the both bare and modified ITO electrodes. The observed indium peak at 3,29 keV, tin peak at 3,44 keV and oxygen peak at 0,52 keV confirm the presence of the indium-tin oxide in the ITO layer. All spectra show a significant peak for silicon at 1,74 keV representing the glass substrate of the ITO electrodes. The small sodium peak at 1,04 keV and chlorine peak at 2,62 keV that appeared in the acquired spectra possibly originated from the PBS buffer used in the electrode modification process.

A significant carbon peak at 0,28 keV was detected in all samples. It may be, most likely, the signal of carbon residues from the not fully removed cellulose during annealing or it may come from the carbon adhesive tape used to anchor the sample to the holder. Nitrogen peak at 0,39 keV may be attributed to the modification layers or to the enzyme. Calcium peak at 3,69 keV may be attributes to an impurity in composition of glass substrate.

The peaks decrease from bare ITO to PEI-CDH and GA-CDH, which is additional indication of the modifying layer present in the electrode surface. Furthermore, by comparing the decrease of the peaks from Si, In, Sn and Ca, it is most likely that GA forms denser thick layer, which penetrates deeper into the interior of the macroporous structure.

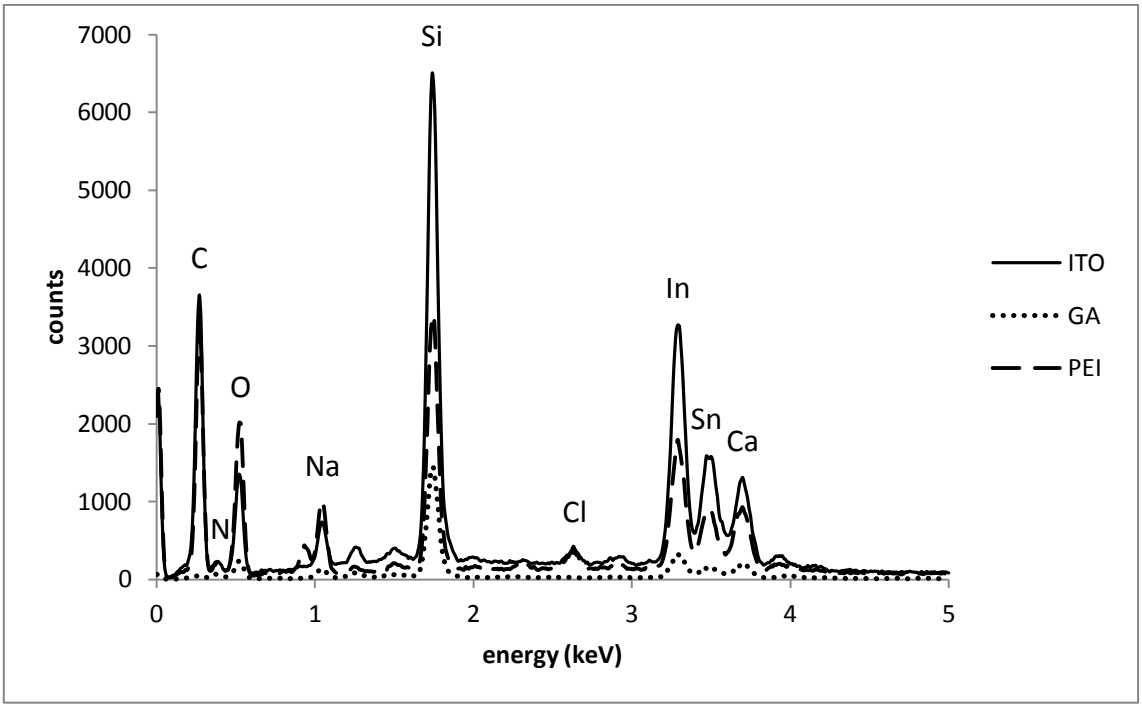


Figure 9: EDX spectra of bare ITO, PEI-CDH modified ITO, CDH-GA modified ITO.

4.2 Characterization of ITO electrode by XPS

Elemental composition of macroporous ITO electrodes before and after surface modification with CtCDH and PEI or GA was studied using XPS. The chemical states of the elements were determined by investigating the binding energy positions and the shapes of the XPS peaks and by comparison the energy values with the ones stated in the NIST database. The elements investigated were indium, tin, oxygen, carbon and nitrogen. In order to prove the presence of enzyme, emphasis was put on nitrogen content, as enzyme is protein and contains amine groups and peptide bonds. The chemical composition of differently modified ITO electrodes is summarised in Table 2.

Table 2: Comparison of chemical composition of differently modified macroporous ITO electrodes.

	Atomic concentration (at.%)				
	Major elements			Middle layer	
	C	**N**	**O**	In	Sn
ITO	52.2	0.0	32.9	1.4	0.4
ITO+PEI+CDH	62.4	12.5	20.1	1.9	0.2
ITO+PEI	42.0	1.9	34.7	11.6	1.1
ITO+CDH+GA	67.9	9.2	19.0	0.6	0.0

The XPS survey spectrum obtained for bare macroporous ITO electrode exhibited a peak formation at 445,0 eV (In3d5) and at 452,6 eV (In3d3) corresponding to indium and at 486,8 eV (Sn3d5) and at 495,2 eV (Sn3d3) corresponding to tin (Figure 10). ITO had a composition of $(In_{10}Sn_1)O$. Detailed scan in the O1s region showed that there are 4 possible chemical states. Two of them belongs to ITO, i.e. indium oxide with more ionic character at 530.4 eV (2.0 %) and tin oxide with more covalent character at 531.7 eV (0.5 %). The other two are silicon dioxide at 532.6 eV (64.5 %) and the organic oxygen groups (531.4 eV, 32.5 %) [52, 53]. Carbon detected on the surface of bare ITO is attributed to cellulose used during ITO preparation process, when cellulose was annealed at 400 °C. The binding energy at 285,2 eV is associated with organic carbon bond (C-C).

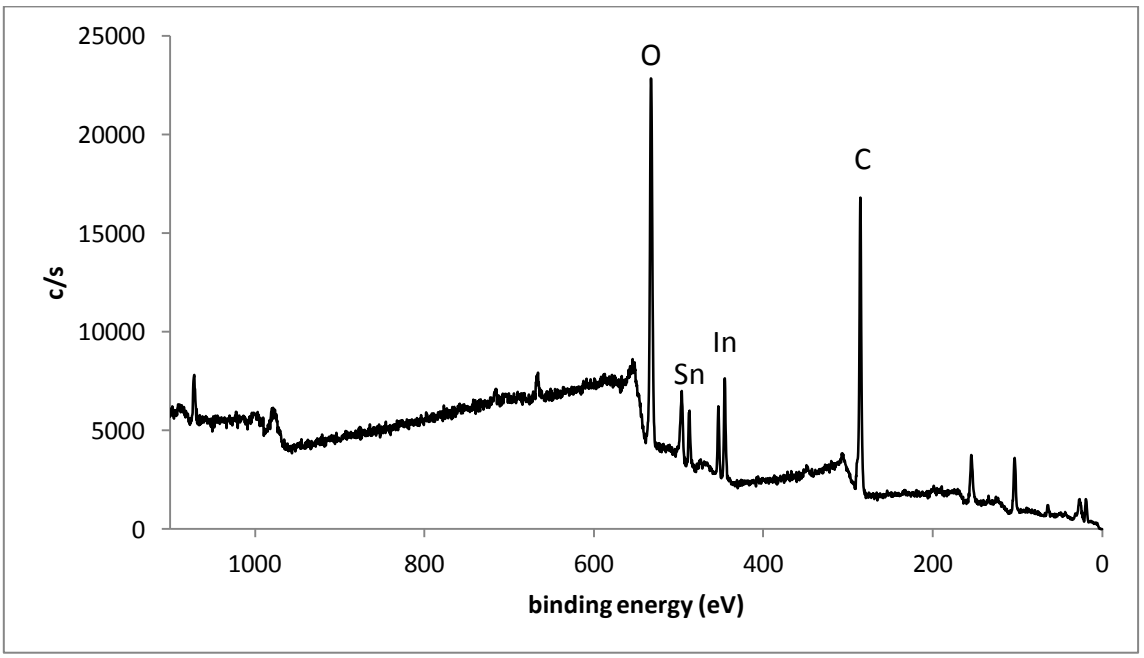


Figure 10: XPS spectrum of bare ITO electrode.

Compared to bare ITO, CDH-PEI modified surface showed a nitrogen peak in XPS spectrum, which may be attributed to PEI as well as to CDH (Figure 11). To distinguish an origin of nitrogen, the XPS spectrum of ITO electrode without CDH was examined (Figure 12). The spectrum obtained from electrode without enzyme had a nitrogen peak at 400,3 eV, which corresponds to binding energy for organic compounds, i.e. PEI (Figure 14). The nitrogen peak observed for PEI was very small in comparison with CDH-PEI, which indicates high amount of enzyme loaded onto the porous ITO surface.

The main carbon peak at 285,5 eV represented C-C bond (Figure 15). These results proved the presence of the polyethyleneimine layer. The C 1s spectrum of CDH-PEI modified electrode consisted of the signals characteristic for amines, amides, C-O and O-C=O bonds (Figure 15). High-resolution N 1s spectrum exhibited two distinct peaks at 400,6 eV and 402,3 eV corresponding to secondary amine (-NH-) or secondary amide (-CO-NH-) and protonated amino groups (NH_3^+) (Figure 14). The NH_3^+ peak may serve as an additional evident of the electrostatic interactions between the oppositely charged polymer surface groups and the proteins molecules [54, 55].

The difference in chemical states of elements confirmed the presence of enzyme on CDH-PEI modified electrode. Additionally, the increased amount of nitrogen indicating the protein molecules adsorbed (Table 2).

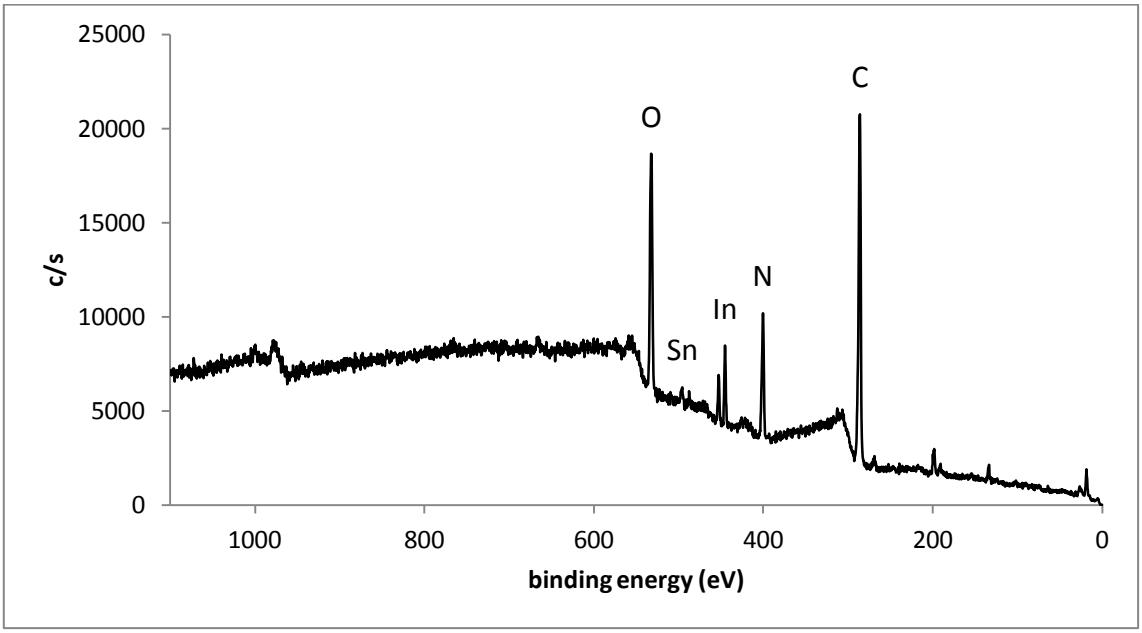


Figure 11: XPS spectrum of PEI-CDH modified electrode.

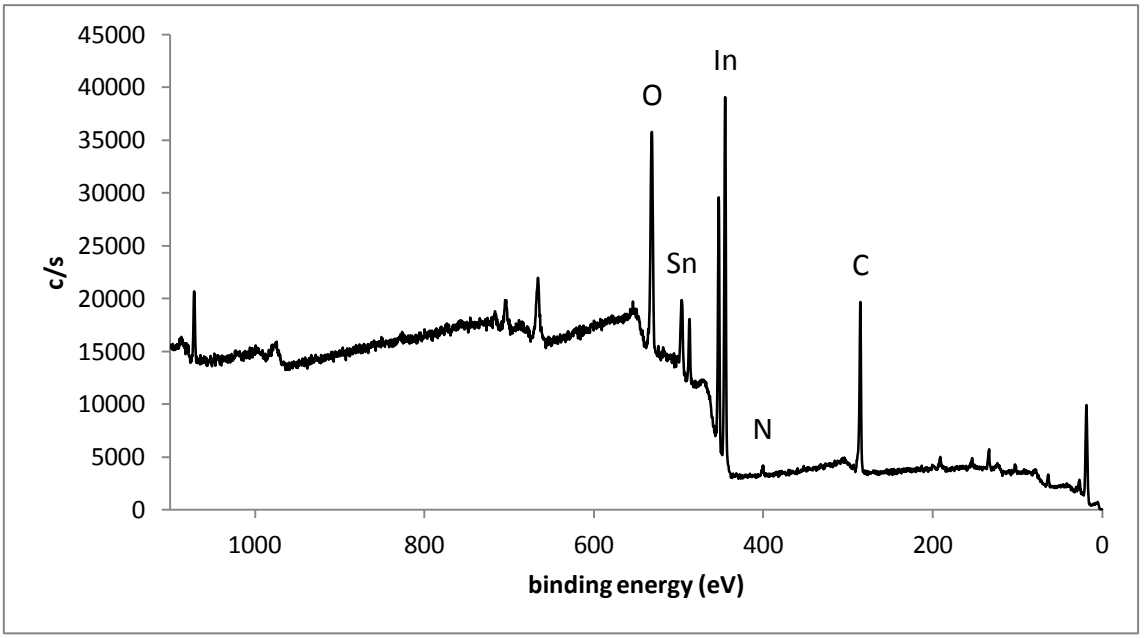


Figure 12: XPS spectrum of PEI modified electrode.

The XPS spectrum of CDH-GA modified electrode exhibited characteristic N 1s peak in the range of 400 eV, which demonstrate the enzyme presence (Figure 12). It exhibited two components having binding energies of 399.3 eV and 400,1 eV, assigned to $–NH_2$ and $–CONH_2$, respectively (Figure 13). Carbon present in the CDH-GA modified electrode originates in CDH and GA. Detailed scan in the C 1s region showed 4 carbon peaks (Figure 14). The peaks at 284,2 eV and 285,2 eV represent C=C and C-C bond, respectively. The peak at 287,6 eV may be attributed to C=O group of GA and the peak at 286,2 eV may be assigned to amide bonds bond resulting from enzyme interactions with glutaraldehyde. The decrease of indium and tin contents was observed, probably due to the presence of denser layer of protein cross-linked with GA, which covers and partially screens the ITO surface [56, 57]. These findings is in a good agreement with the above described data obtained by EDX for the same modified surfaces.

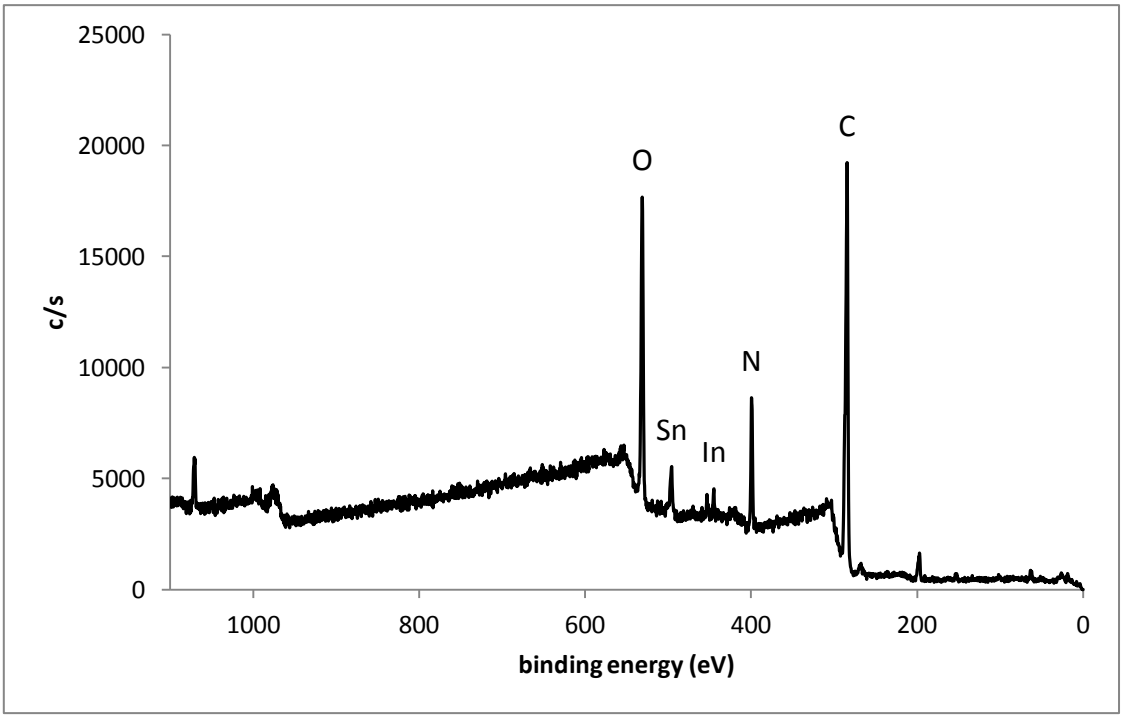


Figure 13: XPS spectrum of CDH-GA modified electrode.

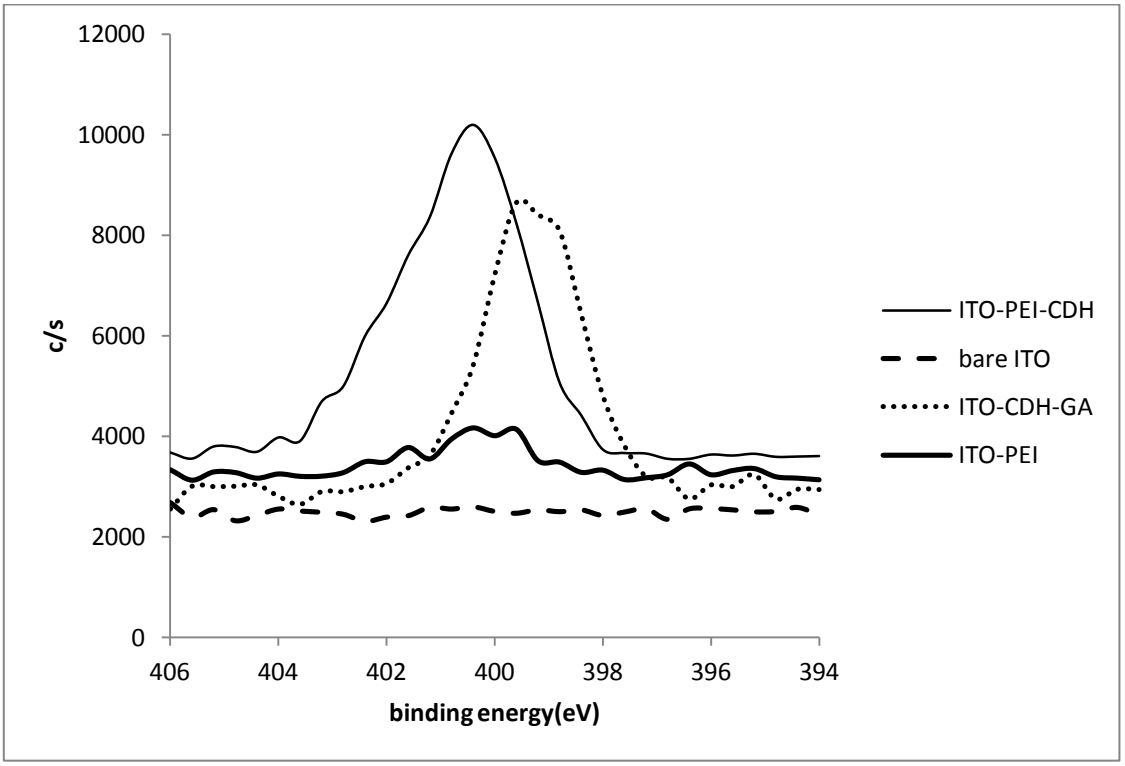


Figure 14: Comparison of N 1s spectra for electrode samples modified with different approaches.

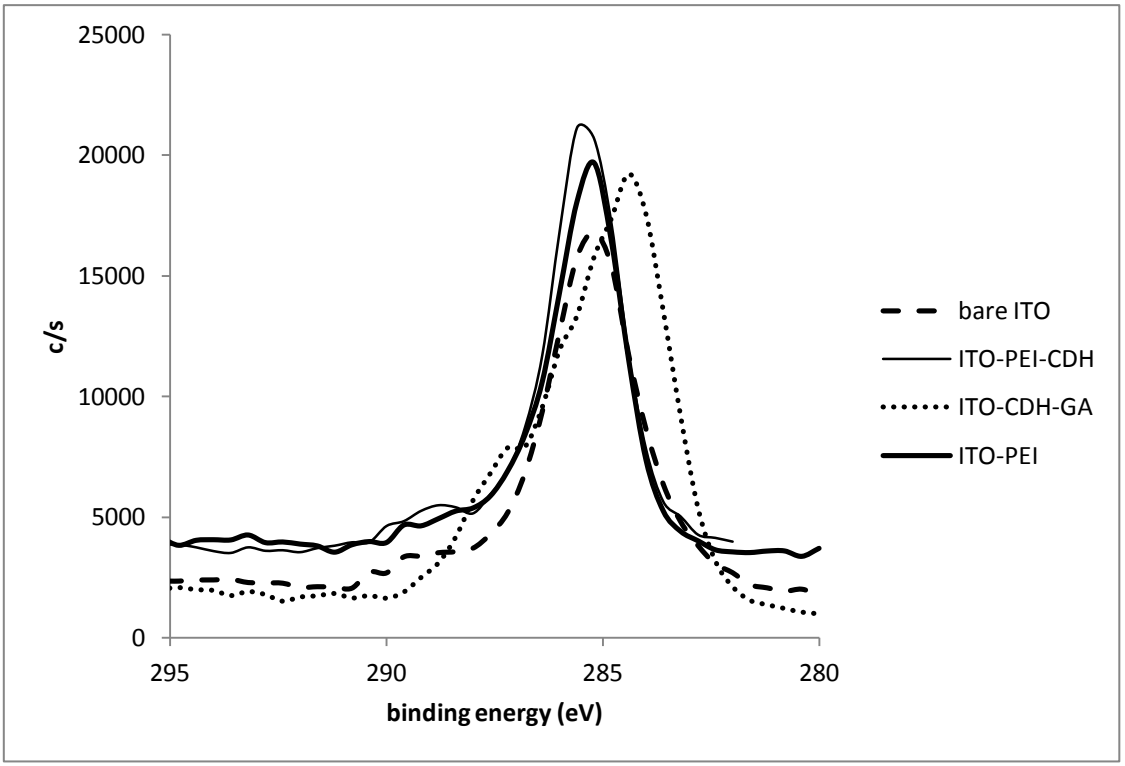


Figure 15: Comparison of C 1s spectra for electrodes modified with different approaches.

Additionally, to investigate CDH-GA covering, depth profile experiment was performed. Depth profiling uses an ion beam to etch thin layers of the material from the surface of the sample. After every etch cycle, a new spectrum is recorded. It was found, that the nitrogen peak decreases after a new etch cycle. The presence of nitrogen in the depth of 50 nm, indicates either formation of relatively thick layer on the electrode surface, or shows penetration of the biomolecule into the pores interior (Figure 16). As the SEM images could still reveal the porous structure of the GA-CDH modified ITO surface, it is most likely that the enzyme penetrates in the macroporous interior of the glass. In contrast, nitrogen signal in PEI-CDH spectrum diminished after sputtering the first layer. This clearly indicates that PEI-CDH form larger aggregates, which accumulate mainly on the surface of the macroporous ITO. This conclusion is also supported by the observations from the SEM images.

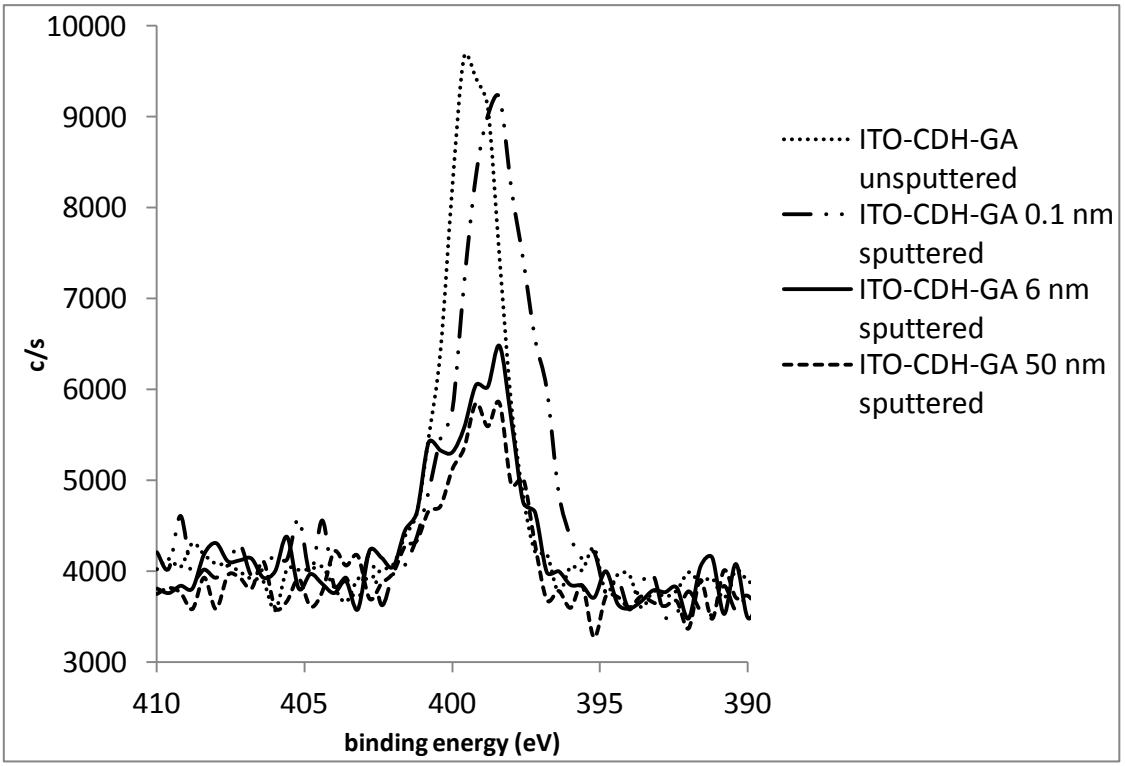


Figure 16: XPS depth profile of ITO-CDH-GA modified electrode in N 1s region.

4.3 Electrochemical measurements

4.3.1 Cyclic voltammetry of flat and porous ITO electrode

The electrochemical behaviour of flat and macroporous ITO electrode was compared by cyclic voltammetry using 1 mM equimolar solution of potassium ferricyanide/ferrocyanide $K_3Fe(CN)_6/K_4Fe(CN)_6$. Resulting voltammograms showed cathodic and anodic peaks for reversible reaction. For nanostructured electrode, a pair of well-defined peaks are observed. The anodic peak potential for oxidation is 0,275 V and cathodic peak potential for reduction is 0,020 V. The redox peak currents are nearly identical in height and are measured as 0,3797 µA and -0,3398 µA, respectively. In comparison, the voltammogram of flat ITO electrode showed only a pair of much less distinct redox peaks (Figure 17).

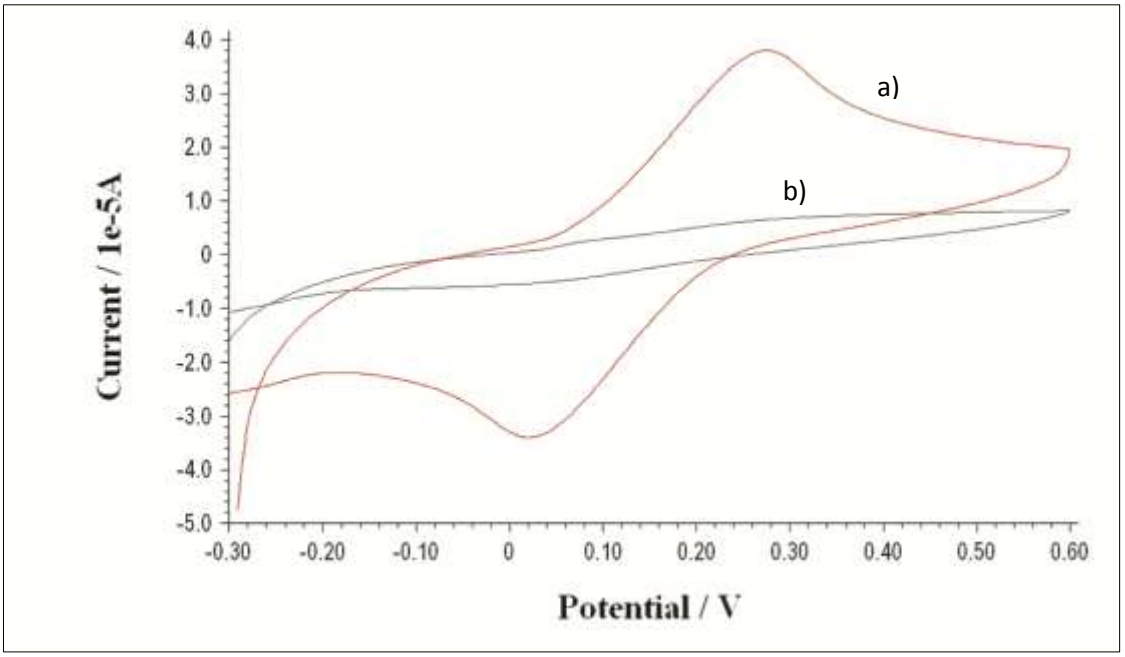


Figure 17: Cyclic voltammograms of a) macroporous ITO and b) flat ITO electrodes in 1mM equimolar solution of $K_3Fe(CN)_6/K_4Fe(CN)_6$. Scan rate 0,1 V/s.

A significant increase in current was observed for macroporous ITO eletrode in comparison to the flat ITO electrode as a result of high interface area. The current response of porous electrode is about 4,5 times higher than that on the flat one.

The active surface area of both electrodes was estimated by using Randles-Sevcik equation [58]:

$$I_p = (2{,}69 \times 10^5) n^{3/2} A D^{1/2} v^{1/2} C_0 \qquad (6)$$

where I_p is the peak current, A is the electroactive area (cm^2), D is diffusion coefficient ($cm^2 s^{-1}$), C_0 is bulk concentration of the redox probe (mol cm^{-3}), v is the scan rate (V/s), and n is the number of electrons transferred in the redox event. The calculated area for the porous ITO electrode was more than 5 times higher that the flat one and was calculated as 0,162 cm^2 and 0,03 cm^2 for macroporous and flat ITO electrodes, respectively.

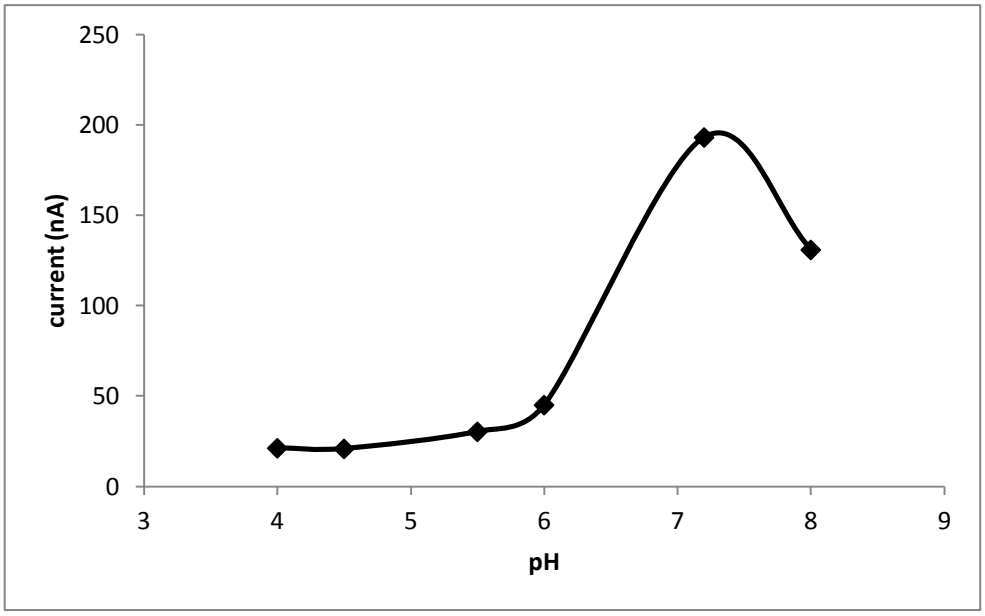
It can be seen that three-dimensional structure of the electrode surface leads to enhanced electrochemical performance and results in higher sensitivity. It makes macroporous ITO electrode beneficial as a conducting platform for construction of biosensors rather than conventional planar ITO glass electrode.

4.3.2 Optimization of working conditions for CDH modified macroporous ITO electrode

Enzyme modified ITO electrodes were used to perform electrochemical analysis. The optimal experimental conditions for amperometric measurements of glucose were found. All measurements were performed in 50 mM glucose solution in a flow-injection mode. The factors considered in optimization process included pH of measuring buffer, applied potential and flow rate.

The effect of the buffer pH was examined in the range of 4,0 to 8,0 (Figure 18). The pH profile shown that the current signal started increasing as the pH was above 4,0 and then slightly decreased in more alkaline conditions. Most CHDs from basidiomycete exhibit optimal activity in an acidic pH range, which is beneficial for efficient electron transfer [59]. However, CtCDH used in this experiment showed pH optimum at 7,2. This result is in good agreement with literature data [60]. The high activity at this pH gives possibility to construct sensing devices operating at physiological conditions or in conditions, which are maximally adjusted to physiological.

a)



b)

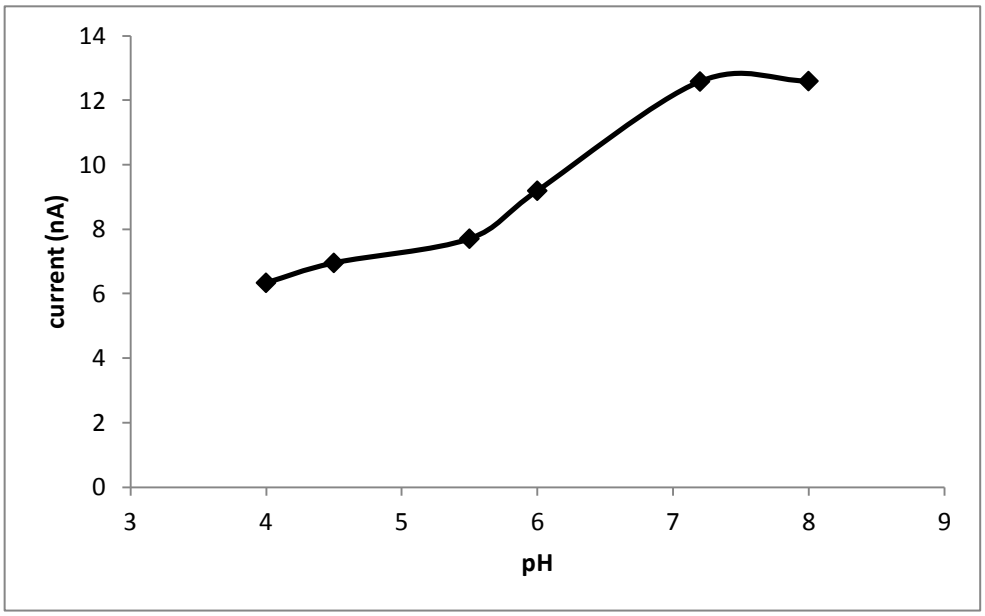


Figure 18: Influence of pH on the current response of the CHD modified macroporous ITO electrode. a) PEI modification b) GA modification.

Then the effect of working potential on the current response was tested. The applied potential varied from -0,1 V to 0,4 V (Figure 18). The maximum current was achieved at 0,3 V versus Ag|AgCl 0,1 M KCl. The current peak at more positive potential decreased, moreover it was previously reported that potential higher than 0,3 V can irreversibly damage the enzyme functioning [34]. Therefore all further flow-injection amperometric measurements were conducted at E=0,3 V.

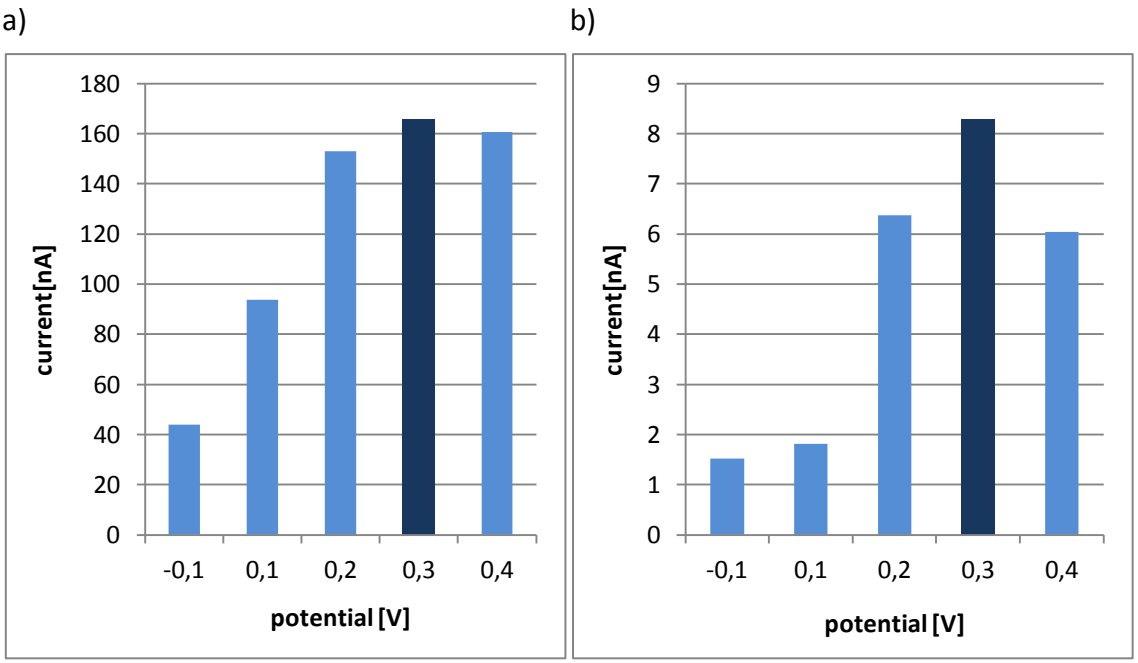


Figure 19: Influence of applied potential on current response of the CDH modified macroporous ITO electrode a) PEI modification b) GA modification.

Finally, the optimal flow rate was determined. The current signal was measured using 4 different flow rates: 0,25, 0,5, 1,0 and 1,5 ml/min (Figure 19). The highest current response was achieved at the flow rate of 0,25 ml/min, but it led to unfavourably long response time. The flow rate of 0,5 ml/min allowed faster analysis while the analytical response was still sufficiently high. Therefore it was selected as optimal for further measurements.

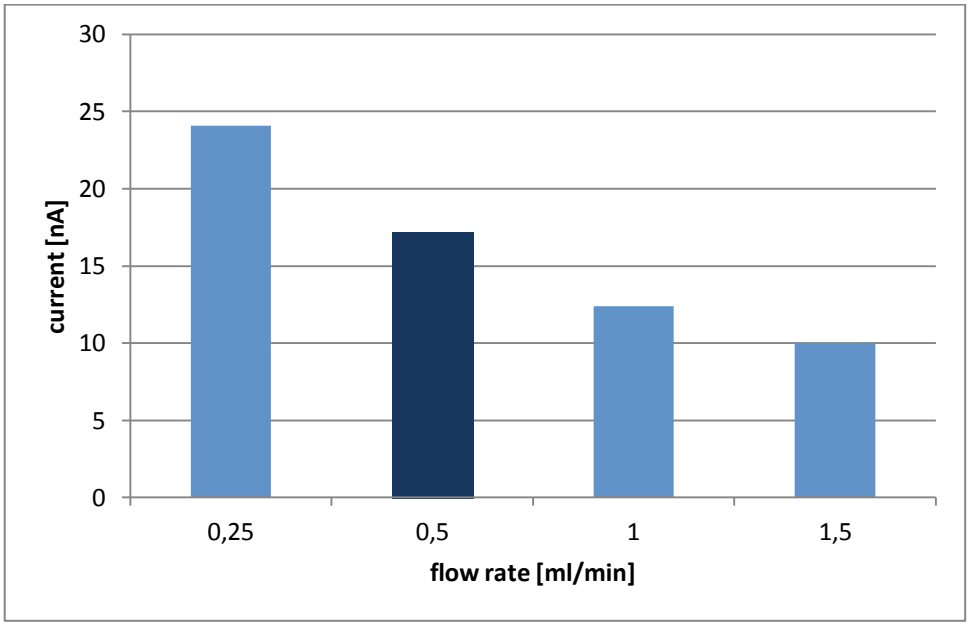


Figure 20: Influence of flow rate on the current response of the CDH modified macroporous ITO electrode.

4.4 Comparison of electrochemical performance of the enzyme functionalized macroporous ITO electrodes

The important aspect in development of enzyme electrode is protein immobilization onto the electrode surface. An ideal immobilization is expected, from one hand, to improve electron transfer between the biorecognition part (redox enzyme) and the electrode surface (in this particular studied case, a direct electron transfer). From the other hand, the immobilized enzyme is expected to maintain its biological activity and provide the biosensor with sufficient stability.

As it was found, CDH showed a poor adhesion to the surface of pristine macroporous ITO without its any additional modification. This is likely due to that the deposited enzyme forms layers loosely bound to the electrode surface and, therefore, leaching from the electrode immediately when it is submerged in the liquid media (i.e. measured sample of buffer) [49]. Moreover, the electrode modified with CDH gave negligibly small signal even with high concentrations of glucose and hence it does not provide sufficient sensitivity for practical applications. It was found that physical adsorption of the enzyme onto the macroporous ITO electrode is inconvenient for construction of a biosensor.

Therefore, in order to efficiently immobilize the enzyme and to preserve its activity, special measures on additional modification of ITO electrodes must be taken. In this work, the following approaches on the electrode functionalization have been applied

1) Cross-linking of the enzyme using glutaraldehyde.
2) Coverage of the electrode surface with polyethyleneimine, a polymer, which carries positive charges, followed by electrostatic binding of the enzyme to the modified surface.

Glutaraldehyde is a bifunctional reagent, which links covalently to the amine groups of enzyme, and hence fixing the protein molecules and creating a stable structure. PEI is a polycation, which is composed of primary amines for linear PEI or secondary and tertiary amines for branched PEI. The charge groups make branched PEI freely miscible with water at room temperature. Due to its positive charges PEI is widely used to

attach cells or biocatalyst onto the functionalized surfaces [61]. The adhesive properties of PEI were used for the construction of biosensors using, e.g. cytochrome c [62], urease [63] glucose oxidase [64], which resulted in the increased stabilities of the biorecognition part or the electron transfer rates.

The electrodes modified with CDH and further treated with PEI or GA were tested using electrochemical three-electrode flow-through cell. The electrodes were placed in the cell and glucose solutions of various concentrations (1, 2,5, 5, 10, 20, 50, 100, 150 and 200 mM) prepared in PBS buffer (pH 7,2) were injected into the flow system. Upon an injection of the glucose substrate, electrodes modified using both approaches show a notable bioelectrocatalytic current, giving proof of the presence of the active enzyme on the electrode surface (Figures 21,22). Bioelectrocatalysis demonstrates efficient electron exchange between the conductive electrode surface and between the redox center of the entrapped enzyme.

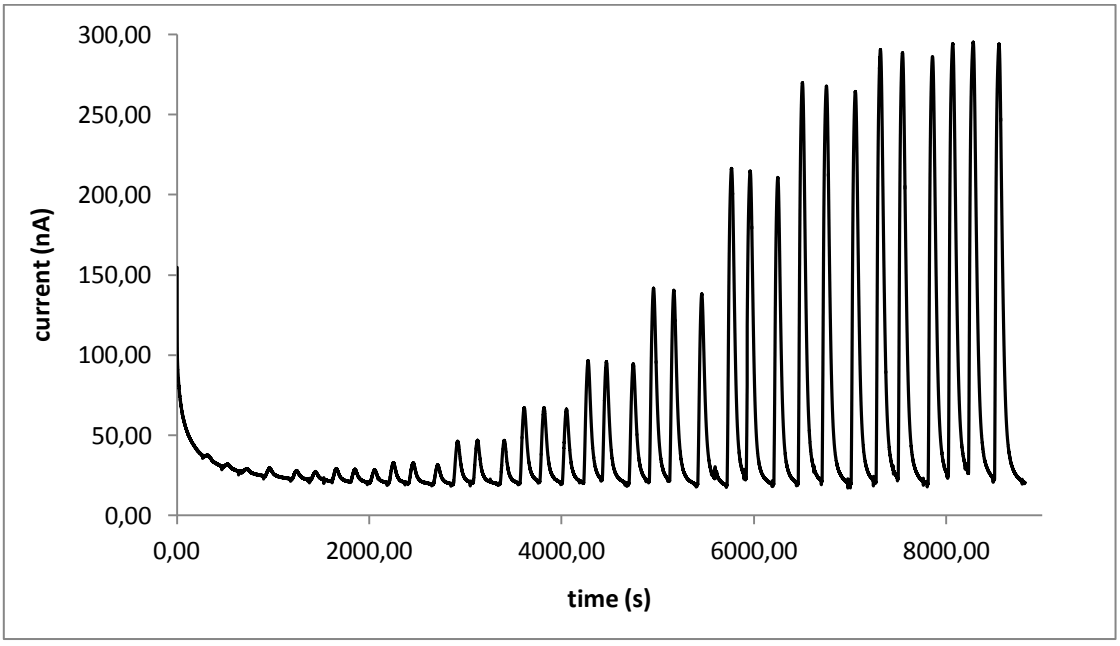


Figure 21: Response curve of PEI-CDH modified macroporous ITO electrode obtained from injection of 0,125 - 200 mM of glucose. Each concentration of the substrate is injected in triplicate.

The calibration curve exhibits typical Michaelis-Menten behaviour, and reaches plateau at 150 mM (Figure 22). The linear range of the calibration curves was 1 – 20 mM for both electrodes (Figure 23). The wide linear range covers the relevant range for monitoring glucose levels in biological fluids, e.g. blood.

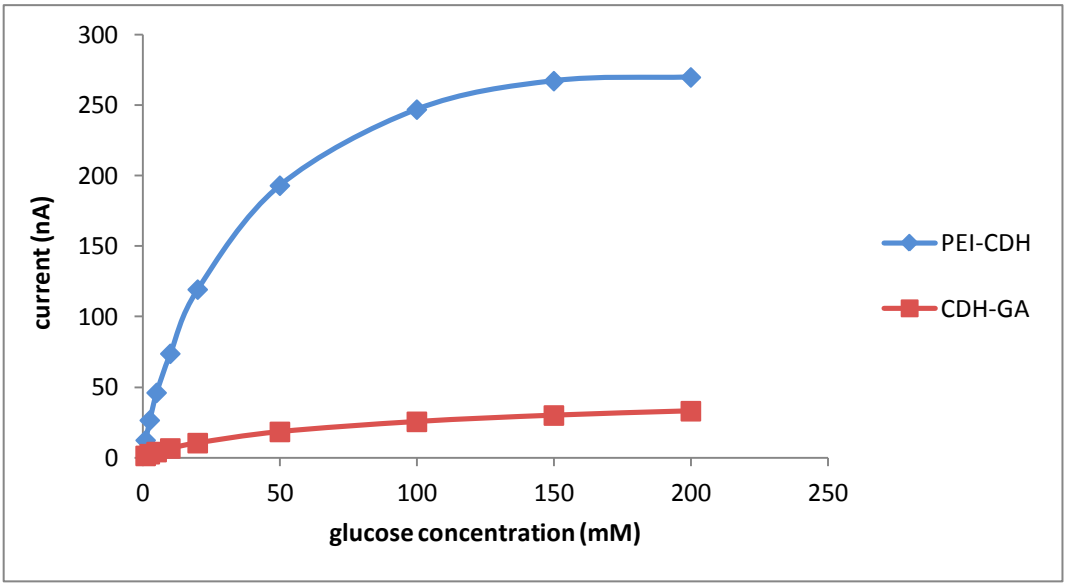


Figure 22: Calibration curves for glucose obtained using PEI-CDH modified electrode and GA-CDH modified electrode. Performed in 10 mM PBS (pH 7,2) with a flow rate of 0,5 ml/min and a working potential of 300 mV versus Ag|AgCl 0,1 M KCl.

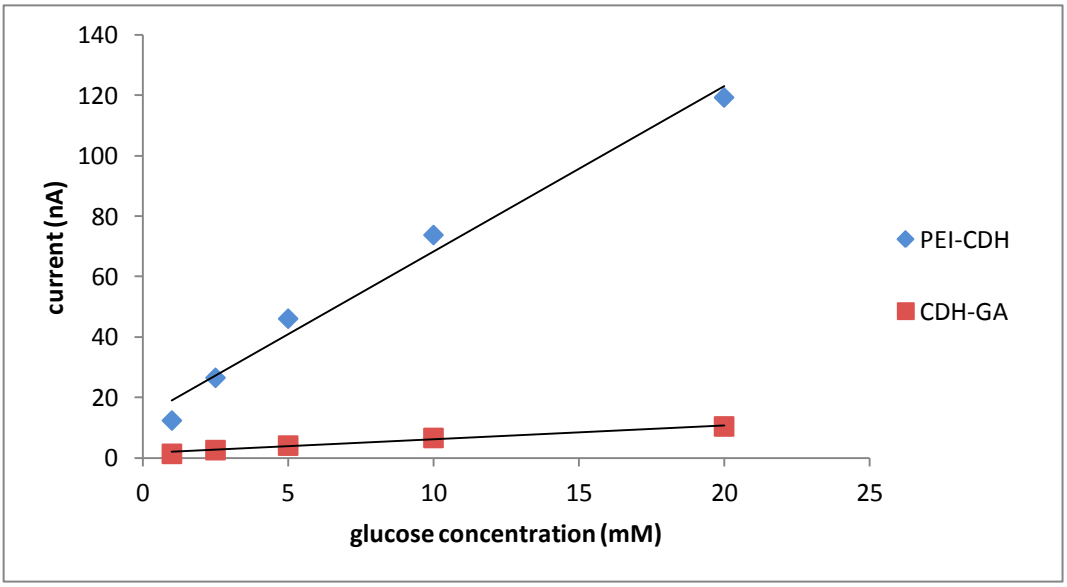


Figure 23: Linear range of glucose calibration curves obtained using PEI-CDH modified electrode and CDH-GA modified electrode.

It was observed that the analytical response obtained from the electrode modified with PEI was about 10 times higher than the one modified with GA. It is most likely that PEI increases the amount of adsorbed enzyme on the electrode surface due to electrostatic interactions between the negatively charged carboxylic groups of the enzyme and the positively charged polycationic polymer. Moreover mild immobilization conditions do not suppress/decrease the activity of the enzyme, as no

covalent bonds are formed between the biocatalyst and the modifier, and therefore the native structure of the enzyme is preserved. Altogether is contributes to higher catalytic response. It was previously reported that PEI may also increase the electron transport between DHCDH and CYTCDH or between CYTCDH and the electrode by screening negative charges, comparable to the previously reported effect of divalent cations on the activity of MtCDH [65].

Lower analytical response observed during cross-linking of the enzyme with GA may be explained by the covalent interaction between the enzyme and GA. Cross-linking with GA firmly "fastens" the enzyme molecule and restricts a degree of conformational changes necessary for the biocatalytic reaction, and hence, decreases its activity. At the same time, the activity of the CDH cross-linked with GA is significantly increased in comparison with the activity of the same enzyme immobilized using simple physical adsorption. CDH-GA modified electrode showed 4 to 5 times higher response than the one without any modifier. This data is in a good agreement with the literature findings [33, 66]. It can, at this stage, only be speculated that GA links the enzyme molecules in a way that makes it possible to keep a larger amount of the enzyme in direct electron transfer contact with the electrode surface.

Further measurements were performed with injecting the lower glucose concentrations of 0,125, 0,25 and 0,5 mM glucose solutions (in 10mM PBS) and limit of detection (LOD) was calculated using the equation:

$$LOD = 3s/S \quad (7)$$

where *s* is standard deviation and *S* is slope. LOD was found to be 0,03 mM for the PEI-CDH modified electrode and 0,37 mM for GA-CDH modified electrode.

4.5 Study the operational stability of the macroporous ITO based enzyme biosensor

The operational stability of the enzyme immobilized on the support depends on the intended use of the biosensor. Disposable sensors may be active from seconds to several minutes, while reusable sensors may require longer stability. Besides the

operational stability of immobilized enzyme system is important for commercial applications. One of the factors influencing operational stability is efficacy of the immobilization process. The adsorbed molecules due to physical forces usually have a tendency to leach from the support, and therefore limited long term stability of the electrode. This problem may be solved by the appropriate modification of the electrode surface, which is more robust and thus less susceptible to the impact of the environment.

Study of stability of the macroporous enzyme modified ITO electrodes was conducted using amperometry at applied potential of 0.3 V vs Ag/AgCl reference electrode. The operational stability in long-term measurements of the ITO electrodes was studied by injecting 10mM glucose in 5 minutes intervals and the current response as a function of time under optimised conditions was observed.

The results of study of operational stability of the CDH modified electrodes after seven hours of continuous injection of the sugar solution are shown in Figure 24.

PEI modified electrode exhibited the decrease of the initial response by 72% within the study interval. In contrast, the electrodes modified with GA were found to be more stable. The decrease in current was 15% and 31% when used 1% GA and 0,1% GA solution, respectively.

The enzyme immobilized on PEI modified electrode suffers from rather low long-term stability under set experimental conditions. Poor stability of the electrode may indicate relatively weak nature of electrostatic interaction between PEI and CDH. The enzyme bound onto the surface of PEI could dissociate and cause leaching, consequently leading to loss of its activity over a period of time.

It was shown that using GA as cross-linking reagent substantially enhanced the operational stability and that is important in possible applications for biosensors. GA treatment has been widely used to avoid the enzyme loss, as it can introduce intermolecular cross-linking in proteins or it can improve the attachment of the enzyme molecule onto the support [67]. The decrease in electrochemical signal may indicate that a certain amount of the enzyme is immobilized electrostatically with the

bare ITO surface. Other possible cause of a long term decline in current response could be leaching of the free or loosely bound enzyme molecules. The decrease of response is quite rapid during first two hours, then it shows a tendency to stabilise and remain at constant level throughout the rest of experiment.

The effect of the concentration of glutaraldehyde solution on the stability was observed. With the concentration of glutaraldehyde solution decreasing, the electrode stability was also decreased. The lower stability seen with the lower concentration of glutaraldehyde (0,1%) may indicate incomplete immobilization of the enzyme.

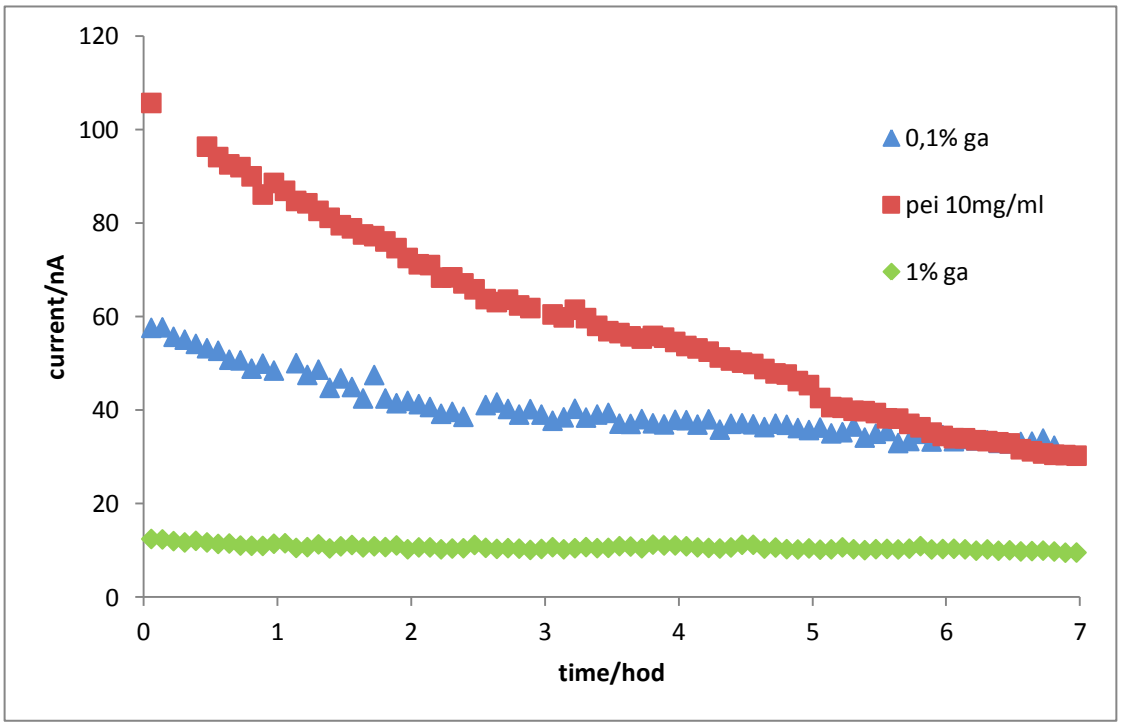


Figure 24: Variation of response for glucose with time obtained for PEI modification, 1% GA modification and 0,1% GA modification.

4.6 Nafion coating reduces interferences in amperometric measurements of glucose

The accuracy of amperometric blood glucose measurements may be affected by various electrochemically active interfering substances present in the blood sample. These include naturally occuring ascorbic acid, which is present in blood in

concentration 34-80 µmol/l, uric acid found in blood in concentration range 178-416 µmol/l, and widely used analgesic acetaminophen in therapeutic concentrations of 130-150 µmol/l. These substances due to their electrochemical activity generate a current, which interfere the detection of analyte of the target compound [68].

The interfernce of ascorbic acid was examined using amperometry with PEI modified electrode. 1 ml of 85 µM solutions of ascorbic acid in 10 mM PBS were injected into flow system. A constant potential of 0,3 V versus Ag|AgCl 0,1 M KCl was applied and the resulting current was measured. The response of ascorbic acid was significantly higher than the detected concentrayions of glucose, and therefore it compromised the accuracy of glucose measurements.

To overcome the interference of anionic species, the electrode surface was first modified with 0,1% Nafion solution. Nafion is fluoropolymer containing negatively charged sulfonate groups, which is permeable for cations and electrostatically repels anionic substrates [69].

It was found that the Nafion solution deposited onto the electrode surface form selective membrane and suppresses the penetration of ascorbic acid to the electrode surface resulting in lower current response (Figure 25). After deposition of the first Nafion layer the decrease in current was not diminished sufficiently. The one layer suppressed the signal by about 58%. Therefore more layers of Nafion coating were applied on the electrode surface.

The three layers of 0,1% Nafion reduced the signal by about 91%. More concentrated solution of Nafion did not form smooth continuous membrane throughout the electrode surface, thus it was unsuitable as an efficient barrier to hinder penetration of ascorbic acid.

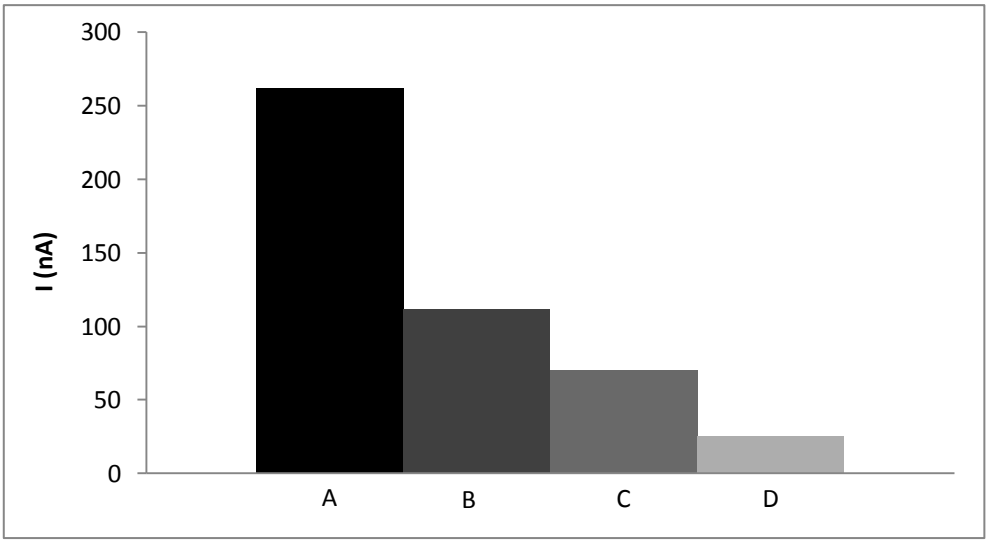


Figure 25: The response of 85 µM ascorbic acid obtained using PEI modified electrode with A) no Nafion, B) 1 Nafion layer, C) 2 Nafion layers, D) 3 Nafion layers. Performed in 10 mM PBS (pH 7,2) with flow rate of 0,5 ml/min and potential of 0,3 V versus Ag|AgCl 0,1 M KCl.

Although the response to ascorbate was attenuated significantly in the presence of three Nafion layers, the response from this interfering compound was not completely eliminated. Therefore it may be necessary to further optimise the coating process in order to suppress interferences completely.

5 Conclusion

Numerous attempts have been made to construct stable, reliable, and sensitive glucose biosensors because of the clinical significance of monitoring blood glucose levels. In this study, macroporous ITO was chosen as the transducing element in the biosensor design. The increased surface area of porous structure was demonstrated comparing the cyclic *voltammetric response* of a flat ITO and a macroporous ITO. Cellobiose dehydrogenase from *Corynascus thermophilus*, which is able to communicate directly with a conductive electrode material, was employed as biorecognition element.

The simple physical adsorption of the enzyme onto ITO platform was not sufficient for construction of sensor. However the enzyme was successfully immobilized using cationic polymer PEI or by cross-linking with glutaraldehyde. Macroporous ITO has been shown as a suitable electrode platform for the incorporation of higher amounts of CtCDH, taking advantage of the large porous surface area. This enhances bioelectrocatalytic response in comparison with flat ITO electrode.

The efficacy of enzyme loading and incorporation into a macroporous ITO interior was studied using SEM, EDX, XPS and electrochemistry.

The electrodes modified using both approaches have provided the same linear range between 1 to 20 mM of glucose. PEI-CDH modified electrode has shown a higher sensitivity and low limit of detection of 0,03 mM, but poor long-term stability and CDH-GA modified electrode has shown significantly improved stability, but lower sensitivity with the limit of detection of 0,37 mM.

Additionally, Nafion coating was applied to reduce the interference of ascorbic acid. Although Nafion has minimized the electrochemical response of ascorbic acid by 90%, it could not be eliminated completely in this study. Therefore more investigations of how to optimize the Nafion coating, which could completely abolish the effect of electrochemically active anionic compounds may be recommended.

Successful immobilization of CtCDH onto macroporous ITO platform enabling DET between enzyme and the electrode surface is a step towards the development of a novel amperometric glucose biosensor.

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7 Apendices

7.1 List of figures

7.2 List of tables