Univerzita Karlova v Praze

Farmaceutická Fakulta v Hradci Králové

Katedra Analytické Chemie

University of Gothenburg

Faculty of Science

Department of Chemistry and Molecular Biology

Charakteristika vertikálně uspořádaných uhlíkových nanotrubic jako platforem pro aplikace v oblasti biosenzorů

Vedoucí diplomové práce: doc. PharmDr. Lucie Nováková, Ph.D.

Školitel specialista: Assist. Prof. Gulnara Safina, Ph.D

Vedoucí katedry: prof. RNDr. Petr Solich, CSc.

Hradec Králové 2014

Tereza Štipková

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry

University of Gothenburg

Faculty of Science

Department of Chemistry and Molecular Biology

Working performance of vertically aligned carbon nanotubes as platforms for future biosensing applications

Supervisor: Assoc. Prof. PharmDr. Lucie Nováková, Ph.D.

Supervisor specialist: Assist. Prof. Gulnara Safina, Ph.D.

Head of the Department: Prof. RNDr. Petr Solich, CSc.

Hradec Králové 2014

Tereza Štipková

ABSTRAKT

Univerzita Karlova v Praze, Farmaceutická fakulta v Hradci Králové

Katedra Analytické chemie

Kandidát: Tereza Štipková

Školitel: doc. PharmDr. Lucie Nováková, Ph.D.

Školitel specialista: Assist. Prof. Gulnara Safina, Ph.D.

Název diplomové práce: Charakteristika vertikálně uspořádaných uhlíkových nanotrubic jako platforem pro aplikace v oblasti biosenzorů

Monitorování glukózy v krvi je nezbytnou součástí moderní terapie diabetu. V této práci byly použity mnohostěnné vertikálně uspořádané uhlíkové nanotrubice (CNT) pro konstrukci nového biosenzoru třetí generace. Hlavním zaměřením práce bylo zjištění možnosti uplatnění senzoru pro monitorování glukosy. Amperometrický biosenzor na bázi CNT byl vyvinut imobilizací celobiosadehydrogenasy z houby *Corynascus thermophilus* na povrch CNT pomocí fyzikální adsorpce. Vysoce hydrofobní povrch nanotrubic byl modifikován 0,0001% (w/v) polyvinylalkoholem (PVA) za účelem umožnění adsorpce enzymu. Výsledky měření pomocí rentgenové fotoelektronové spektroskopie potvrdily úspěšnou imobilizaci enzymu na povrch CNT modifikován PVA. Topografie povrchu a elementární složení nanomateriálu byly zkoumány rastrovacím elektronovým mikroskopem a metodou energiově disperzní spektroskopie. Optimální podmínky pro elektrochemické měření v průtokové cele s nástřiky glukosy zahrnovaly konstantní průtok 0,5 ml/min a konstantní napětí 0,3 V. Za těchto podmínek poskytoval biosenzor linearitu v rozmezí 1 – 25 mM glukosy, s limitem detekce 1 mM. Tento rozsah zahrnuje všechny koncentrace, které by mohly být nalezeny ve vzorcích lidské krve, a tedy senzor je způsobilý pro zamýšlené budoucí použití. Při měření v průtokové cele po dobu 6 hodin s nástřiky relativně vysoké koncentrace glukosy (10 mM) v pěti minutových intervalech vykazoval senzor výbornou stabilitu, s maximální variací odezvy 17,7 % a se zachovávanou počáteční odezvou na konci měření.

Klíčová slova: amperometrický senzor, uhlíkové nanotrubice, celobiosadehydrogenasa, glukosa

ABSTRACT

Charles University in Prague, Faculty of Pharmacy in Hradec Králové

Department of Analytical Chemistry

Candidate: Tereza Štipková

Supervisor: Assoc. Prof. PharmDr. Lucie Nováková, Ph.D.

Supervisor specialist: Assist. Prof. Gulnara Safina, Ph.D.

Title of thesis: Working performance of vertically aligned carbon nanotubes as platforms for future biosensing applications

Blood glucose monitoring is a crucial part of a modern diabetes therapy. In this work, the multi-walled vertically aligned on-chip grown carbon nanotubes (CNT) were applied in construction of a novel third-generation biosensor. The main focus of this work was to investigate the possibility of future application of the sensor in glucose monitoring. The CNT-based amperometric biosensor was developed by immobilising cellobiose dehydrogenase from *Corynascus thermophilus* onto the CNT surface by physical adsorption. The highly hydrophobic surface of the nanotubes was functionalized with 0.0001% (w/v) polyvinyl alcohol (PVA) in order to enable the enzyme adsorption. X-ray photoelectron spectroscopy measurements confirmed the successful enzyme immobilization onto PVA modified CNT surface. The surface topography and elemental composition of the nanomaterial were examined by scanning electron microscopy and energy-dispersive X-ray spectroscopy respectively. The optimal working conditions for flow-injection electrochemical measurements were found as follows: flow-rate of 0.5 ml/min and working potential applied of 0.3 mV. Under these conditions, the biosensor had a linear range between 1 - 25 mM glucose with the limit of detection of 1 mM. The range covers all concentrations likely to be found in human blood samples. Hence the sensor is eligible for the intended future application. A good operational stability of the sensor was observed when tested in flow-injection mode for the duration of 6 hours with relatively high concentration of glucose (10 mM) injected in 5 minute intervals. The maximum observed variation of current was 17.7 % and, by the end of the measurement, the sensor kept 100 % of its initial response.

Keywords: amperometric sensor, carbon nanotubes, cellobiose dehydrogenase, glucose

PROHLÁŠENÍ / STATEMENT

„Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.“

“I declare this thesis is my original copyright work. All literature and other sources I used for writing the thesis are listed in the bibliography and properly cited in the work. The work has not been used in order to obtain other or equal degree.”

Hradec Králové, 05.09.2014 _____

Tereza Štipková

ACKNOWLEDGEMENT

I would like to thank Assist. Prof. Gulnara Safina, Ph.D. for her enormous help and outstanding supervision during my stay at the University of Gothenburg and during the time I was working on this thesis. Leading me through my project and introducing the world of electrochemistry to me, she was the biggest help for completing my measurements. I cannot emphasize how grateful I am for her tirelessness in answering all of my questions. Working in the Ewing group was a tremendous pleasure, as the amazing spirit of the team pushed me through the hard times and was a great support for me. Therefore I would like to thank all the members of the Ewing group for making my work so much more enjoyable.

I am deeply grateful to Assoc. Prof. Lucie Nováková, Ph.D. for her prompt comments, corrections, help and very professional supervision of my work. With her never-ending enthusiasm for science, she inspired me to choose the field of analytical chemistry for my thesis.

Honest thanks go to my family for their huge support and to Huyen and Veronika for their absolute trust in me during my studies and especially during the final year.

CONTENTS

List of abbreviations

CGMS	Continuous Glucose Monitoring Systems
CNT	Carbon Nanotubes
CtCDH	*Corynascus thermophilus* Cellobiose degydrogenase
CYT_{CDH}	Cytochrome Domain of Cellobiose Dehydrogenase
CV	Cyclic Voltammetry
DET	Direct Electron Transfer
DH_{CDH}	Flavodehydrogenase Domain of Cellobiose Dehydrogenase
EDX	Energy-dispersive X-Ray Spectroscopy
ESEM	Environmental Scanning Electron Microscope
FAD	Flavine Adenine Dinucleotide
GOx	Glucose Oxidase
IDDM	Insulin Dependent Diabetes Mellitus
IET	Internal Electron Transport
NGM	Non-invasive Glucose Monitoring
NIDDM	Non-insulin Dependent Diabetes Mellitus
PBS	Phosphate Buffer Saline
PVA	Polyvinyl alcohol
SEM	Scanning Electron Microscope
SMBG	Self Monitoring of Blood Glucose
XPS	X-Ray Photoelectron Spectroscopy

1 Introduction

With the recent significant life-style changes, diabetes has become a world-wide epidemic and one of the major causes of deaths [1]. Current number of approximately 150 million diabetic patients will rise up to alarming 30 million in next two decades [2]. Therapy of diabetes includes administration of insulin and /or oral antidiabetic drugs in order to reach and maintain physiological blood glucose levels. The management of the disease also requires frequent monitoring of blood glucose to assess the effects of therapy and prevent both long term and acute complications [3, 4]. Invasive devices for self monitoring of blood glucose are nowadays most commonly used due to their accuracy and relatively low cost comparing to other devices. These are most frequently based on electrochemical detection employing enzymatic reaction of glucose oxidase with glucose [3]. Despite numerous sensors have been developed and used commercially, there is a need for improved robust and precise sensors to be introduced. The usage of glucose oxidase is a cause of drawbacks due to its intrinsic redox activity, thus not allowing the direct electron transfer from enzyme redox centre to the transducer, i.e. sensing electrode [5]. Moreover, some mediators used to address this problem are toxic and thus new sensors' construction efforts try avoiding them [4].

Recent discovery and research of *Corynascus thermophilus* cellobiose dehydrogenase (CtCDH) show the promising use of this enzyme in construction of third generation glucose biosensors. Unlike glucose oxidase, CtCDH allows direct electron transfer and therefore no mediators are necessary, while allowing the measurements of glucose at physiological pH [5, 6].

Carbon nanotubes (CNTs) have unique electrochemical properties for successful application in biosensor field and therefore the interest in their research has been increasing in the recent decades. Compared to traditional electrodes, CNTs show faster response time and higher sensitivity attributed to their hollow structure with ability to receive electrons from analytes. They have been specifically interesting for demonstrating high electrical conductivity, great chemical stability and extremely high mechanical strength [4, 7].

2 Objective of the thesis

Objective of this thesis was to characterize the properties and working performance of biosensor based on vertically aligned multi-walled carbon nanotubes grown on-chip functionalized with immobilized cellobiose dehydrogenase from fungi *Corynascus thermophilus* in the physiological conditions. The thesis aims at investigating the possibility of using this amperometric sensor in the field of glucose monitoring. The main focus is to construct a third-generation CNT-based biosensor with successfully immobilized enzyme, characterize the elemental composition of the surface and examine the properties of the sensor by electrochemical measurements.

3 Theoretical part

3.1 Diabetes Mellitus

A complex metabolic disorder called diabetes mellitus is currently considered a world-wide epidemic of the 21st century. With the lifestyle changing rapidly in the developed countries, the significance of this chronic disease has been proven by alarming rise of prevalence and being one of the major causes of mortality and morbidity [1]. Studies show that the number of patients diagnosed with diabetes has doubled over the past 20 years [8] and the total number of diabetes patients is estimated to increase by 50.7% more over the next two decades [2].

From more than 150 million diabetics world-wide, 10% suffer of type 1 diabetes (IDDM – insulin-dependent diabetes mellitus), which can be defined as insulin deficiency caused by pancreatic β-cells destruction, most frequently expressed from early childhood or adolescence. Etiology of IDDM can be either autoimmune or idiopathic [3, 9, 10].

The majority of the diabetic cases fall on insulin resistance or dysfunctional secretion gained through life, type 2 diabetes (NIDDM – noninsulin-dependent diabetes mellitus) [3, 9]. Usually the onset of this type of disease is in overweight patients aged over 40 year [3].

The physiological glucose levels in whole or capillary blood are primarily maintained by secretion of insulin and glucagon, and are within the range of 4.9 to 6.9 mmol/l [9]. The main characteristic of both types of diabetes is a chronically raised blood glucose level [11]. Long term exposure to concentrations out of this range due to insulin dysfunction can cause severe conditions. Untreated long-term hyperglycemia leads to neuropathy, macrovascular complications such as atherosclerosis and risk of other cardiovascular diseases, peripheral vascular disease and microvascular complications including nephropathy and retinopathy, possibly resulting in renal failure and blindness. There is evidence that the severity and length of hyperglycemia affect the microangiopathy [11]. Due to late diagnosis and thus a long period of high glucose levels with lack of glycaemia control, these complications are more likely to be found in patients with type 2 diabetes.

The blood glucose level below the physiological range is called hypoglycemia and if untreated, can result in severe acute complications from confusion, weakness and fainting, to coma or even death [9].

The therapy of diabetes is aimed at maintaining the glucose concentration in physiological range as much as possible. Since its discovery in 1921, the patients are treated with insulin administered parenterally, followed by short and long acting insulin analogues, continuous subcutaneous insulin injection (CSII) and oral antidiabetic drugs. The control and maintenance of normoglycemia is a key step in therapy and a crucial process in order to enable the patient to live a normal life avoiding the short- and long term complications [3].

Especially in the patients with type 1 diabetes and the insulin-treated type 2 patients, there is a strong need of glycaemia monitoring several times per day in order to achieve a good management of the disease. Through monitoring, the efficacy of the insulin therapy is checked and treatment can be adjusted in order to reach the optimal therapy plan. More importantly, the life-threatening hypoglycemic or hyperglycemic emergencies can be avoided with a precise blood glucose control [4]. Frequent monitoring can help identify factors that cause glucose swings, such as activity, diet or medication and measure their impact as well as identify trends in glucose levels [12].

3.2 Glucose monitoring and Glucose biosensors

Throughout the history of glucose monitoring, numerous methods and tools have been proposed and developed. These can be classified into three groups – invasive, minimally invasive and non–invasive methods.

3.2.1 Invasive and minimally invasive methods

The invasive monitoring devices have been so far the most commercially successful and thus the most frequently used due to their excellent accuracy and low price [3, 10].

3.2.1.1 Self monitoring of blood glucose

Nowadays the blood glucose is most often measured by healthcare professionals and patients themselves (SMBG – self monitoring of blood glucose) using the invasive finger-pricking method with a lancet to obtain the capillary blood sample. The blood sample of a volume of less than 1 µl is transferred onto a strip and measured in the glucometer with electrochemical detection [10, 11].

Having made the blood glucose monitoring possible for home usage since the 1970s, it became the integral part of the therapy with obvious advantage and progress. However, the intermittent monitoring only few times per day appears insufficient for recognizing glucose swings therefore unrecognized hypo- or hyperglycemic events may occur in patients, leading to emergency events. Moreover, the pain associated with obtaining blood samples is considered a disadvantage and is a reason for lack of compliance in many patients. Despite these drawbacks, SMBG devices have not yet been overcome by any other methods. Smaller lancets, smaller blood sample volume requirements and alternate site testing makes the testing less painful and more bearable by patients [10, 13, 14].

3.2.1.2 Continuous glucose monitoring systems

The continuous glucose monitoring systems (CGMS) have been the most efficient in providing the information about the glycaemia throughout the whole day. While intermittent measurements mostly focus on measuring the blood glucose before mealtime and bedtime, the continuous monitoring eliminates the risk of hyperglycemia or hypoglycemia also in the periods when glucose levels are not measured or cannot be measured, e.g. postprandial or overnight and the glucose swings that otherwise are not identified. The glucose swings are often associated with illnesses or sport activities. This approach increases the patients' safety especially in the cases of overnight and/or the unrecognized hypoglycemia, which mostly occurs in children aged 6 years or less. The continuous glucose levels data can be used to minimize the risk of the acute complications as well as improve the therapy according to the long term glycaemia trends. According to the studies, the use of CGMS reduces glucose levels' variability and hypoglycemia events as well as improves Hemoglobin (Hb) A1c levels [14]. The reduced Hb A1c levels reflect on

improved glycemic control and the reduction of micro- and macrovascular complications are observed as a result [15].

The current commercially available CGMS devices approved by Food and Drug Administration are based on monitoring the glucose level of the interstitial fluid while having been calibrated for the capillary blood glucose. The disposable sensor, consisting of amperometric enzyme-coated electrode, is inserted into the subcutaneous tissue for up to 7 days. The detection method is electrochemical, the interstitial glucose concentration is dependent on the current produced by the sensor. The enzyme immobilised at the positively charged electrode is glucose oxidase, being a catalyst in the hydrogen peroxide production, which is the target of the analysis [11]. The data are collected by a transducer and sent wirelessly to the receiver, where the glucose concentration is calculated and the result is shown with symbols or alarm in case of possible hypoglycemia or hyperglycemia risk. The data are collected as frequently as every 1 to 5 minutes. The real time data are used to prevent the acute events while the availability of the retrospective review of glucose values for at least several days helps identifying the trends and changing the basal and bolus insulin dosages accordingly. The importance of assessing the trends is higher in children due to growth and changing insulin sensitivity [11, 14].

The usage of CGMS devices has several drawbacks such as the warm-up time required for the sensor after being inserted into tissue (varying from 2 to 10 hours) and the need of calibration with the capillary blood sample obtained by finger pricking to enhance accuracy, usually several times per day. There is also the need of the time lag between the glucose values in blood and interstitial fluid which can result in errors [15]. Patients have to commit to the therapy which requires the knowledge of data interpretation and correct usage of the complex device. Moreover, patients find having the device constantly inserted into their body rather uncomfortable and the high costs may be unaffordable if no reimbursement is provided [14].

3.2.2 Non-invasive methods

The painful usage of the current invasive monitoring devices is considered their major disadvantage by patients. The idea of non-invasive glucose monitoring (NGM) offers a more comfortable management of the disease and the possibility to measure glycaemia more often, thus having a better control of the disease. In recent decades, there has been a

significant focus on developing pain-free glucose monitoring methods. Several devices based on different methods have been designed so far. However, more research is required in order to develop robust, precise and stabile devices. Currently the NGM devices are not used due to lack of precision, significant interferences and costliness in comparison to the conventional invasive devices [10].

There have been multiple techniques used for the construction of NGM devices with substantiate claims. Reverse iontophoresis with the application of electric potential between an anode and a cathode on the skin surface is used to generate the electric current, while glucose molecules are collected at the cathode and consequently measured by a sensor. The major drawback of this device designed for continuous glucose monitoring is the strong interference of sweating, physical activity, cold temperatures and skin irritation caused by the long-term usage [10, 11]. The bioimpedance spectroscopy technique is based on measuring the impedance of tissue. The changes in the red blood cells membrane potential caused by varying glucose concentration are determined by the impedance spectrum. However, the membrane is affected also by the disease state and the whole technique is dependent on the content of water. Other technologies employed include ultrasound, electromagnetic and heat capacity, occlusion NIR spectroscopy or laser microporation [10, 11].

3.2.3 Glucose biosensors history

The first commercially available device for measuring glucose, based on the technology of a reflectometer, was developed and launched on the market in the late 1960s. With a tremendous progress in the area of biosensor developing, the devices that followed up included a biosensor with mostly electrochemical detection, which proves as the most sensitive, reproducible and most importantly a cost-effective method. These sensors include the use of enzymes with redox groups that change their redox state during the biochemical reactions. Most commonly used enzymes are glucose oxidase (GOx) or glucose dehydrogenase (GDH). The mechanism of substrate oxidation by GOx is based on the enzyme accepting electrons thus being reduced and followed by its reactivation into the oxidised state by transferring electrons on molecular oxygen producing hydrogen peroxide

as a target of detection [4, 12]. The reaction can be summarised by following equation (see Equation 1):

$$\text{Glucose} + O_2 \rightarrow \text{gluconolactone} + H_2O_2 \quad (1)$$

$$\text{GOx (FAD)} + \text{glucose} \rightarrow \text{GOx } (FADH_2) + \text{gluconolactone} \quad (2)$$

$$\text{GOx } (FADH_2) + O_2 \rightarrow \text{GOx (FAD)} + H_2O_2 \quad (3)$$

The equation (see Equation 2) shows the mechanism of reduction within the enzyme. Flavin adenin dinucleotid (FAD) is reduced to its reduced form ($FADH_2$) whereas glucose is being oxidised forming gluconolactone. Oxygen is used in the process of flavin reoxidation with hydrogen peroxide being produced (see Equation 3) [4, 12].

3.2.3.1 First generation biosensors

The constructions of the first generation glucose biosensors used the enzymatic reactions with a suitable detection method and a transducer. With GOx being immobilised close to the surface of the electrode, the depletion of oxygen molecules can be measured with a Clark oxygen electrode. Similarly the production of hydrogen peroxide is proportional to the concentration of glucose and therefore can be measured amperometrically. For this purpose usually the platinum working electrode is used with a potential of +0.7 V versus Ag|AgCl applied. Using this approach, the first biosensing device measuring glucose was developed by Clark and commercially launched in 1975 by Yellow Spring Instrument Company (YSI).

The construction of the sensor includes an enzyme immobilised between two membranes, polycarbonate outer and cellulose acetate inner. The outer one allows the glucose molecules from the sample to pass and excludes other larger molecules from passing through. After hydrogen peroxide is produced, the molecules pass through the inner membrane onto the surface of the electrode. Both membranes help preventing the interfering substances from reaching either enzyme or electrode. However, there have been several drawbacks recognized such as costs of platinum electrode and relatively high voltage used increasing chances of interference of endogenous substances such as ascorbic acid, uric acid or commonly used drug acetaminophen [4, 12]. As the equation suggests, the whole process is

oxygen dependent and the oxygen supply is a limiting factor of the response. Using mass-transport limiting films of polyuretan or polycarbonate enables the flow of oxygen alongside with glucose. This problem was also addressed by using oxygen-rich carbon paste electrodes which are internal source of oxygen [4, 12].

3.2.3.2 Second generation biosensors

Glucose oxidase redox centre with FAD molecule is buried inside the protein structure and therefore cannot react directly with the electrode surface and the direct electron transfer (DET) becomes difficult. The second generation of biosensors address this difficulty with applying artificial electron accepting mediators to facilitate the electron transfer between FAD and electrode. The enzyme is specific regarding the substrate but does not show specificity regarding the electron acceptor, therefore mediators are applicable. Substances suitable for this use have to react with FAD faster than oxygen, should posses good electrochemical properties, be non-soluble in aqueous solutions and chemically stable. Particularly ferrocene derivatives as well as ferricyanide and transition-metal complexes have been used [4,12].

$$GOx_{(red)} + 2M_{(ox)} \rightarrow GOx_{(ox)} + 2\ M_{(red)} + 2\ H^{+} \quad (4)$$

$$2M_{(red)} \rightarrow 2M_{(ox)} + 2e^{-} \quad (5)$$

After GOx is reduced by glucose ($GOx_{(red)}$), the reoxidation process is based on transferring electrons onto oxidised mediator, creating its reduced form $M_{(red)}$ (see Equation 4). This step is followed by mediator reoxidation at the electrode surface with creating current response estimating glucose concentration (see Equation 5). As the equations demonstrate, using mediators addresses the first generation drawback of oxygen dependence [4, 12].

There have been also other approaches used to facilitate electron transport such as connections through redox polymers covalently bond with osmium-complex electron relays, gold nanoparticles or carbon nanotubes [4].

3.2.3.3 Third Generation Biosensors

Recently, numerous attempts in creating a next generation of biosensors have been performed. Developments in the field focus mainly on accomplishing DET and omitting the leachable toxic mediators. Several approaches could possibly solve the problem. While redox centre of GOx is spatially blocked from reacting by protein layers, the alternative enzymes with the ability of DET are being tested. Other approach suggests excluding enzyme detection and having it replaced by metals or metal oxides, creating a non-enzymatic electrode. This way, the problems of enzyme instability and tendency to degradation can be avoided [4, 12].

3.3 Cellobiose dehydrogenase

Cellobiose dehydrogenase (CDH; EC 1.1.99.18; cellobiose: [acceptor] 1-oxidoreductase) is a fungal enzyme with its attractive electrochemical properties giving it a potentially advantageous application in the field of biosensors. Enabling direct electron transfer (DET) from catalytic side to the electrode surface, CDH has been applied in cellobiose, cellodextrins, maltose, lactose, diphenolic compounds and catecholamines detection as well as in versatile biofuel cell anodes. The enzyme is naturally produced in various ascomycetes and basidiomycetes fungi as a lignocelluloses-degrading agent and its production is induced in presence of cellulose or cellobiose [6].

3.3.1 CDH structure

CDH belongs to flavocytochromes of the haemoflavoprotein family and is composed of two distinguishable domains, the larger flavodehydrogenase domain (DH_{CDH}) and smaller cytochrome domain (CYT_{CDH}), connected via a linker protein (Figure 1). Each domain contains a cofactor, flavine adenine dinucleotide (FAD) being a prosthetic group of DH_{CDH} and haem b of CYT_{CDH}. The tertiary structure of the enzyme allows internal electron transport (IET) between the two domains, being in a close distance to each other. The active sites of both domains are connected by a built-in electron transfer pathway thus IET becomes possible. The unusual sterical location of haem b catalytic side on the surface of the domain gives the

enzyme its specific qualities through enabling both IET and DET from the CYT_{CDH} to the electrode [5, 6].

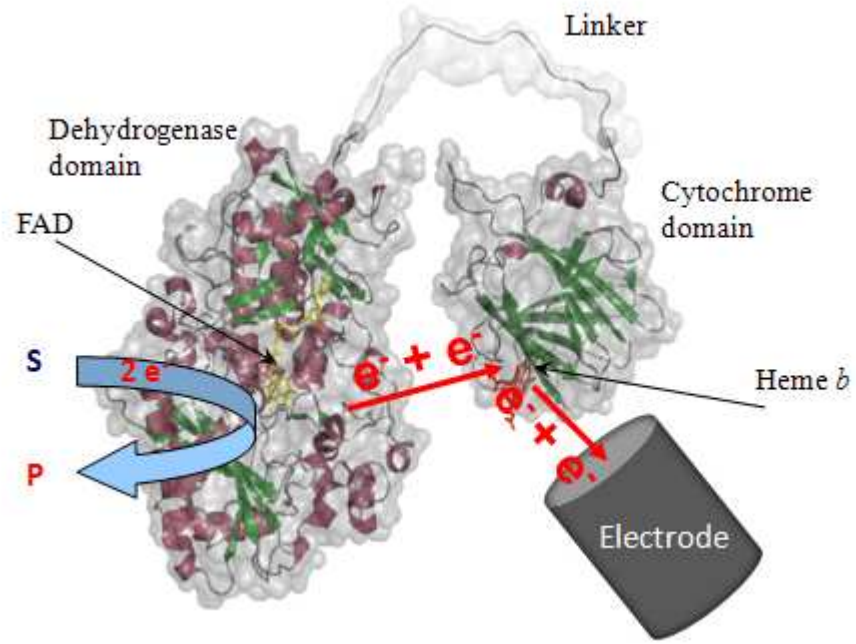

Figure 1: Cellobiose dehydrogenase and the electron transport. Figure was adapted from [6].

3.3.2 Properties and classification

CDH produced by different fungi species have different catalytic properties and are classified in three different classes. Class I is produced by basidiomycetes and is characterized by longer amino acid sequence and oxidation of only cellodextrins and disaccharides (e.g. lactose). The IET between the domains is only active in acidic pH. The classes II and III are products of ascomycetes and oxide various mono-, di- and oligosaccharides with IET more efficient at neutral and alkaline pH. This characteristic allows them to be more likely applied in measurements at physiological pH [5].

One of the promising enzyme subtype oxidizing saccharides at this pH range is CDH produced by ascomycete Corynascus thermophilus (CtCDH) with a perspective usage in the field of third generation glucose biosensors [5].

It has been suggested that DET is also important for the enzyme's natural function in the process of wood degradation, where CDH is expressed with other wood-degrading enzymes

that show DET to an electrode. The biological wood degradation is based on lignocellulose being attacked by hydroxyl radical as a subsequent product of Fe^{2+} and H_2O_2 reaction. The other theory suggests direct production of oxygen radicals with the active role of CYT_{CDH}in the process [6].

3.3.3 Electron Transfer Mechanism

The substrate oxidation and subsequently, electron transfer within the enzyme and from the enzyme to an electrode surface, is a complex multi-step process. In the first step, substrate (e.g. cellobiose) is oxidized at the DH domain while FAD cofactor (Fo - oxidized FAD) accepts two electrons from the substrate resulting into reduced FAD (Fr - reduced FAD). No changes occur at the haem domain in this stage (Ho – oxidized Haem). The reaction can be summarized as following (see Equation 6):

CDH (Fo Ho) + cellobiose → CDH (Fr Ho) + cellobionolactone (6)

This step is followed by reactions that enable sequential transfer of electrons to the electrode. The fully reduced FAD of DH domain is reoxidized while haem cofactor of CYT_{CDH} is reduced in two steps. The mechanism includes two single-electron reactions and is based on IET. Products of the first part are partially reduced FAD semiquinone radical (Fr·) and reduced haem (see Equation 7), which has to be reoxidized before accepting the second electron from FAD. Reoxidization of haem occurs at the electrode surface via DET (see Equation 8). After haem has been reoxidized, second IET reaction takes place resulting in production of fully oxidized FAD and reduced haem (see Equation 9), which is again reoxidized at the electrode via DET (see Equation 10). The electron transfer mechanism is described in the following reactions [6]:

CDH (Fr Ho) → CDH (Fr· Hr) + H^+ (7)

CDH (Fr· Hr) → CDH (Fr· Ho) + e^- (electrode) (8)

CDH (Fr· Ho) → CDH (Fo Hr) + H^+ (9)

CDH (Fo Hr) → CDH (Fo Ho) + e^- (electrode) (10)

3.4 Carbon nanotubes

3.4.1 Characteristics

Since their discovery in 1991 by Iijima [16], the properties of carbon nanotubes (CNTs) have been extensively studied in various research fields and found numerous applications, from which the usage in the field of biosensors appears as one of the most interesting and promising one. With their unique electrochemical, mechanical and structural properties, such as high electrical conductivity, high surface to volume ratio, outstanding chemical stability and extremely high mechanical strength and robustness, they have shown a tremendous potential in future biosensing applications [7, 16, 17, 18, 19]. Moreover, the studies show that CNTs applied in biosensors with biomolecules can cause enhanced electrical activity of these molecules due to electrocatalytic properties on CNTs. These properties are attributed to the CNT dimensions, electronic structure and topological defects of the CNT surface [7, 16].

Carbon nanotubes consist of sp^2 carbon units, creating seamless closed hollow tubes. Based on their structure, CNTs can be divided into two groups. Single-walled CNTs (SWCNTs) are composed of a single graphite sheet forming a tube of 0.4-2 nm in diameter [7, 16, 17]. Another type of nanotubes, multi-walled CNTs (MWCNTs) contain several concentric graphite tubes with diameters varying from 2 to 50 nm. Unlike many other carbon platforms, the tubes of CNTs are spatially well-oriented. The orientation gives them bigger surface accessible for reactions and their unique electrochemical properties [7, 16].

3.4.2 Fabrication of CNT

There have been several methods of fabricating aligned CNTs introduced. Arc-discharge technique was used for the synthesis. However, this approach led to several undesired side products. With laser ablation, CNTs of relatively high purity and adjustable quality can be produced [4].

Thermal chemical vapor deposition (CVD) and plasma enhanced CVD have been mostly applied in the fabrication process as the superior methods, either followed by transferring the CNTs onto the surface of electrode or using them for direct growth on the surface with

appropriate catalytic substrates. The growth on prepared substrates using CVD assures the high purity production of well-aligned CNTs [4, 7].

3.4.3 Application of CNT in biosensors and their modifications

CNTs have been studied in application in various biosensors, e.g. amperometric electrodes, DNA sensors and immunosensors. The majority of reported biosensors are designed for measurements of biomolecules in biomedicine and environmental pollutants, based on enzymatic reactions. In this type of sensors, CNTs role is both the attachment of biomolecules and enhancement of electrochemical response as well as mediating the electron transfer from the enzyme to the electrode surface. The tremendous advantage of including CNTs into the sensor's construction is their ability to lower the overpotential encountered for oxidation of NADH, which is a common cofactor of many enzymes employed in the biosensor field. Using CNTs enables lowering the working potentials in biosensors containing dehydrogenase enzymes. For glucose detection, potential of -0.2 V can be used, which significantly eliminates the interference of impurities and other substances present in the sample and allows the sensor to be used for measurements in biological fluids. Moreover, higher peak current in voltammetric response of numerous molecules was observed on CNT modified electrodes [7, 16]. In comparison with traditional carbon electrodes, sensors involving CNTs have generally higher sensitivities, lower limits of detection, and faster electron transfer kinetics [20]. These properties make CNTs an attractive detecting platform for future biosensor constructions. With the need of new developments for the glucose control and diagnostics in diabetic patients, CNTs have been extensively studied for preparation of glucose biosensors. Numerous glucose biosensors involving CNTs and glucose oxidase have been introduced [7, 16, 17].

With the necessity of attachment of a biomolecule (e.g. enzyme) to the surface of CNTs, several aspects have to be taken into account, which play a big role in the properties of a future biosensor. Biomolecules can be attached either covalently or by a non-covalent bond. Covalent bond assures higher stability and durability of the attachment, however can sterically interfere with the active site of the molecule and thus lower its activity. For covalent attachment, carbodiimide chemistry is often used, while CNTs are usually chemically modified [7, 20].

Methods of non-covalent attachment of a biomolecule on CNTs include entrapment into gel and physical adsorption. Besides direct physical adsorption, techniques with adsorption onto CNT functionalized with polymers or biomolecules and techniques employing surfactants are used [20]. In comparison to sensors with covalently attached biomolecules, these show lower stability and durability [7]. The non-covalent attachment techniques preserve the conformation of the immobilized enzyme, thus are considered to be more promising [20].

To enable the attachment of most biomolecules, the surface of CNTs must be chemically modified to more hydrophilic, as pure CNTs are not water-soluble. Several methods have been introduced, such as plasma oxidation, chemical or electrochemical methods, allowing the surface to contain functional groups that play crucial role in further modification of CNTs and attachment of biomolecules [7, 19].

On the surface of electrode, CNTs can be either immobilized randomly, using entrapment in a polymer or physical adsorption. This type is referred to as the non-aligned CNTs. For the fabrication of biosensors with non-aligned CNTs, several strategies have been introduced. The most commonly used approaches include forming pastes of CNTs dispersed in various solvents and polyelectrolytes or incorporating CNTs within composite matrices with polymer materials such as Teflon or polyvinyl acetate. CNTs have also been used as intermediates between platinum, gold or glassy carbon electrodes and biomolecules [4, 16].

The aligned CNTs are regularly arranged on the electrode surface and their density and length can be modified. Due to their higher conductance compared to non-aligned CNTs, the electron transfer is facilitated [7].

3.5 Basic principles of Voltammetric techniques

Voltammetry is a commonly used analytical technique based on application of a potential (E) to an electrode and monitoring of the resulting current (i) flowing through the electrochemical cell. It has been widely used for quantitative analysis of organic and inorganic compounds, such as pharmaceuticals or substances in high-performance liquid chromatography (HPLC) and flow-injection analysis. The technique is also used to determine

the electron transfer and reaction mechanisms, thermodynamic properties and in studies of oxidation and reduction processes as well as reaction kinetics studies [21].

When the potential is applied to an electrode, several electrochemical phenomena, described by Nernst equation are occurred. Considering the reversible redox reaction of n electrons involved (see Equation 11), the potential affects the concentration of both oxidized and reduced specimen at the surface of the electrode according to Nernst equation (see Equation 12):

$$Red \leftrightarrow Ox + ne^- \quad (11)$$

$$E = E^0 - \frac{RT}{nF} \ln \frac{[\mathrm{Re}\, d]}{[Ox]} \quad (12)$$

where E is the applied potential, E^0 the standard redox potential of the redox couple; [Ox] and [Red] are surface concentrations at the electrode-solution interface, R is the molar gas constant [8.3144 J mol^{-1} K^{-1}], T is the absolute temperature [K], n is the number of electrons transferred, F is Faraday constant [9.6485 x 10^4 C mol^{-1}] [21].

The concentrations of reduced and oxidized species change depending on the potential value. With negative potential applied, the reduction occurs, represented by larger red/ox ratio. Vice versa, with applying more positive potential, the red/ox ratio is lowered indicating the oxidation process. Both oxidation and reduction result in generation of electric current (i) as a result of mass transport to the working electrode surface [21].

3.5.1 Cyclic Voltammetry

Cyclic voltammetry (CV) is a type of voltammetric technique based on applying varying potential at the working electrode at certain sweep rate [V/s], while the potential is applied in both forward and reverse directions and changed at specific switching potential. When sweeping the potential, the current is measured and the result of the experiment is a cyclic voltammogram with current shown on y-axis and potential on x-axis (Figure 2) [21, 22].

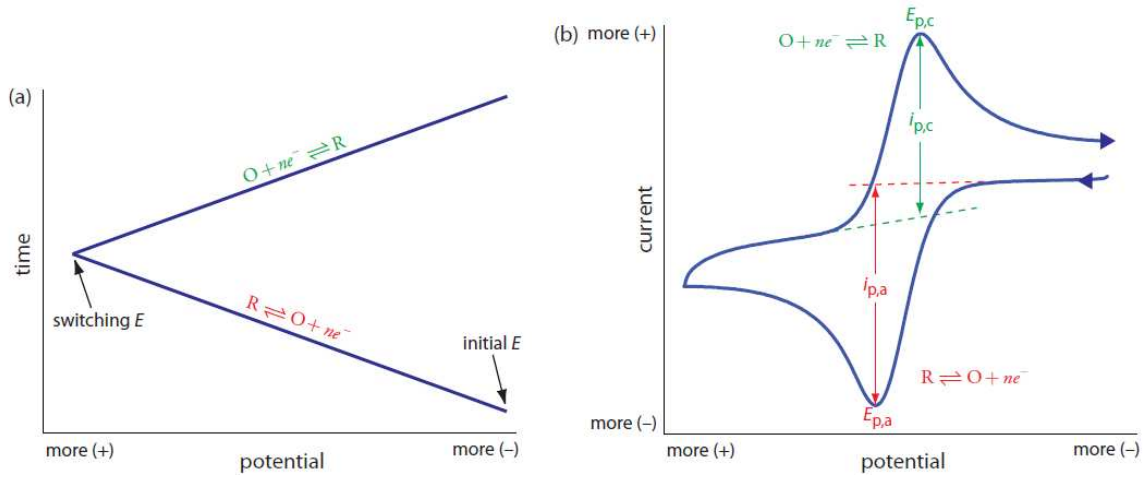

Figure 2: Cyclic voltammetry. a) A cycle of triangular potencial-excitation signal with initial and switching potential marked. b) Cyclic voltammogram. Peak currents ($i_{p,a}$; $i_{p,c}$) and peak potentials ($E_{p,a}$; $E_{p,c}$) are marked for oxidation and reduction segments respectively. Figure is adapted from [22].

In voltammograms, several parameters are measured. The cathodic and anodic peak currents (i_{pc} , i_{pa}) are given by the Rendles – Sevcik equation (see Equation 13):

$$i_p = 2.686\ 10^5\ n^{3/2}\ A\ D^{1/2}\ v^{1/2} C \qquad (13)$$

where i_p is the peak current [A], n is the number of electrons passed per molecule of analyte oxidized or reduced, A is the electrode area [cm^2], D is the diffusion coefficient of analyte [cm^2/sec], v is the potential sweep rate [V/s], and C is the concentration of analyte in bulk solution [mol/cm^3].

The peak potentials (E_{pc}, E_{pa}) of the cathodic and anodic peaks for reversible reactions are important characteristics of the voltammogram. These can be measured and the peak separation is determined as (see Equation 14, 15):

$$\Delta E_p = E_{pa} - E_{pc} = 2.303\ RT/nF \qquad (14)$$

$$\Delta E_p = 0.0592/n\ V \qquad (15)$$

For one electron, the ΔEp equals 60 mV in reversible reactions in 25°C. This theoretical value varies in real measurements.

The halfway potential $E_{1/2}$ for a redox couple in a reversible reaction is described as (see Equation 16):

$$E_{1/2} = \frac{(Epa + Epc)}{2} \tag{16}$$

CV has been mostly applied in studies of redox reactions, their intermediates and products stability. The experiments require instrumentation consisting of a potentiostat, an electrochemical cell and a computer and are carried out in immobile solutions with three electrodes, working, reference and auxiliary electrode [21, 22].

3.5.2 Amperometry

Amperometry is a voltammetric technique where, unlike in previously mentioned CV, the potential applied is constant and is not changed during the measurements. Thus, as a result, the current as a function of time is measured. The method has been mostly applied in sensor construction, where the current is proportional to the concentration of the substance measured. The glucose biosensors based on enzymatic reactions of GOx are an example of amperometric sensors (see section 3.2.3) [22].

3.6 Basic principle of Scanning Electron Microscopy

Scanning Electron Microscopy (SEM) is an advanced technique used in biological and material sciences for examining topography of materials. SEM measurements provide images with a high spatial resolution and magnification range up to the nanometer scale [23].

ESEM (environmental scanning electron microscopy) has broadened the spectrum of materials possible to be observed by electron microscopy by allowing mainly biological samples, hydrated materials and insulating materials to be imaged. For these materials, no or minimal sample preparation is needed, in comparison to conventional SEM [24].

The principle of the method is the surface of the sample being irradiated by electron beam. This primary electron beam is generated by an electron source (either thermal or field emission type). When the surface is being scanned by the primary beam, the secondary electrons and backscatter electrons are generated as a response of the interaction of the surface with primary electrons emitted. The secondary electrons are consequently detected and processed to form a high-resolution image of the sample [25].

3.7 Basic principle of Energy Dispersive X-ray Spectroscopy

Energy dispersive X-ray spectroscopy (EDX) is a non-destructive method allowing obtaining the information on chemical composition of the sample, usually connected to SEM measurement. The X-rays with specific energies corresponding to each element are generated by the electron beam irradiating the sample, hence interacting with its atoms. In this process, the X-rays of specific energies are emitted from the sample atoms, as the outer shell electrons substitute the vacancies in inner shells that were formed after inner shell electrons had been removed. The energy of the X-rays emitted depends on the energies of the two shells and thus is a characteristic of each element and a criterion for distinguishing the chemical composition. In order to determine the quantity of the element, the intensity of X-ray signal is measured. EDX measurements provide spectra of X-ray intensity on y-axis and X-ray energy [eV] on x-axis [26].

3.8 Basic principles of X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) is the most broadly applicable surface analysis technique today, providing information on chemical composition, chemical states and depth profile of element distribution. It is based on measuring the energy distribution of photon-excited electrons from atoms in the surface region of the sample. It is suitable for measuring gaseous and liquid samples. However, solids are the most frequently used. With XPS measurements, all elements except hydrogen can be detected. Compared to other methods,

it is a generally non-destructive technique. However, with the possibility of organic material destruction by X-ray beams [27].

The technique is based on a primary beam of soft X-rays (with the sources of Al Kα of 1486.6 eV or Mg Kα of 1253.6 eV) irradiating the sample. The interaction results in the excitation and subsequent ejection of low-energy electrons, as the photoionization of the atoms, molecules, or ions of the specimen occurs. The incoming photon of energy hν will photo-excite an electron of binding energy E_B. The sample work function ϕ_{sample} is necessary to remove the electron from the sample. This energy is gained as the electron enters the spectrometer where it has to overcome the work function ϕ of the spectrometer [27]. As seen in equation 17, the conservation of energy is assured:

$$E_B = h\nu - E_K - \phi \qquad (17)$$

where E_B is the binding energy of an electron, hν is the energy of the X-ray photon, E is kinetic energy of emitted electron and ϕ is the spectrometer work function. An XPS measurement provides a spectrum with the binding energies on the x-axis and the intensity on the y-axis [27].

4 Experimental part

4.1 Chemicals and Reagents

Enzyme

recCorynascus thermophilus CDH (cellobiosedehyrogenase) with volumetric activity (DCIP assay, pH 5.5, 30 C) = 29 U/mL, (Cyt c assay, pH 7.5, 30 C) = 20 U/mL

Solutions

All solutions were prepared using deionized (Milli-Q) water purified by a Millipore purification system (18.2 MΩcm) Purelab Classic, ELGA LabWater, Lane End Industrial Oark, High Wycombe, Bucks, UK.

Phosphate Buffer Saline (PBS) 10 mM

Buffer was prepared using PBS tablets pH 7.2

One tablet dissolved in 1000 ml of water yields:

0.14 M NaCl, 0.0027 M KCl, 0.010 M phosphate buffer pH 7.2

Medicago AB, Uppsala, Sweden

PBS was stored at room temperature.

D-(+)-Glucose

ACS reagent purity grade

Obtained from Sigma-Aldrich, St. Louis, MO, USA

Glucose solutions (0.01 mM – 100 mM) for electrochemical measurements were prepared in 10 mM PBS. Stored at 4-8 °C.

Nafion®perfluorinated ion-exchange resin 5 wt. % solution in a mixture of lower aliphatic alcohols and water

Obtained from Sigma-Aldrich, St. Louis, MO, USA

Ferrocenemethanol

Obtained from Sigma-Aldrich, St. Louis, MO, USA

The 1 mM solution prepared in 10 mM PBS was used. Stored at room temperature.

L-Ascorbic acid

Reagent grade

Obtained from Sigma-Aldrich, St. Louis, MO, USA

85µM solutions for electrochemical measurements were prepared in 10 mM PBS. Stored at 4-8 °C.

Poly (vinylalcohol) (PVA) solution

Poly (vinylalcohol) 99+% hydrolyzed

Obtained from Sigma-Aldrich, St. Louis, MO, USA

The 0.0001% w/v solution was used. Solution was heated at 90 °C for 1 hour with constant stirring, cooled down to room temperature. The prepared solution was stored at room temperature.

4.2 Electrochemical Measurements

4.2.1 Instrumentation for electrochemical measurements

CHI Potentiostat was used to perform all amperometric and voltammetric measurements, Model 1000B Series Multi-Potenciostat, manufactured by CH Instruments, Inc., Austin, TX, USA. Potentiostat was directly connected with a cell stand, Model CS-3A Stand cell Ver. 1.1, ALS Co., Ltd, Tokyo, Japan.

All measurements were performed at room temperature.

The continuous flow amperometry was performed using a flow injection system consisting of:

- Peristaltic pump, purchased from Harvard apparatus, Holliston, MA, USA. Operational flow rate used was 1.0 ml/min.

- Six port valve with manual injection, manufactured by Rheodyne, Cotati, CA, USA
- Loop connecting the valve and the cell with dimensions of d=0.025 mm, l=123.4 cm, V=0.5 ml
- Faraday cage
- The flow-through amperometric cell for inserting the CNT-covered electrode with a rubber ring of 0.5 cm of inner diameter (Figure 3)

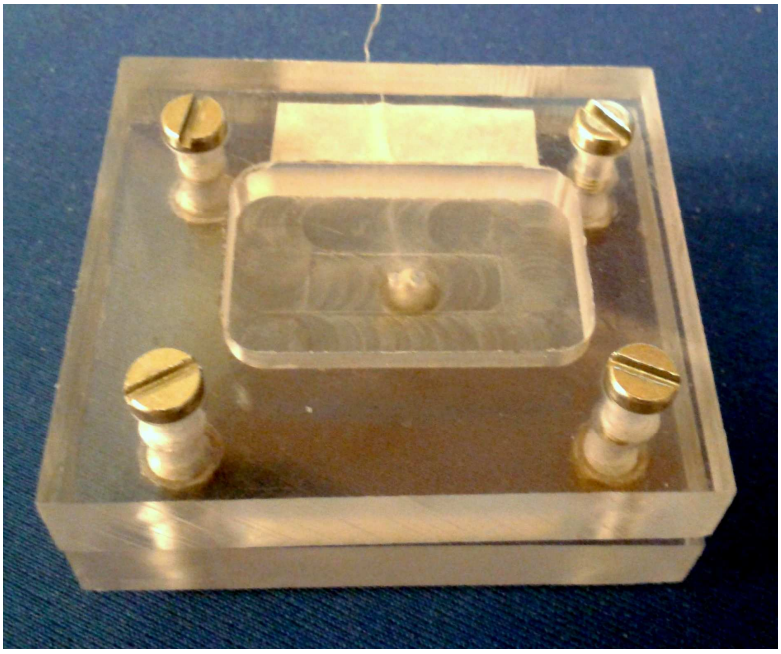

Figure 3: Home-made flow-through cell for inserting the CNT electrode to perform electrochemical measurements.

All measurements were carried with CNT-covered electrode as the working electrode. Electrode consisted of vertically aligned multi-walled CNT (MWCNT) fabricated using plasma enhanced chemical vapour deposition (CVD) on Si wafer with metal catalyst layer (Ni).

An Ag|AgCl 0.1 M KCl electrode was used as the reference electrode and a platinum electrode (platinum wire) was used as the auxiliary electrode. Electrodes were connected to the CHI potentiostat operated with its own software (Electrochemical Software, CH Instruments, Inc., Austin, TX, USA).

4.2.2 Working electrode preparation

The electrode surface containing the CNT was modified with the solution of CtCDH by physical adsorption of the enzyme onto the CNT surface. The amount of 5 µl of the enzyme solution was spread on the surface of the electrode, letting the solution evaporate. The electrode was subsequently kept at 4-8 °C overnight before performing measurements and continuously stored at the same temperature under constant humidity after every use.

In order to achieve a more hydrophilic surface of CNTs, the modification of the electrode with PVA solution was carried out by immersing the electrode into 0.0001% w/v PVA solution for the period of 24 hours at room temperature. After letting the surface dry, the electrode was modified with the enzyme solution as described above.

For the surface modification with Nafion to avoid the interference of anionic compounds, 1 µl of 5% solution was used and deposited evenly on the unmodified electrode surface. Electrode was then heated at 70 °C for 10 minutes and cooled down to room temperature before any further modifications were performed. Electrode was stored at room temperature unless modified with CtCDH.

4.3 Characterization of CNT-based electrode by SEM and EDX

Surface structure and chemical composition of the working CNT-based electrode were characterized by several advanced techniques. ESEM was used to evaluate the shape and dimensions of CNTs. All ESEM measurements were performed using Quanta 200 ESEM FEG, manufactured by FEI (Hillsboro, OR, USA). It is equipped with a Schottky field emission gun (FEG). Measurements were provided in both high and low vacuum modes.

ESEM was connected to Oxford Inca 300 Energy Dispersive X-ray (EDX) system for the analysis of elemental composition of the nanotubes (Oxford Instruments, Oxon, UK).

No sample preparation was required for either of the measurements. The electrode was secured on a metal stage using the sticky carbon tape.

4.4 Characterization of CNT-based electrode by XPS

X-ray photoelectron spectroscopy was used to obtain information about chemical composition and chemical states of the unmodified and enzyme modified electrode surfaces. PHI5500 instrument was used to perform the XPS measurements. The X-ray source used was a monochrom Al Kα with energy of 1486.6 eV. The power was 350 W and the standard take-off angle used was 45°.

No sample preparation was required for the XPS measurements. When measuring the CtCDH modified electrode, the surface was washed with PBS solution in order to eliminate the possible excessive amount of enzyme on the surface that was not immobilized.

5 Results and discussion

5.1 Characterization of CNTs using Scanning Electron Microscope

CNTs as the surface layer of the working electrode were characterised using high resolution ESEM. The images were taken from the area with deliberately made scratches in order to show the vertically aligned structure of the nanomaterial.

The images obtained by ESEM technique provided a detailed image of the topography and shape of the pristine CNTs, as seen in Figures 4-8. CNTs used for biosensor construction are clearly vertically aligned tubes with the dimensions of several nanometers. The uniform forest-like structure and length of the nanotubes (approximately 30 μm) are observed (Figure 4). Varying the time of the synthesis, the desired length of the nanotubes can be obtained. There are no signs of impurities, e.g. amorphous carbon in form of granules or other impurities, present in any of the pictures, indicating the high quality of CNTs. The shape and structure of the individual CNTs can be observed in detail in Figure 5. The dense structure of the CNT layer with individual nanotubes can be clearly seen when the edge of the layer is observed (Figures 4, 6, 7).

As it can be seen in Figure 8, the CNT surface is prone to deterioration with mechanical damage. The surface observed had been previously mechanically damaged, while performing electrochemical measurements. The examined part of the sensor indicates the collapse of the nanotubes while wetting during electrochemical experiments. The phenomena of collapsing after exposure to water can potentially cause the instability and impairment of the biosensor's performance in electrochemical measurements. Further investigation on improvement of attachment of the nanotubes to the substrate is recommended to be conducted.

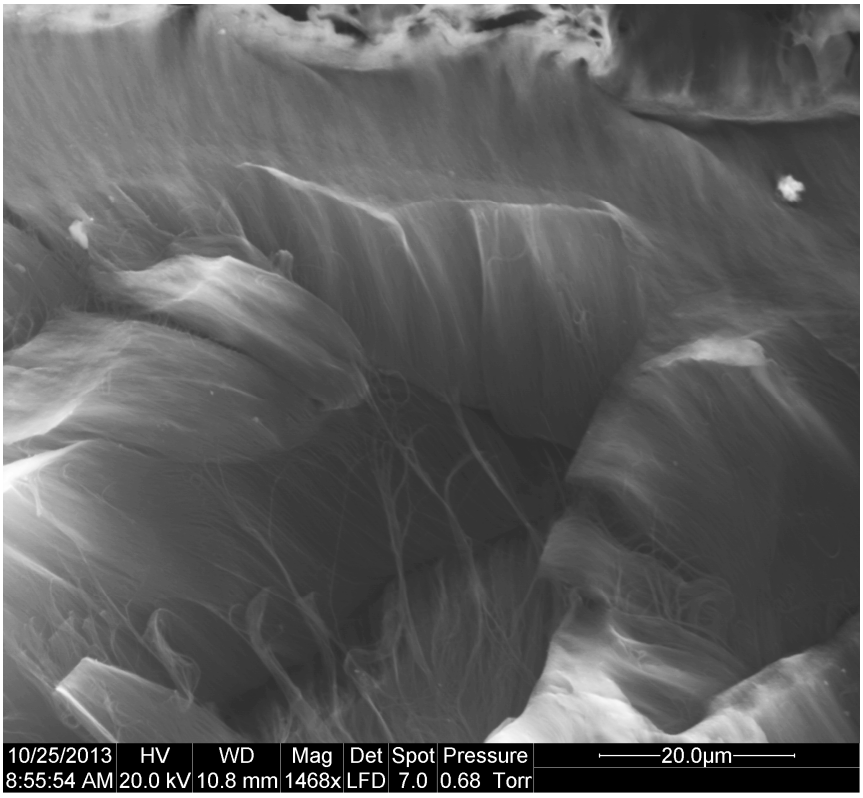

Figure 4: CNT layer of the working electrode. Nanotubes are vertically aligned creating a dense layer.

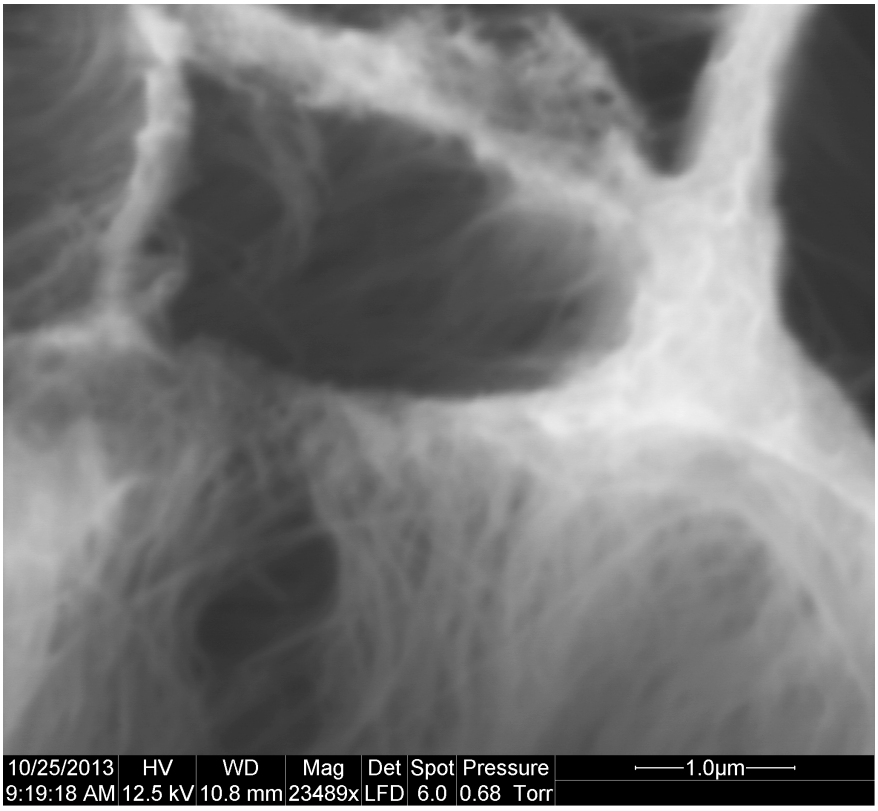

Figure 5: Zoom-in on individual nanotubes of the CNT layer.

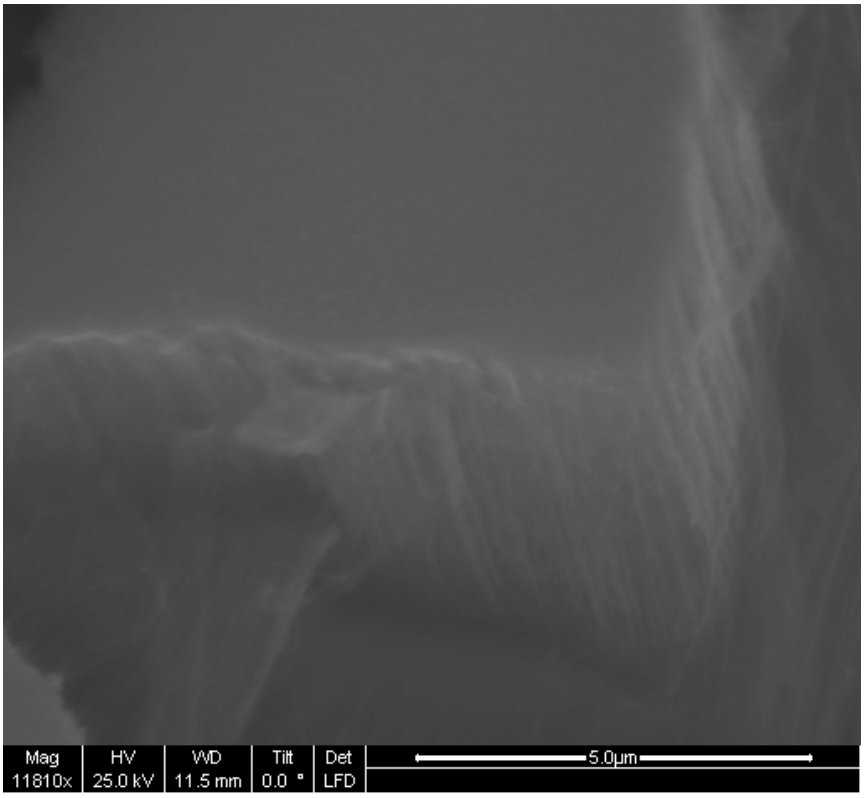

Figure 6: The edge of the CNT layer.

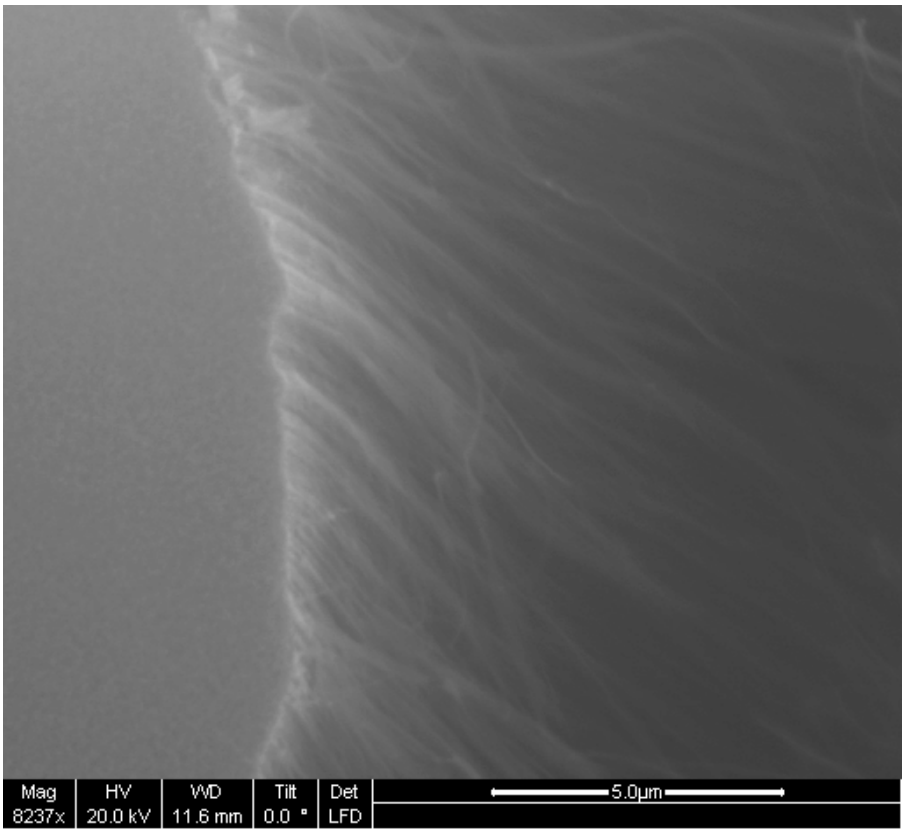

Figure 7: CNT layer with clearly visible nanotubes.

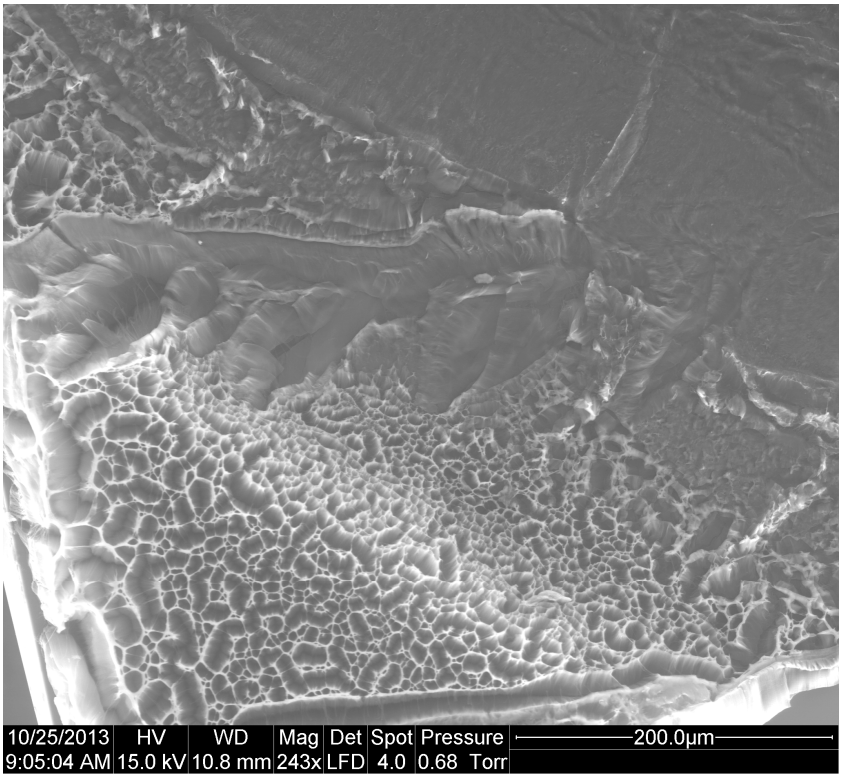

Figure 8: Partially collapsed CNT layer after the contact with water.

5.2 EDX characterisation

Energy dispersive X-ray spectroscopy was used to obtain elemental composition of the CNT working electrode. The spectrum (Figure 9) showed a significant carbon peak at 0.27 keV overlapping with oxygen peak at 0.52 keV, both representing the CNTs. The silicon peak at 1.74 keV represents the wafer used as the surface for catalyst deposition and CNT growth. Aluminium peak at 1.48 keV may be attributed to the material that the microscope stage is made of.

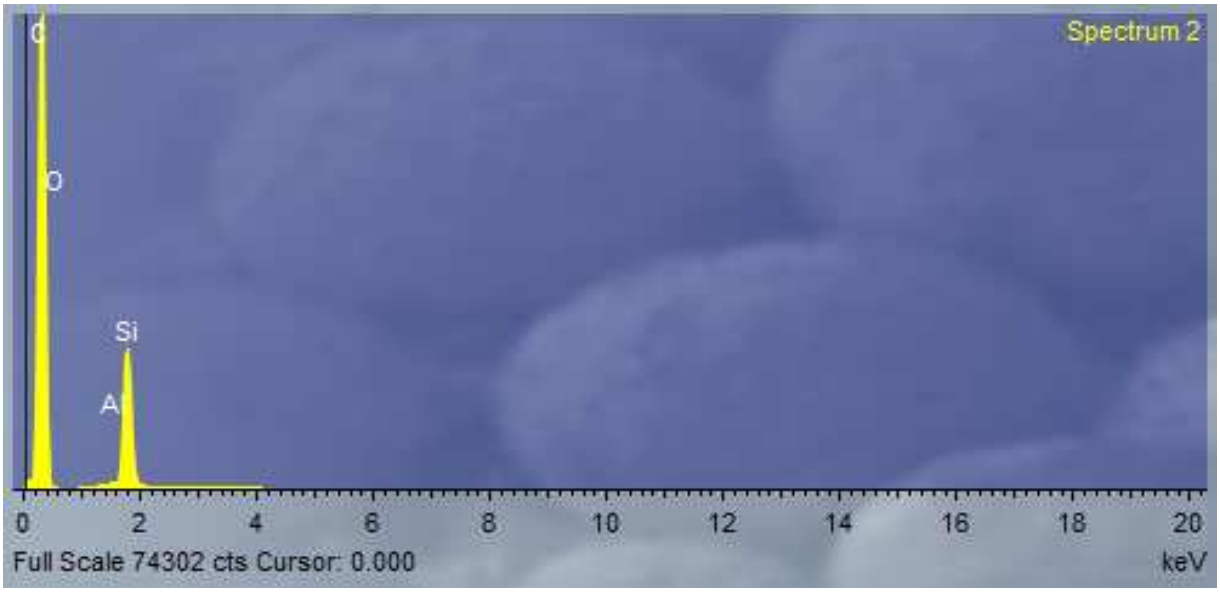

Figure 9: EDX spectrum of CNT electrode.

5.3 XPS characterization and comparison of differently modified electrodes

To obtain the information about chemical composition and chemical state of the elements of the material, XPS measurements were performed. First, unmodified pristine CNT was measured (Figure 10). Consequently, the chemical spectrum of electrode with PVA modification was obtained (Figure 11). Lastly, the electrode modified with both PVA and CtCDH was measured to acquire the data about chemical composition (Figure 12), with emphasis on chemical state of C, N and O atoms and compared with spectra of electrodes without enzymatic modification (Figure 10, 11). The main objective was to confirm the presence of enzyme as a proof of successful immobilization, being a critical step in biosensor construction. XPS spectrum (Figure 12) and detailed spectra of peak regions of CtCDH modified electrode (Figure 14, 15, 16) prove the presence of organic molecules, for the enzyme being a protein specimen.

5.3.1 Chemical composition

As shown in Figure 10, 11, 12, all three CNT electrodes contain carbon and oxygen as elements of highest significance. Carbon peak is generally attributed to the carbon nanoutubes and also organic carbon of the enzyme present in Figure 12. Presence of oxygen atoms in Figure 10 is due to the oxidation of the surface which has been in contact with the oxygen in the air. In Figures 11 and 12 the higher oxygen peak is attributed to PVA modification and enzyme molecules. Traces of impurities present in spectrum of CtCDH modified electrode (Figure 12), are due to the contact of the electrode with the enzyme or PBS buffer used to remove the excessive amount of loosely attached enzyme (see section 4.4). None of the traces found interferes with the performance of the sensor.

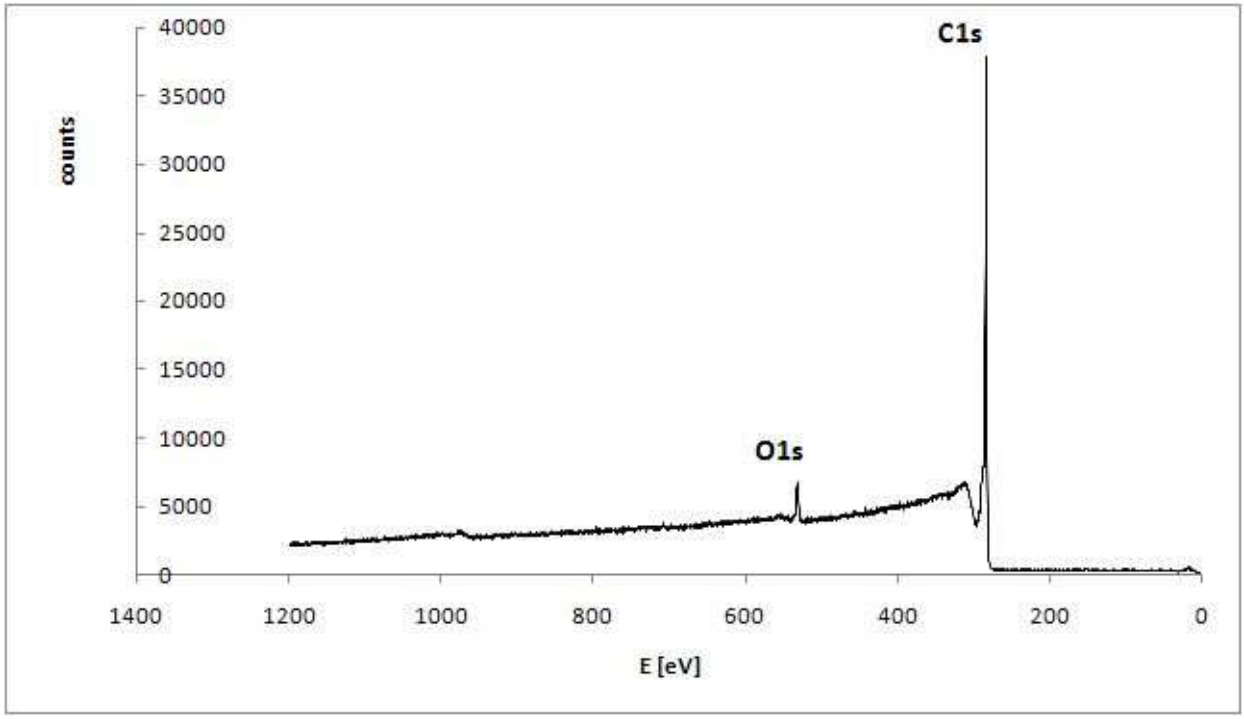

Figure 10: CNT surface of the unmodified working electrode. The dominant carbon 1s peak is observed. Oxygen 1s peak is attributed to surface oxidation when exposed to the oxygen in the air.

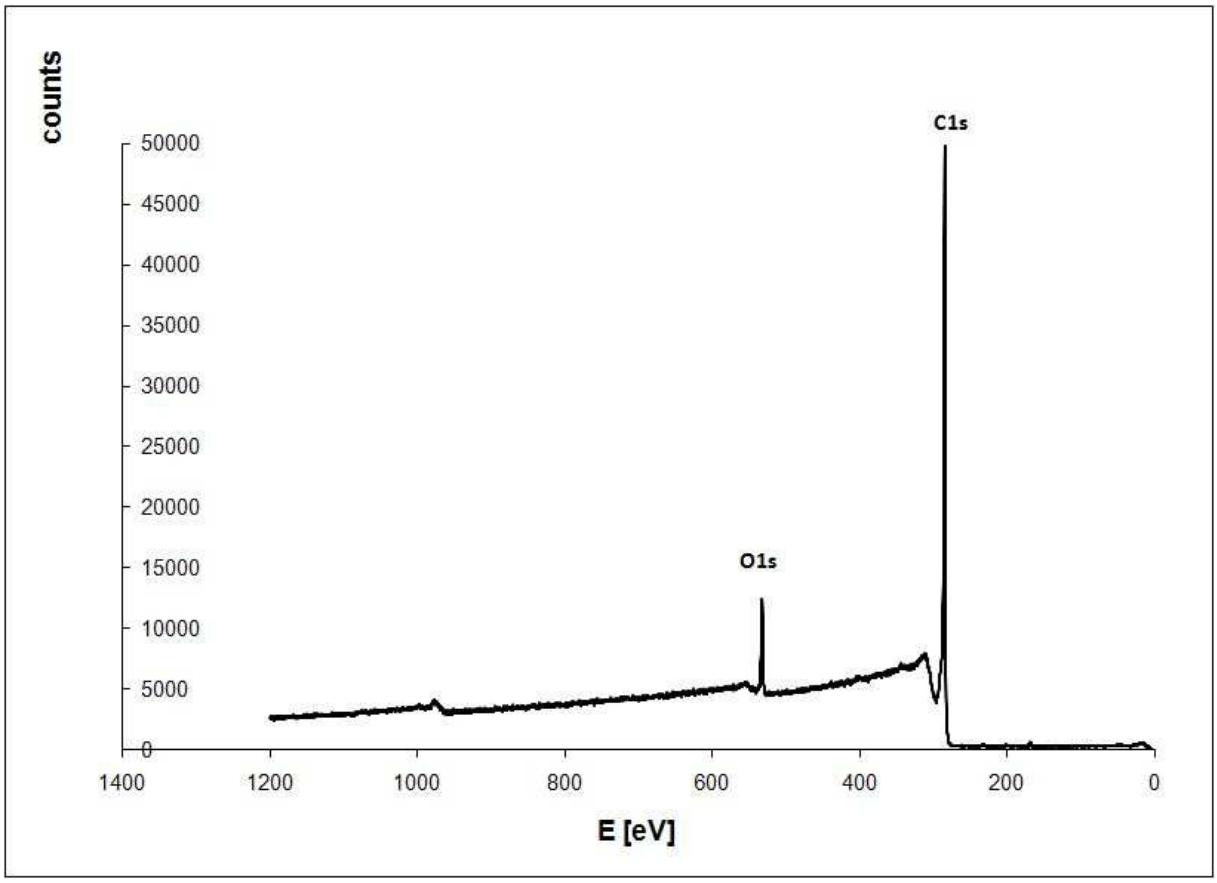

Figure 11: CNT surface of the working electrode modified with PVA solution. Higher oxygen 1s (O 1s) peak is observed as the result of CNT surface modification with PVA.

When comparing the pristine CNT electrode (Figure 10) with PVA functionalized electrode (Figure 11), significantly higher oxygen (O1s) peak is observed as a proof of adding hydrophilic OH groups onto the surface of CNTs.

As it can be seen in Figure 12, the immobilization of enzyme by physical adsorption onto PVA modified CNT layer was successful. The presence of nitrogen (N1s) peak and dramatically higher oxygen peak (O1s) suggest an organic molecule being present in the sample.

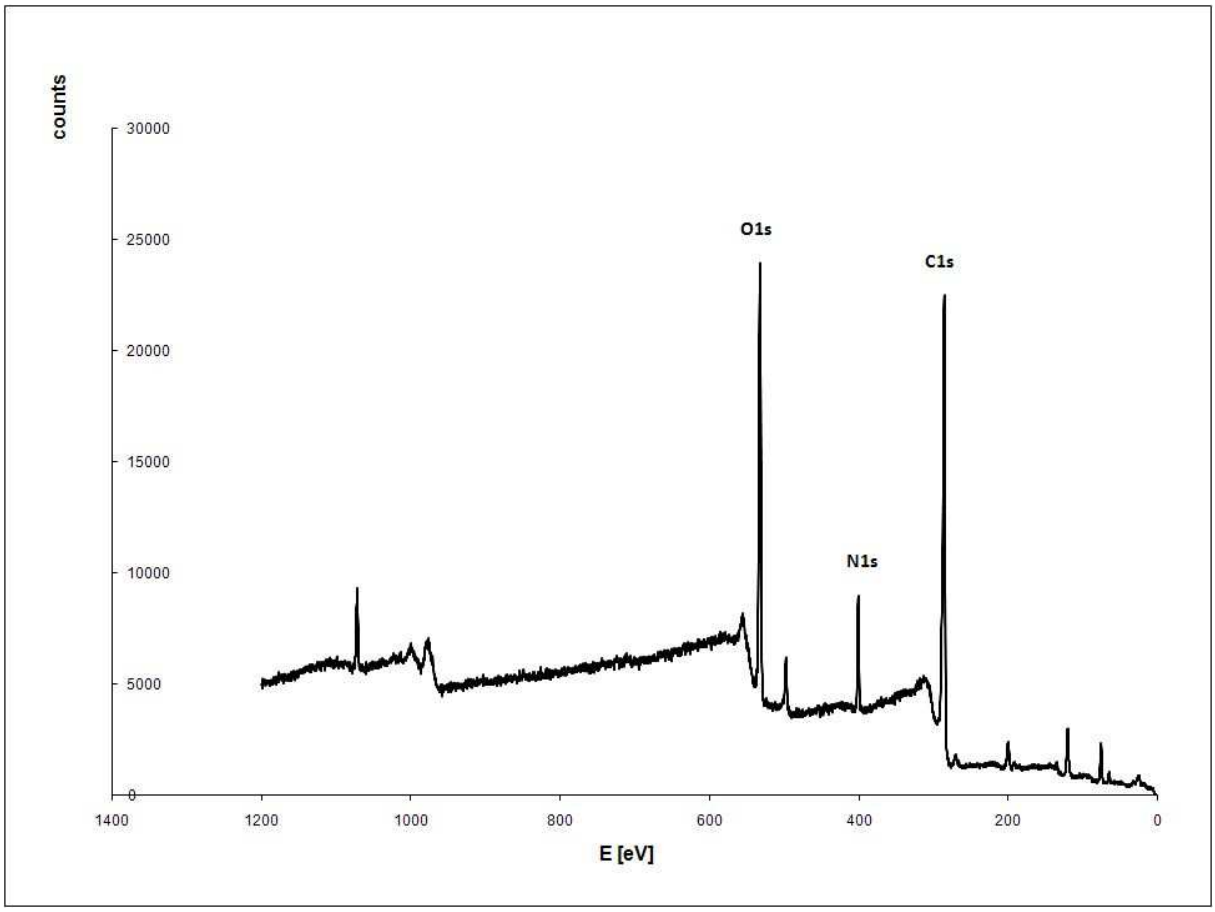

Figure 12: CNT surface of the working electrode modified with PVA and CtCDH. The peaks of significance are C1s, N1s and O1s, proving the enzyme immobilization onto PVA modified CNT. Other peaks are attributed to various impurities.

5.3.2 Chemical state data

For chemical state analysis, the spectra of individual elements of the unmodified CNT electrode and the CtCDH modified electrode were measured (Figures 13-16) and compared. The chemical states were determined from the peaks of XPS spectra and binding energies.

As seen in Figure 13, the carbon 1s spectrum of pristine CNT shows one major peak at binding energy of 284.7 eV, corresponding to the C-C bond, see in detail in Table 1. There are no other significant bands visible (Figure 13).

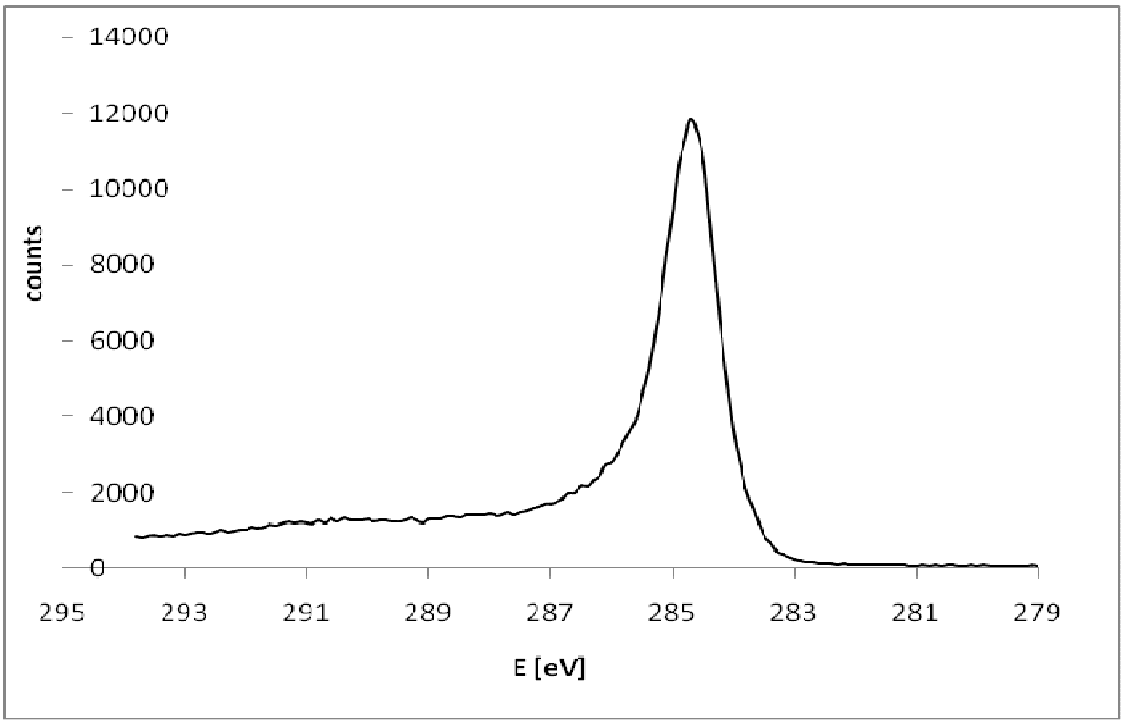

Figure 13: Carbon 1s XPS spectrum of unmodified CNT.

Band	Position	Height	Area	%Area	Bond
1	284.68	10330	13259	70.84	C-C
2	285.78	1777	2443	13.03	
3	287.08	997	1228	6.56	
4	289.18	619	852	4.55	
5	291.01	432	936	5.00	

Table 1: XPS spectrum of C1s region of unmodified CNT electrode. Band 1 represents the single bond of two carbon atoms, the graphitic carbon. Bands 2-5 represent different types of bonding between carbon and oxygen atoms of no significance.

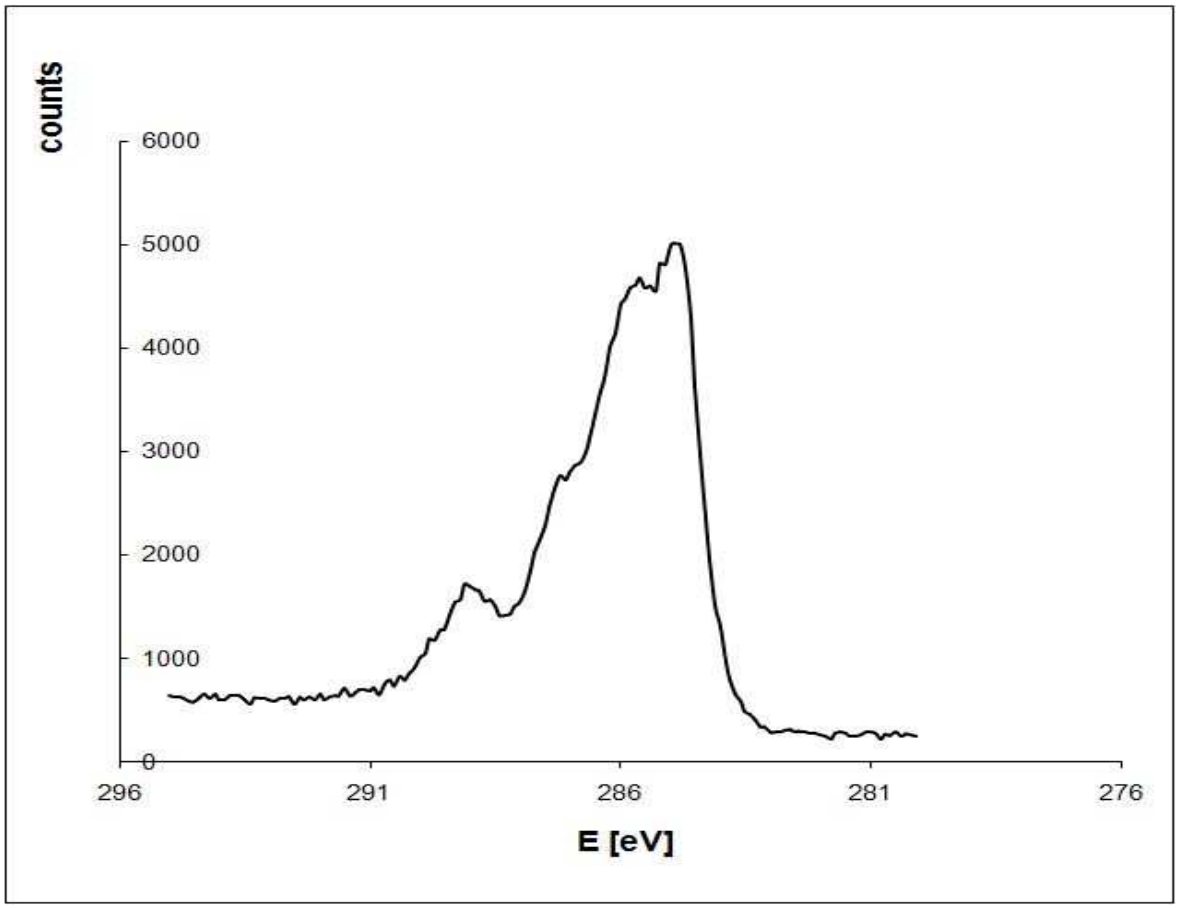

Figure 14: Carbon 1s XPS spectrum of CNT modified with both CtCDH and PVA.

Band	Position	Height	Area	%Area	Bond
1	284.87	3946	5557	37.29	C-C
2	285.97	3221	4862	32.63	C-O
3	287.27	1843	2756	18.50	CO-NH
4	289.12	1081	1724	11.57	CO-O

Table 2: XPS spectrum of C1s region of CNT electrode modified with CtCDH and PVA. Band 1 corresponds to graphitic carbon of the CNT. Bands 2-4 represent the organic carbon – oxygen bonding, indicating the enzyme presence.

The carbon 1s peak on Figure 14 clearly shows the change of chemical state, as the percentage of carbon – oxygen bonding is significantly higher compared to unmodified CNT (Figure 13) and the peak corresponds to variety of bonds, representing the various functional groups present (see Table 2). In comparison, C1s spectrum of pristine CNT (Figure 13) does not have as many significant bands as CtCDH modified sensor (Figure 14). Main peak in both C1s spectra is attributed to graphite.

The XPS spectrum of oxygen 1s region of the enzyme modified electrode (Figure 15) indicates both inorganic and organic bonds being present in the sample. According to Table 3, oxygen seems to be mainly in form of organic compounds in the enzyme modified electrode.

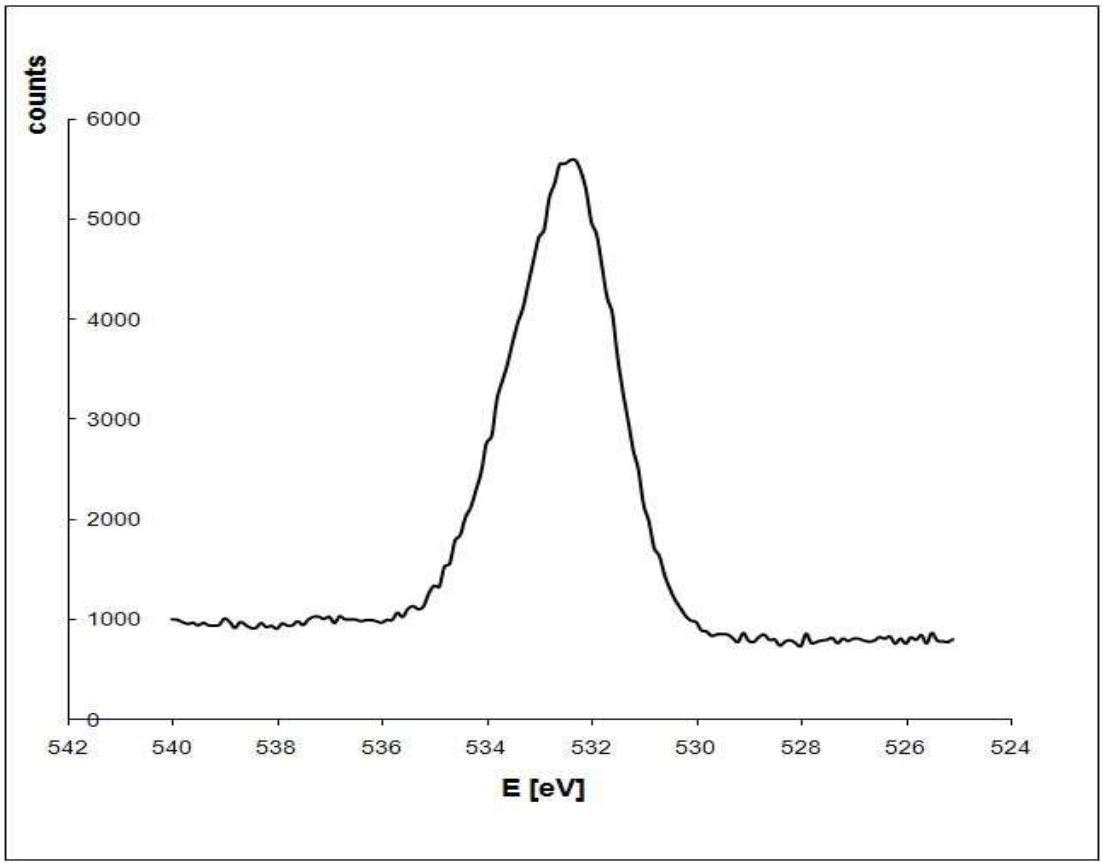

Figure 15: Oxygen 1s spectrum of CNT modified with both CtCDH and PVA.

Band	Position	Height	Area	% Area	Bond
1	529.93	36	88	0.72	
2	532.43	4547	10553	86.67	C=O
3	533.93	605	1482	12.17	H-O-C
4	536.95	29	53	0.44	

Table 3: XPS spectrum of O1s region of CNT electrode modified with CtCDH and PVA. Band 1 indicates the chemical state of inorganic oxides, whereas bands 2-4 represent the oxygen content in the state of organic oxides.

In the same sample, the nitrogen chemical state was acquired from XPS spectra (Figure 16) of the region around 400 eV. This indicates the nitrogen present in organic binding.

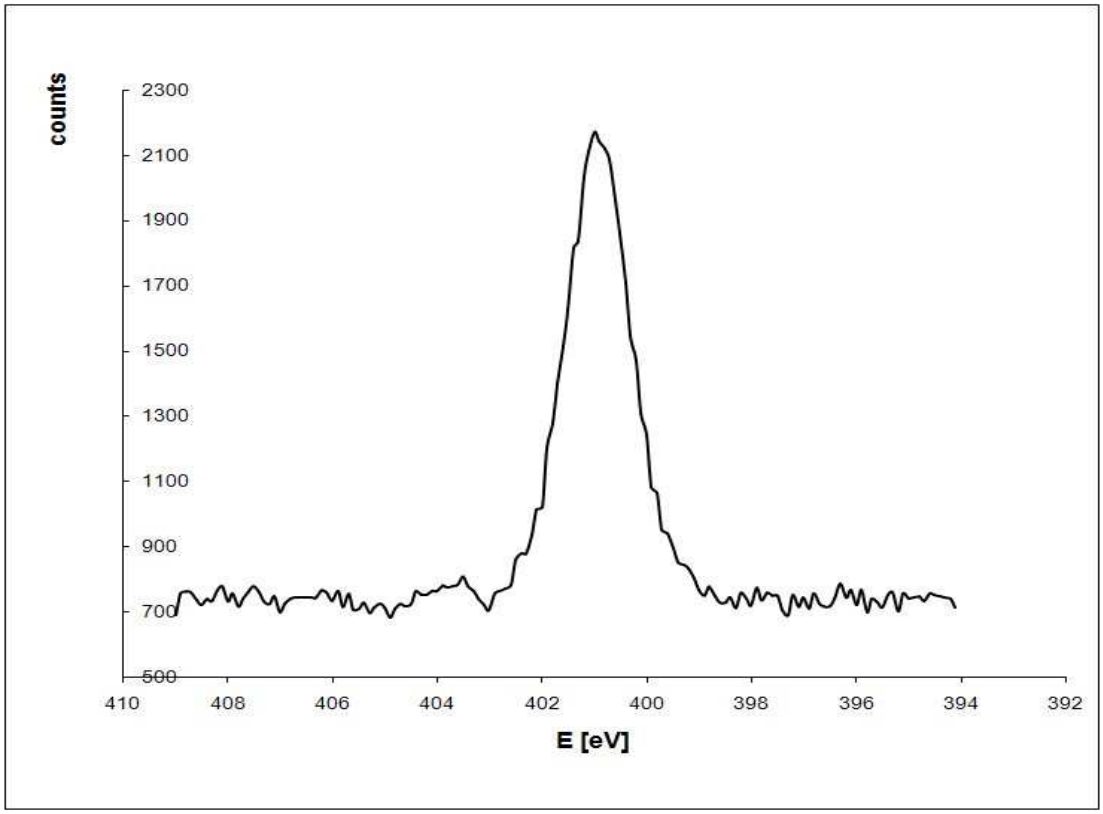

Figure 16: Nitrogen 1s spectrum of CNT modified with both CtCDH and PVA.

Band	Position	Height	Area	% Area	Bond
1	400.92	1402	2468	100.00	N-C

Table 4: XPS spectrum of N1s region of CNT electrode modified with CtCDH and PVA.

All nitrogen present in the sample originates in CtCDH, as spectra of both unmodified and PVA modified electrodes did not include the nitrogen peaks. The presence of the nitrogen 1s peak in the case of CtCDH modified electrode and the absence of this peak in the cases of pristine CNT and PVA functionalized electrodes is the clear evidence of the successful enzyme immobilization.

5.4 Cyclic voltammetry measurements

The electrochemical behaviour of pristine CNT electrode and modified CNT electrodes was characterised by cyclic voltammetry in unstirred PBS, ferrocene methanol and glucose solution, prepared as described in section 4.1.

First, the voltammetric behaviour of the CNT electrode was measured. Figure 17 shows the voltammogram with well defined oxidation and reduction peaks. The peak potentials are 0.349 V for the oxidation peak of segment 1 and 0.236 V for the reduction peak of segment 2, the oxidation and reduction peak currents are 1.229 e^{-4} A and -5.079 e^{-5} A respectively.

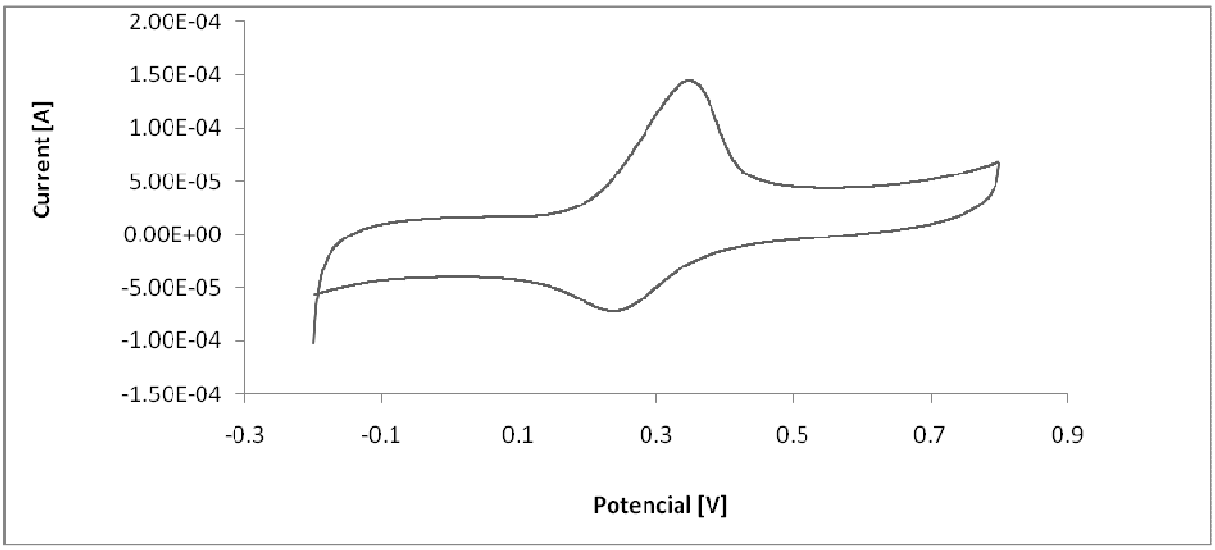

Figure 17: Cyclic voltammogram of unmodified CNT electrode in 1mM ferrocene methanol solution. Scan rate 0.1 V/s.

As seen in Figure 18 (b), the unmodified electrode showed a typical capacitive behaviour with featureless voltammogram [28]. The highly hydrophobic surface caused difficulties in enzyme immobilization and, as a result, glucose could not be measured. The modification with polyvinyl alcohol was performed as demonstrated in [29]. The CNT surface was functionalized with hydrophilic hydroxyl groups and as seen on Figure 19 it clearly became more hydrophilic. In comparison, as seen on Figure 18 (a), the PVA modified electrode showed a pair of redox peaks, as oxidation peak at 0.18 V and reduction peak at 0.07 V are observed. Those peaks are more likely attributed to reduction and oxidation of haem group of the enzyme.

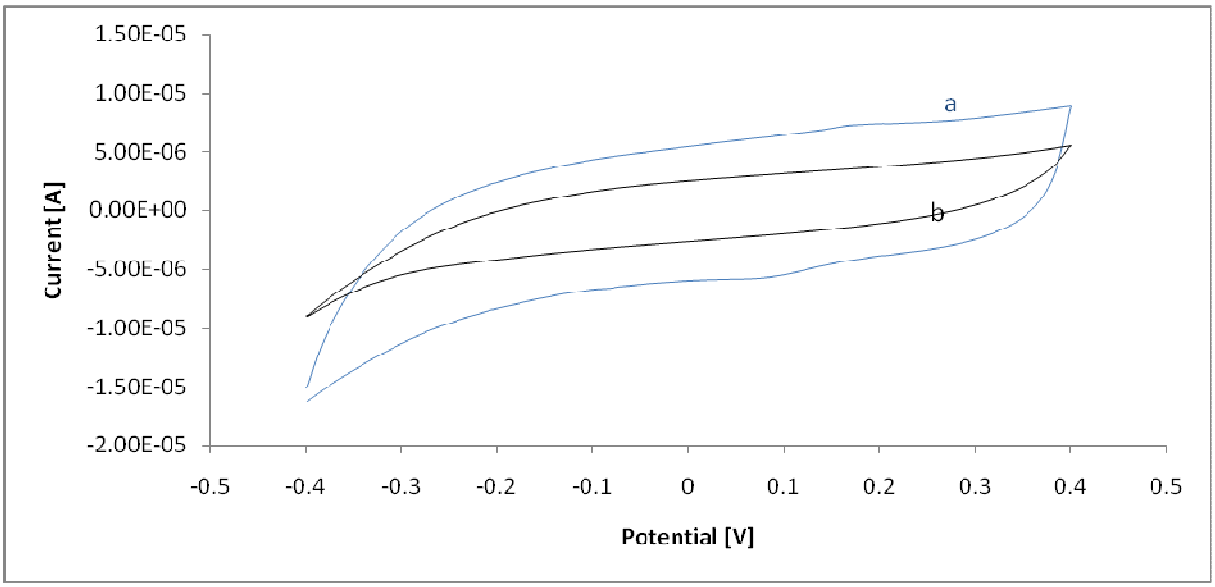

Figure 18: Cyclic voltammograms of CtCDH modified CNT electrode in 10 mM PBS solution as background electrolyte: (a) electrode modified with PVA, (b) no PVA modification performed. Scan rate 0.05 V/s.

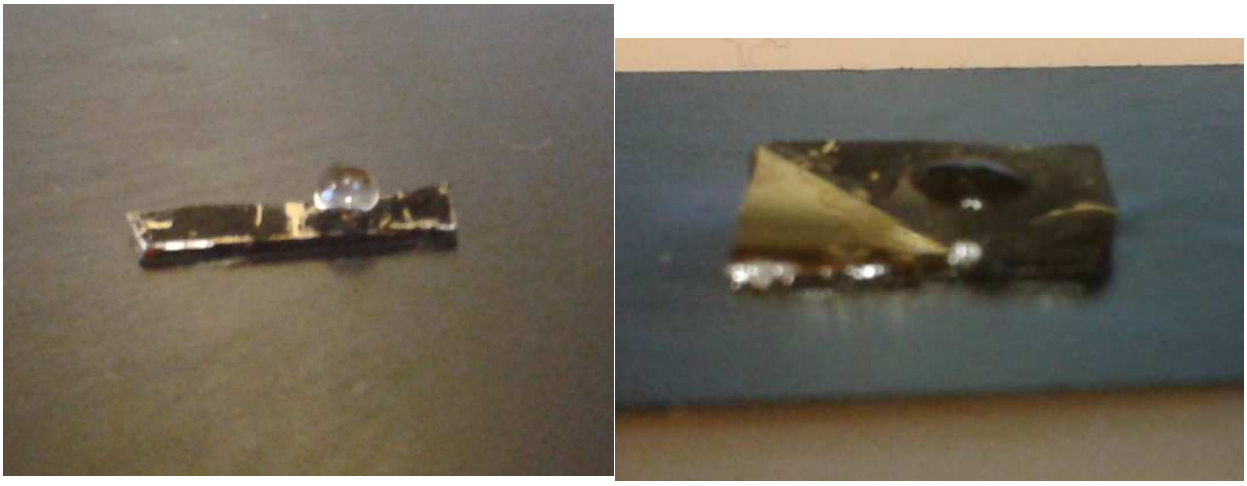

Figure 19: Modifying the CNT electrode with PVA. Unmodified CNT (left), CNT modified with PVA (right). The larger contact angle indicates that the surface has become more hydrophilic upon modification.

Further voltammetric experiments were performed by the PVA functionalized CtCDH modified CNT. Figure 20 shows the response of the electrode in (b) PBS solution as the background electrolyte and (a) with addition of glucose (156 mM solution in 10 mM PBS) as a substrate of CtCDH. As seen in Figure 20 (a), the addition of glucose causes a significant increase of the current, evolving electrocatayltic current, starting at around -0.05 V.

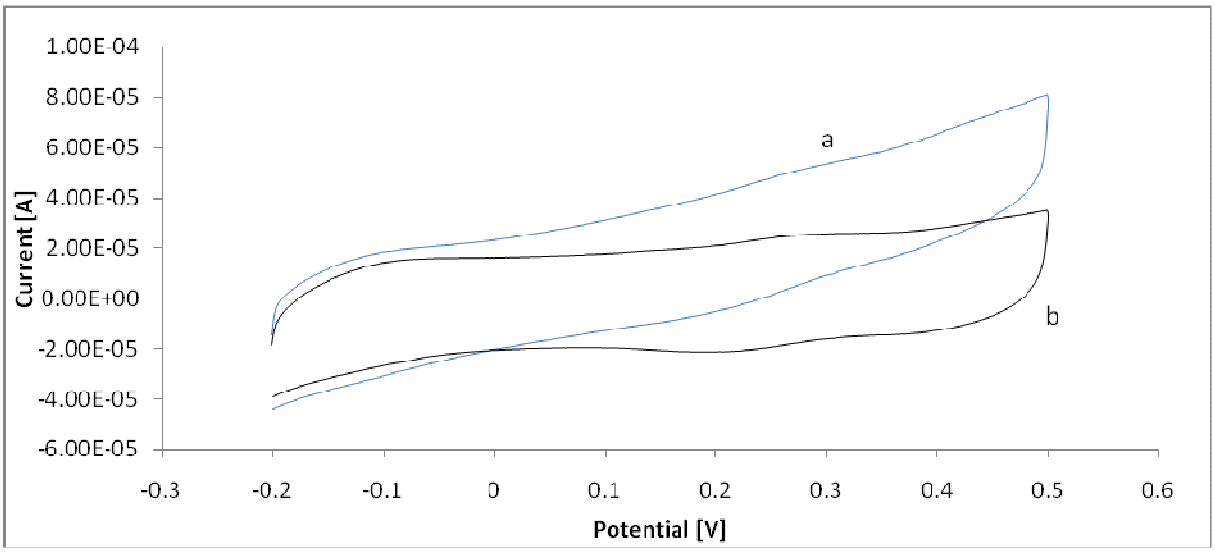

Figure 20: Cyclic voltammograms of CtCDH modified PVA functionalised CNT electrode in (a) 156 mM glucose in 10 mM PBS and (b) 10 mM PBS. Scan rate applied was 0.1 V/s.

As previously demonstrated in XPS measurements (section 5.3), Figure 20 confirms the hypothesis of modifying CNTs with PVA being a crucial step in enabling of successful immobilization of CtCDH onto the CNT surface.

5.5 Optimization of the working conditions of the biosensor

Amperometric measurements were carried out in order to study the behaviour and characteristics of the biosensor for measurement of glucose.

5.5.1 Working conditions optimalisation

First, the optimal conditions for measurements were found. Experiments with CNT electrode were performed in flow-injection mode to optimise the flow rate and the working potential.

Working potentials of 100 mV, 200 mV and 300 mV were applied and the current response was measured. Measurements were carried out in 50 mM glucose solution using the flow rate of 1 ml/min. Working potential of 300 mV versus Ag|AgCl 0.1 M KCl appeared to show more than double the response of other potentials (Figure 21). Lower potentials of 100 mV used in previous work [5, 30, 31] did not show satisfactory response to provide

measurements with lower glucose concentration. Higher potentials than 400 mV applied for long time can cause irreversible damage to the enzyme [32] and therefore were not tested.

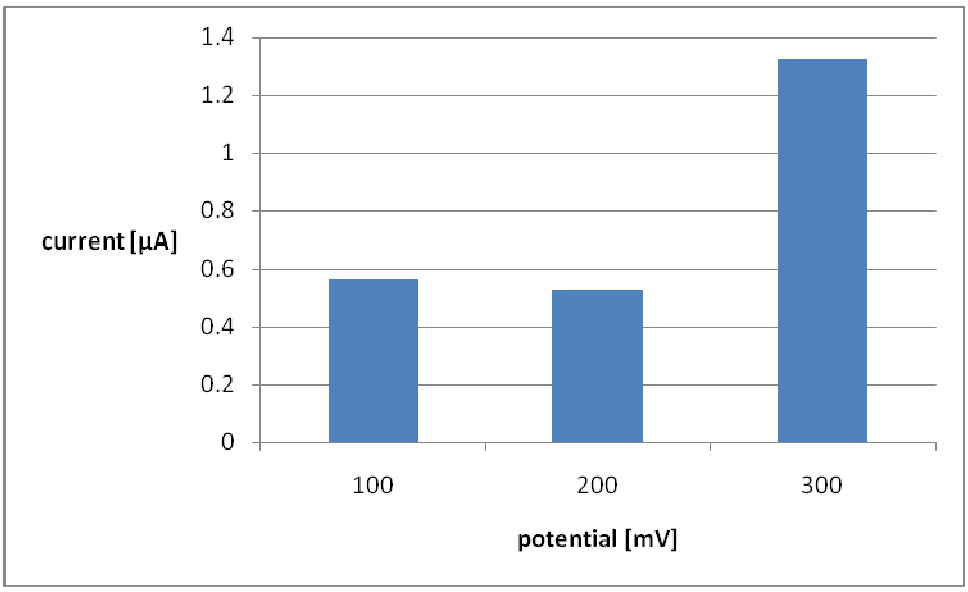

Figure 21: Dependence of the response current for glucose on the applied potential. Injections of 50 mM glucose in 10 mM PBS were measured at flow rate of 1 ml/min using CtCDH modified and PVA functionalized CNT electrode.

Further measurements were performed to optimise the flow rate. As suggested by previous work [5, 30], the rates of 0.5, 1.0 and 1.5 ml/min were tested by measuring the current response of different glucose concentration injected. Figure 22 shows the flow rate of 0.5 ml/min provides the highest analytical response. However, the response is not rapid enough and the measurement time is inconveniently long for the purpose of the intended application. Therefore the flow rate of 1.0 ml/min was chosen for further measurements, being a combination of both satisfactory current response and the response time.

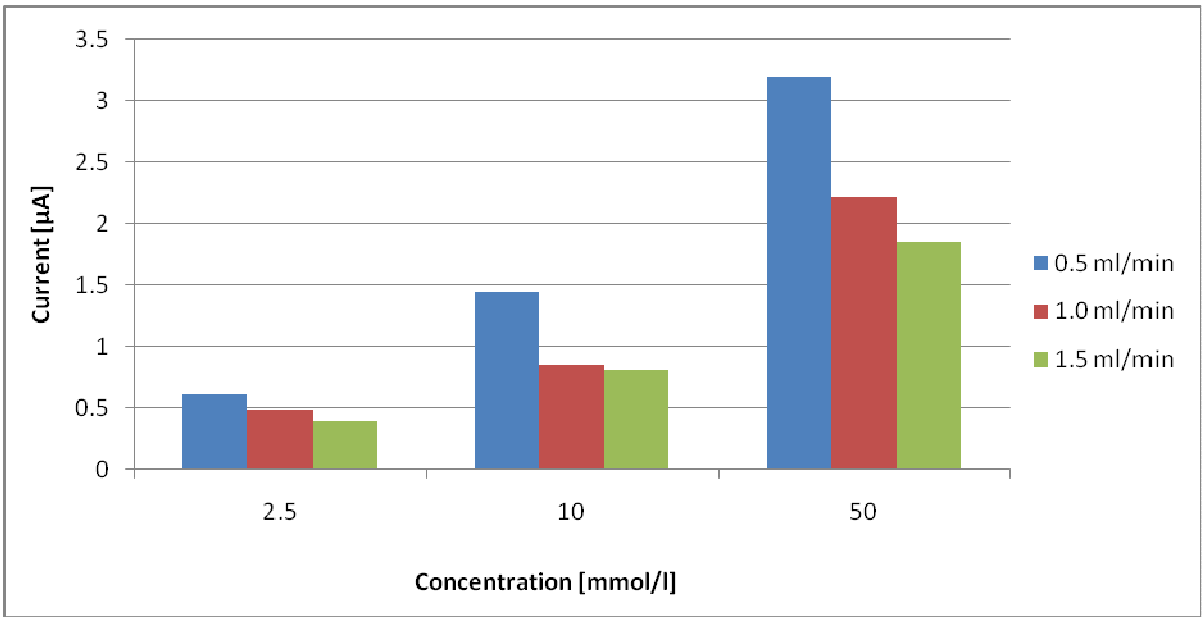

Figure 22: Dependence of the current response on glucose concentration. Three different flow rates are compared (0.5, 1.0, 1.5 ml/min). Injections of 2.5, 10 and 50 mM glucose in 10 mM PBS were measured. Potential applied was 300 mV versus Ag|AgCl 0.1 M KCl on the CtCDH modified and PVA functionalized CNT electrode.

5.5.2 Glucose calibration

To obtain the values of catalytic current depending on glucose concentration, the flow-injection amperometric measurements were carried with CNT electrode modified with both PVA and CtCDH. Glucose solutions (in PBS) of concentrations 1, 2, 5, 10, 25, 50 and 100 mM were injected and the current was measured, as seen in Figure 23. The calibration obtained by this method shows the linearity in the range of glucose concentrations of 1 – 25 mM (Figure 24). The linearity range of the electrode is in accordance with previously reported biosensors employing CtCDH [5].

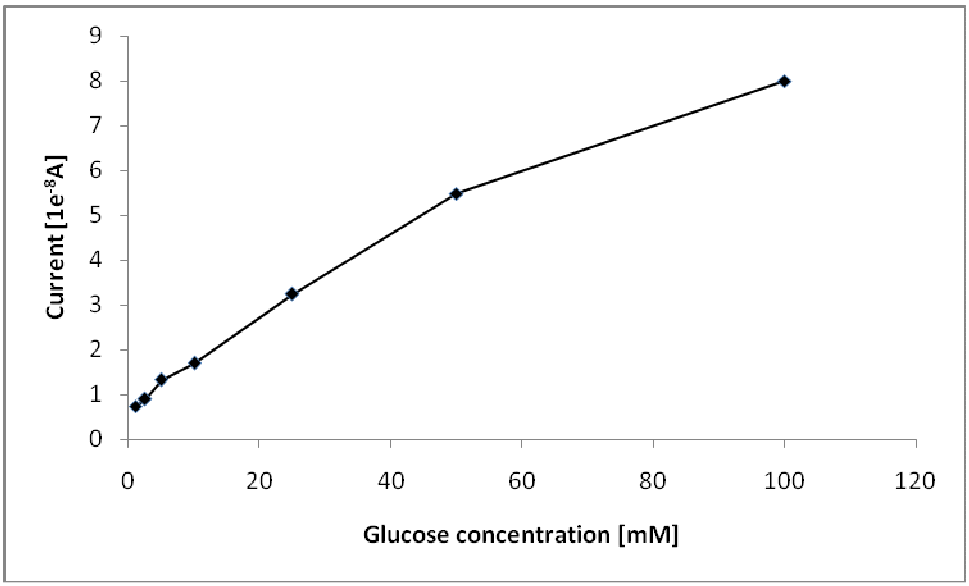

Figure 23: Calibration curve of the catalytic current response depending on glucose concentration. Performed in 10 mM PBS (pH 7.2) with a flow rate of 1 ml/min and a working potential of 300 mV versus Ag|AgCl 0.1 M KCl.

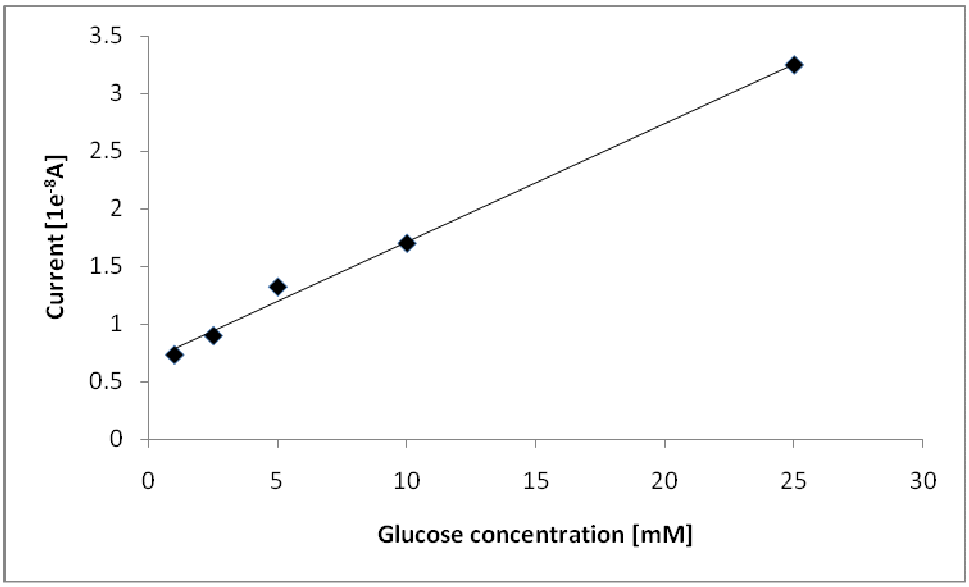

Figure 24: The linear range of the catalytic current response depending on glucose concentration. Experiments were performed in 10 mM PBS (pH 7.2) with a flow rate of 1 ml/min and a working potential of 300 mV versus Ag|AgCl 0.1 M KCl. The calibration shows the linearity range (R^2=0.995) in glucose concentrations of 1 – 25 mM.

As seen in Figure 23, the sensor shows linear response in limited range of the tested glucose concentrations. Similar behaviour was observed in previously reported sensor [5]. However,

unlike many reported sensors applying GOx [33], the linearity range of the biosensor presented in this work covers broad range of glucose concentrations. It corresponds with both physiological glycaemia levels (4.9 - 6.9 mM) [9] and pathophysiological levels in diabetic patients that can reach up to 25 mM [5] in the event of hyperglycaemia. Symptoms of hypoglycaemia are developed with glucose levels of 3.3 mM and the episode of hypoglycaemia is defined as blood glucose levels below 2.8 mM [34]. Therefore the application of developed biosensor is also relevant in these extreme conditions.

Furthermore, the control experiments with PVA functionalized CNT electrode without CtCDH modification were performed. The same glucose concentrations were injected in flow injection amperometric analysis, however without any catalytic current response. This indicates that all the responses are attributed to catalytic current generated by the enzymatic reaction between CtCDH and the substrate.

The calibration range of the CtCDH modified CNT electrode is a presumption for its promising application in third-generation glucose biosensor construction for direct measurement of glucose.

Further measurements were carried out in the same conditions with injecting lower glucose concentrations of 0.01, 0.1 and 0.5 mM glucose solutions (in 10mM PBS). None of the concentrations provided a current peak with S/N of 3 or higher. Therefore, the LOD of this sensor was estimated as 1 mM. Compared to previously reported biosensors [5, 30], this is a drawback when considering the application of the sensor in detection of glucose concentrations below 1 mM. However, such low concentrations of glucose are highly unlikely to be present in human blood samples [9] and therefore this fact does not prevent the sensor to be potentially applied in blood glucose measurements.

5.5.3 Operational stability

In order to obtain the information on the electrode's behaviour and stability of catalytic current response in long-term measurements, the amperometric experiments were carried out. The flow-through analysis was performed with injecting 1 ml of 10 mM glucose solution (in 10 mM PBS) in 5 minute intervals for the duration of 6 hours (Figure 25). The sensor showed a stable response with the maximum decrease of initial current of 17.7 %. After 6

hours of continuous measurements, the sensor kept 100 % of its initial activity. In comparison with the previously reported CNT sensors with CtCDH [5, 30, 31, 33], the CtCDH modified CNT biosensor shows similar behaviour and thus a satisfactory stability for its future application.

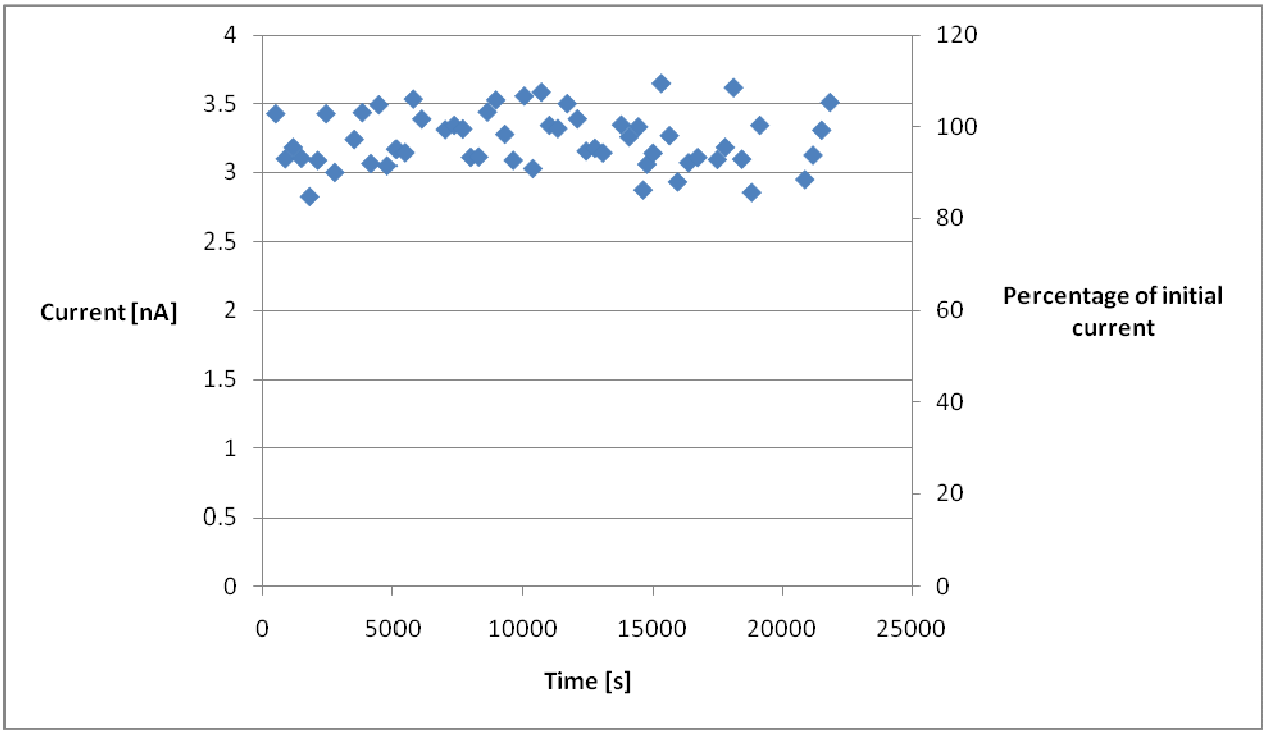

Figure 25: Variation of response for glucose with time obtained for a PVA functionalised CtCDH modified CNT electrode. Experiments were performed with 10 mM glucose (in 10mM PBS) at the flow rate of 1 ml/min and a working potential of 300 mV versus Ag|AgCl 0.1 M KCl.

5.5.4 Interferences of other substances and Nafion coating

One of the major difficulties of providing measurements with human blood samples is the interference of other substances present in the sample. The common interfering agents are naturally occurring ascorbic acid (34-80 µmol/l) and uric acid (178-416 µmol/l) as well as commonly used drug acetaminophen (therapeutic concentrations of 130-150 µmol/l) [35].

In this work, only the interference with ascorbic acid was examined performing the flow-through amperometric measurements in 10 mM PBS injecting 1 ml of 85 µM solutions of ascorbic acid in PBS at the potencial of 300 mV versus Ag|AgCl 0.1 M KCl. Ascorbic acid is

oxidized at the minimum potencial of 200 mV [35]. The measurements showed a significantly higher response than the one of glucose, up to 10 times, overlapping with the response of glucose. As a result, glucose could not be measured in the presence of ascorbic acid. Hence, ascorbic acid critically interferes with the measurements.

To avoid the interference of anionic specimen, additional functionalization of the CNT surface using Nafion coating of the CNT electrode was performed (see section 4.2.2). Nafion is a perfluorosulfonated cation-exchange polymer, selectively incorporating positively charged ions and repelling anion species [36]. Various sensors with Nafion film have been previously introduced [28, 36, 37, 38]. The terminal sulfonate group is crucial for the ion-exchange properties of Nafion (Figure 26).

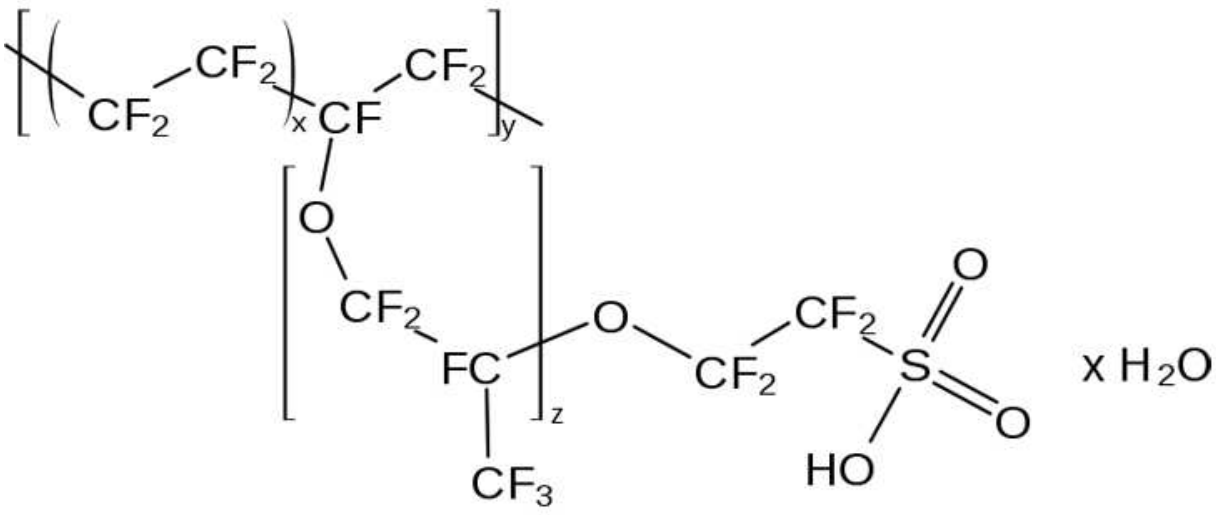

Figure 26: Chemical structure of Nafion.

Nafion-coated electrode was used for same measurements with 85 µM solutions of ascorbic acid. No current response was observed, similarly to previously stated in [37]. However, no response of glucose was detected either. Possible explanation may be that due to high hydrophobicity of Nafion film covering CNT, the enzyme seems appearing to be not immobilized, thus no glucose oxidation catalysis is occured [36]. Therefore, this coating method didn't lead to successful elimination of interference of ascorbic acid with glucose measurements and is not applicable for this purpose. Since Nafion has been widely and successfully used for various selective cationic detections in biosensor construction, it is

advised to perform further measurements with different coating method to enable the enzyme immobilization.

6 Conclusion

A novel type of third-generation glucose biosensor was developed by simple physical immobilization of *Corynascus thermophilus* cellobiose dehydrogenase onto the on-chip grown vertically aligned multi-walled carbon nanotubes. The surface structure and element composition of the nanomaterial was examined by multiple advanced methods including ESEM, EDX and XPS and the electrochemistry performed in both batch and flow-injection modes. The CNT surface modification with polyvinyl alcohol resulted in enhanced hydrophilicity. It was the crucial step in enabling the CtCDH to efficiently attach to the CNT. Under optimal working conditions, the electrochemical measurements revealed a linear range of glucose calibration of 1-25 mM, covering the whole physiological and pathological glycaemia range and thus making the biosensor eligible for future application in the field of blood glucose monitoring. Although the limit of detection of 1 mM showed significantly lowered sensitivity than reported in previous works, it does not interfere with applying the biosensor in the suggested field, as the concentrations of glucose below 1mM are highly unlikely to be found in the samples. A good operational stability was observed, similar to previously introduced CtCDH-based biosensors. The selectivity towards interfering substance was examined. Ascorbic acid significantly interfered with the measurements, resulting in disabled glucose detection. The modification with Nafion coating was carried out in order to eliminate the ascorbic acid electrochemical response. However, this approach led to the loss of sensitivity towards glucose, thus the successful elimination of interference was not achieved. Further investigation is recommended to be performed in order to both efficiently eliminate the interference and preserve the sensitivity towards glucose.

Hence, with exploiting the simple immobilization method, broad linear concentration range, acceptable detection limit and great operational stability, the new biosensor has a potential to be applied in the glucose monitoring devices and other relevant biosensing fields.

7 References

[1] Guariguata L., Whiting D., Weil C., Unwin N., The International Diabetes Federation diabetes atlas: Methodology for estimating global and national prevalence of diabetes in adults. Diabetes Research and Clinical Practice 2011, 94, (3), 322–332

[2] Whiting D., Guariguata L., Weil C., Shaw J., IDF Diabetes Atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Research and Clinical Practice 2011, 94 (3), 311–321

[3] Wright J., Diabetes Mellitus Etiology. Encyclopedia of Food Sciences and Nutrition (Second Edition) 2003, ISBN: 978-0-12-227055-0, 1776–1782

[4] Zhu Z., Garcia-Gancedo L., Flewitt A., Xie H., Moussy F., Milne W., A Critical Review of Glucose Biosensors Based on Carbon Nanomaterials: Carbon Nanotubes and Graphene. Sensors 2012, 12, 5996-6022

[5] Zafar M. N., Safina G., Ludwig R., Gorton L., Characteristics of third-generation glucose biosensors based on Corynascus thermophilus cellobiose dehydrogenase immobilized on commercially available screen-printed electrodes working under physiological conditions. Analytical Biochemistry 2012, 425, 36–42

[6] Ludwig R., Harreither W., Tasca F., Gorton L., Cellobiose Dehydrogenase: A Versatile Catalyst for Electrochemical Applications. ChemPhysChem 2010, 11, 2674 – 2697

[7] Wang J., Lin Y., Functionalized carbon nanotubes and nanofibers for biosensing application. Trends Analyt Chem. 2008, 27 (7), 619–626

[8] Zimmet P, Magliano D., Herman W., Shaw J., Diabetes: a 21st century challenge. The Lancet Diabetes & Endocrinology 2014, 2 (1), 56–64

[9] Ferrante do Amaral C., Wolf B., Current development in non-invasive glucose monitoring. Medical Engineering & Physics 2008, 30 (5), 541–549

[10] Vashist S. K., Non-invasive glucose monitoring technology in diabetes management: A review. Analytica Chimica Acta 2012, 750, 16– 27

[11] Pickup J., Hussain F., Evans N., Sachedina N., In vivo glucose monitoring: the clinical reality and the promise. Biosensors and Bioelectronics 2005, 20, 1897–1902

[12] Newman J., Turner A., Home blood glucose biosensors: a commercial perspective. A review. Biosensors and Bioelectronics 2005, 20, 2435–2453

[13] Szymborska-Kajaneka A., Psureka A., Heseb R., Strojeka K., Self-monitoring of blood glucose in treatment of type 2 diabetes. Diabetes Research and Clinical Practice 2009, 86, S49-52

[14] Cox M., An Overview of Continuous Glucose Monitoring Systems. J Pediatr Health Care 2009, 23, 344-347

[15] Moser E., Morris A., Garg S., Emerging diabetes therapies and technologies. Diabetes Research and Clinical Practice 2012, 97, 16 – 26

[16] Rivas G. A., Rubianes M. D., Rodriguez M. C., Ferreyra N. F., Luque G. L., Pedano M. L., Miscoria S. A., Parrado C., Carbon nanotubes for electrochemical biosensing. Talanta 2007, 74, 291–307

[17] Vairavapandian D., Vichchulada P., Lay M. D., Preparation and modification of carbon nanotubes: Review of recent advances and applications in catalysis and sensing. Analytica chimica acta 2008, 626, 119–129

[18] Palanisamy S., Cheemalapati S., Chen S., Amperometric glucose biosensor based on glucose oxidase dispersed in multiwalled carbon nanotubes/graphene oxide hybrid biocomposite. Materials Science and Engineering C 2014, 34, 207–213

[19] Gutierrez F., Rubianes M. D., Rivas G. A., Dispersion of multi-wall carbon nanotubes in glucose oxidase: Characterization and analytical applications for glucose biosensing. Sensors and Actuators B 2012, 161, 191– 197

[20] Feng W., Ji P., Enzymes immobilized on carbon nanotubes. Biotechnology Advances 2011, 29, 889–895

[21] Settle F. A., Handbook of Instrumental Techniques for Analytical Chemistry, ISBN 0131773380, Chapter 37, 709-719

[22] Harvey D., Analytical Chemistry 2.0, ISBN 0072375477, Chapter 11, 729-747

[23] Paredes A. M., Encyclopedia of Food Microbiology (Second Edition) 2014, ISBN: 978-0-12-384733-1, 693–701

[24] Donald A. M., Polymer Science: A Comprehensive Reference. (Volume 2: Polymer Characterization) 2012, ISBN: 978-0-08-087862-1, 539–545

[25] http://www.google.com/patents/US5389787

Patent US 5389787, A Scanning electron microscope. 06.07.2014

[26] Scoutaris N., Vithani K., Slipper I., Chowdhry B., Douroumis D.,SEM/EDX and confocal Raman microscopy as complementary tools for the characterization of pharmaceutical tablets. International Journal of Pharmaceutics 2014, 470, (1–2), 88–98

[27] Tougaard S., Encyclopedia of Analytical Science (Second Edition) 2005, ISBN: 978-0-12-369397-6, 446-456

[28] Arvinte A., Westermann A. C., Sesay A. M., Virtanen V., Electrocatalytic oxidation and determination of insulin at CNT-nickel–cobalt oxide modified electrode. Sensors and Actuators 2010, B 150, 756–763

[29] Somenath R., Harindra V., Choi W., Vertically aligned carbon nanotube probes for monitoring blood cholesterol. Nanotechnology 2006, 17, 14–18

[30] Safina G. , Ludwig R., Gorton L., A simple and sensitive method for lactose detection based on direct electron transfer between immobilised cellobiose dehydrogenase and screen-printed carbon electrodes. Electrochimica Acta 2010, 55 (26), 7690–7695

[31] Tasca F., Ludwig R., Gorton L., Antiochi R., Determination of lactose by a novel third generation biosensor based on a cellobiose dehydrogenase and aryl diazonium modified single wall carbon nanotubes electrode. Sensors and Actuators B 177, 2013, 64–69

[32] Trashin S. A., Haltrich D., Ludwig R., Gorton L., Karyakin A. A., Improvement of direct bioelectrocatalysis by cellobiose dehydrogenase on screen printed graphite electrodes using polyaniline modification. Bioelectrochemistry 2009, 76, 87–92

[33] Gao Q., Guo Y., Zhang W., Qi H., Zhang Ch., An amperometric glucose biosensor based on layer-by-layer GOx-SWCNT conjugate/redox polymer multilayer on a screen-printed carbon electrode. Sensors and Actuators B: Chemical, 2011, 153 (1), 219–225

[34] Lai J. C. Y., Leung F. H. F., Ling S. H., Hypoglycaemia detection using fuzzy inference system with intelligent optimiser. Applied Soft Computing 2014, 20, 54-65

[35] Nien P. C., Wang J. Y., Chen P. Y., Chen L., C., Ho K., C., Encapsulating benzoquinone and glucose oxidase with a PEDOT film: Application to oxygen-independent glucose sensors and glucose/O_2 biofuel cells. Bioresource Technology 2010, 101 (14), 5480–5486

[36] Shahrokhian S., Zare-Mehrjardi H. R., Application of thionine-nafion supported on multi-walled carbon nanotube for preparation of a modified electrode in simultaneous voltammetric detection of dopamine and ascorbic acid. Electrochimica Acta 2007, 52 (22), 6310–6317

[37] Mueller K., In vivo voltammetric recording with nafion-coated carbon paste electrodes: Additional evidence that ascorbic acid release is monitored. Pharmacology Biochemistry and Behavior 1986, 25 (2), 325–328

[38] Rocha L. S., Carapuça H. M., Ion-exchange voltammetry of dopamine at Nafion-coated glassy carbon electrodes: Quantitative features of ion-exchange partition and reassessment on the oxidation mechanism of dopamine in the presence of excess ascorbic acid. Bioelectrochemistry 2006, 69 (2), 258–266

8 Apendices

8.1 List of figures

8.2 List of tables

9 Souhrn

Cílem této práce bylo charakterizovat vlastnosti nového amperometrického biosenzoru, vyvinutého s použitím mnohostěnných vertikálně uspořádaných uhlíkových nanotrubic (CNT), syntetizovaných na čipu metodou plazmou iniciované chemické depozice z plynné fáze. Enzymatický biosenzor pozůstával z vrstvy CNT, na které byl imobilizován enzym celobiosadehydrogenasa, produkován houbou *Corynascus thermophilus* z oddělení Ascomycota. Pro imobilizaci enzymu byla zvolena jednoduchá metoda fyzikální adsorpce na povrch CNT, zajišťující zachování konformace a aktivity enzymu. Vlastnosti CNT a enzymu, umožňující přímý elektronový transfer mezi enzymem a nanomateriálem, byly předpokladem k vyvinutí biosensoru třetí generace bez použití elektrochemických mediátorů. Tato práce se zaměřila na zjištění možnosti budoucího uplatnění tohoto amperometrického biosenzoru pro monitorování koncentrace glukosy ve fyziologických podmínkách.

K charakterizaci struktury povrchu CNT a jejich chemického složení byly využity moderní instrumentální metody. Měření rastrovacím elektronovým mikroskopem potvrdilo vertikální uspořádání trubic a umožnilo pozorovat tvar a rozměry CNT vrstvy. Elementální složení bylo popsáno pomocí energiově disperzní spektroskopie.

Vzhledem k výrazné hydrofobicitě nanomateriálu byla provedena modifikace povrchu CNT polyvinylalkoholem (PVA) o koncentraci 0,0001 % (w/v). Funkcionalizací CNT pomocí PVA byla docílena zvýšená hydrofilie povrchu, která znamenala předpoklad k umožnění imobilizace enzymu na CNT. Metodou rentgenové fotoelektronové spektroskopie (XPS) bylo pozorováno chemické složení nanomaterialu. Výsledky měření potvrdily úspěšnou imobilizaci na povrch CNT modifikován PVA.

Elektrochemické vlastnosti senzoru byly zkoumány pomocí amperometrie a cyklické voltametrie. Voltametrické měření potvrdily nutnost modifikace CNT povrchu s použitím PVA pro docílení úspěšné imobilizace enzymu a tedy možnosti měření glukosy. Optimální podmínky pro amperometrické měření v průtokové cele s nástřiky glukosy zahrnovaly konstantní průtok 0,5 ml/min a konstantní napětí 0,3 V. Za těchto podmínek poskytoval senzor linearitu v rozmezí 1-25 mM glukosy, s limitem detekce 1 mM. Uvedený rozsah

pokrývá jak fyziologické tak patologické rozmezí hodnot glykémie a proto dává senzoru předpoklad pro budoucí využití pro měření krevní glukosy. Ačkoliv senzor vykazuje výrazně nižší senzitivitu, než je popsáno u dřívějších výsledků, tento fakt není v rozporu s použitím senzoru v navrhované oblasti, jelikož je výskyt koncentrací nižších než 1 mM v lidské krvi nepravděpodobný.

Při měření v průtokové cele po dobu 6 hodin s nástřiky relativně vysoké koncentrace glukosy (10 mM) v pěti minutových intervalech vykazoval senzor výbornou stabilitu, s maximální variací odezvy 17,7 % a se zachovávanou počáteční odezvou na konci měření. Operační stabilita korespondovala s dříve popsanými senzory.

Vzhledem k popsané interferenci některých substancí byly provedeny měření s kyselinou askorbovou. Za přítomnosti kyseliny askorbové byla detekce glukosy znemožněna. Následně byl povrch CNT modifikován vrstvou ionto-výměnného polymeru Nafionu za účelem odstranění elektrochemické interference kyseliny askorbové. Uvedenou modifikací povrchu CNT avšak došlo ke ztrátě senzitivity pro glukosu. Ve výsledku proto nebyla tato metoda aplikovatelná pro eliminaci interferencí.

S využitím jednoduché metody imobilizace enzymu, širokým rozsahem linearity pro měření glukosy, přijatelnou mezí detekce a dobrou operační stabilitou má vyvinutý biosenzor potenciál být využit v zařízeních pro monitorování glukosy a jiných příslušných oblastech.