บทที่ 1
บทนำ (Preface/Introduction)

1.1. ความสำคัญและที่มาของปัญหาการวิจัย (Rationale/Motivation)

Amperometric enzyme-based biosensors are routinely used tools in clinical diagnosis, personal health care, the process control in food industry and biotechnology and environmental monitoring. The variety and complexity of potential samples and the need of the sensor market for economical mass fabrication request constant advances in the simplicity/reliability of sensor manufacture and permanent progress of sensor quality as to sensitivity, selectivity, detection limits, interference resistance and stability.

This project intended to tackle this challenge and work was proposed for optimizing the architecture of the polymeric immobilization layer that keeps in place (immobilizes) enzymes as the active key sensor component on top of inert working electrodes. Use of a dense network of electrically conductive microfilaments or -spheres was proposed not only to simplify the formation of well-working immobilization layers on electrochemical detector surfaces but at the same time also to considerably improve the effectiveness of the amperometric signal transduction scheme that is normally used for monitoring ongoing substrate-enzyme interaction and thus substrate concentration. Easier fabrication and good response to the analyte (substrate) were aimed at as significant achievements and project contribution to the highly active field of biosensor research. Potential interest is seen in the related scientific and industrial communities.

1.2. วัตถุประสงค์ของโครงการวิจัย (Objectives)

1. General goal of the project was the establishment of an easy to perform and highly reproducible procedure for the fabrication of amperometric enzyme biosensors. Simpler to prepare, the targeted sensors should display the same or even better sensitivity, detection limits and long-term stability as currently available ones.

2. More specifically the target is the utilization of a dense network of, for instance, electrically conductive carbon nanotubes (CNTs) as "immobilization matrix of amperometric enzyme biosensors.

Enzyme fixation in a conductive network would ensure that instead of only the carrying electrode surface the complete immobilization layer could serve as electrochemical detector platform for analyte quantification, which obviously would enhance detection efficiency and thus be advantageous for the sensor performance.

1.3. ขอบเขตของการวิจัย (Framework)

Modern (amperometric) enzyme-based biosensors are highly developed analytical tools [1-4]. However, manufacturers are permanently forced to improve sensor performance in order to meet the user's demands due to the complexity and variety of their samples of interest and economic restrictions. Firm but gentle (biocompatible) fixation of enzymes into thin polymer coatings on a transducer surface, a polarized electrode in case of amperometric biosensors, and efficient detection of enzyme/substrate interaction are at the heart of biosensor construction since it is the immobilized biomolecule and its impact on particular substrates (analytes) that gives this type of analytical device its selectivity and sensitivity for the matching substrate [5, 6]. Optimizing immobilization and tailoring the chemical architecture of the immobilization layer hence are among the strategies for increasing biosensor sensitivity, lowering detection limits and response time and expanding long-term stability. Within this area of activities the planned experiments aimed on (i) simplification of the deposition of the top coats of biosensors and (ii) modification of the functional layers with a network of electrical conduits and an associated improvement of sensor performance.

1.4. ประโยชน์ที่ได้รับจากการวิจัย (Proposed output) ประโยชน์ที่คาดว่าจะได้รับจากงานวิจัยนี้มี 4 ประการหลักคือ

1. Two publications in peer-reviewed international journals with a good impact factor, one for each year of funding.
2. Generation of local human resources (Graduate students, Research assistants) with skills in electrochemical biosensor development
3. New options of protein immobilization for enzyme biosensors with a potential to be commercialized

หน่วยงานที่จะนำผลงานวิจัยไปใช้ประโยชน์

The following institutions may benefit for the project deliveries:

1. Public and private hospitals and their medical laboratories
2. National and international academic research institutions

บทที่ 2
วิธีดำเนินการวิจัย (Methodology)

Figure 1 is a schematic representation of the sequence of actions that lead to the proposed enzyme biosensors with a CNT-based highly porous matrix for enzyme immobilization. In the study glucose oxidase (GOD) served as the model enzyme, and glucose biosensors thus were result and characterized in terms of linear range, detection limit, sensitivity, long-term stability and performance for glucose quantification in model and real samples.

The following text is a short description of the procedure(s) of the making and characterization of the biosensors of this study:

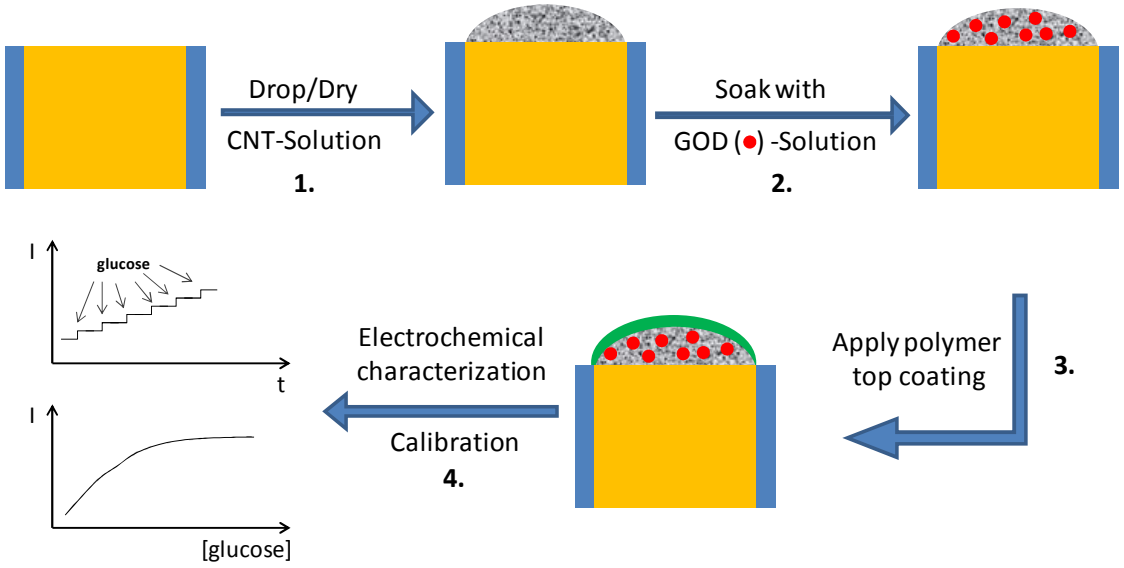

Figure 1: Schematic representation of the steps carried out first for the fabrication of CNT/GOD/EDP-modified Pt disk electrodes that are capable to function as highly sensitive amperometric glucose biosensors and then their characterization via amoperometroc calibration measurements. CNT: carbon nanotube; GOD: glucose oxidase; EDP: electrodeposition paint.

- Substrate electrode for the immobilized enzyme entities were either 3-mm-diameter platinum disk electrodes or 2-mm-diameter carbon disk electrodes.
- Before use, electrodes were cleaned via thorough mechanical polishing; end result had to be a smooth regular surface
- Solutions to be drop-coated on the electrodes (CNT and GOD solution), the cathodic electrodeposition paint (EDP), the glucose stock solution, were all kept in the fridge until use.

- Biosensor preparation starts with the drop-coating with a CNT layer, then came the GOD loading of the CNT deposit, and at the end the electrochemically induced deposition of a polymer film of EDP paint.
- Completed electrodes were stored for 30 minutes in phosphate buffer (removal of loosely bound protein or not precipitated EDP paint) and then overnight in phosphate buffer at 4°C in the refrigerator to allow equilibration.
- Equilibrated biosensors were tested in a beaker-type electrochemical cell. Used for the measurements was a Gamry Reference 600 potentiostat operated in the constant-potential current recording mode (for the construction of I vs. conc. plots), or in cyclic voltammetry mode (for other purposes). Working electrode for the measurements was of course the biosensor (polarized to, for instance, + 600 mV to facilitate the detection of the hydrogen peroxide that is the product of GOD-glucose interaction in the active sensor layer), counter electrode a platinum wire spiral and (pseudo-) reference a Ag/AgCl wire.
- In the calibration measurements the baseline current was allowed to establish wih the biosensor immersed in bare measuring solution (phosphate buffer) , then incremental increases in glucose concentration were applied and the step-like current increases acquired. Calibration curves were constructed by plotting the step-height of current increases vs. actual glucose level of the solution.
- Calibration curves were used to derive the linear range and I_{max}, and K_m values. The sensor sensitivity was measured as the slope of the linear part of the calibration curve.
- Long-term stability was determined with sensors that were stored in buffer solutions in the refrigerator up to a few weeks.
- The analytical properties of the constructed biosensors (e.g. linear range, sensitivity) was compared to published examples and discussed in terms of competitiveness.

บทที่ 3
ผลการทดลองและข้อวิจารณ์ (Results and Discussion)

This summarizing presentation of the outcome of the project is divided in two parts each of them representing the content of a publication that will be prepared to disseminate the related set of specific results on the developed novel biosensor immobilization schemes. The parts are:

I. Untainted Carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors.

II. Carbon nanotube-chitin blends as easy-to-form but well-working enzyme biosensor Immobilization matrices.

3.1 Untainted Carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors

Pure, highly porous networks of CNT (type: P3-SWCNT (purified, high functionality, > 90% carbonaceous purity; source: Carbon Solutions, Inc. Riverside, CA, USA) were placed on the surface of the substrate electrodes via simple application of small droplets of 5 mg/mL COOH-functionalized CNT dispersions in water and let the water evaporate. Repetition of the CNT drop/dry procedure allowed adjustments of the thickness of the CNT deposits and optimization for the purpose of enzyme immobilization. GOD was implemented into the CNT electrode coatings via application of a 5 mg/mL aqueous solution of the enzyme and evaporation of solvent. Repetition of the GOD drop/dry procedure allowed variation of the final load of the CNT network with active enzyme molecules. Loss of the loaded enzyme via outward diffusion was avoided by placement of a top polymer coating. This diffusion barrier was actually established with an industrial cathodic electrodeposited paint that was deposited on top the CNT/GOD electrode modification via application of a constant negative potential. Variables to play with for obtaining different EDP film thicknesses were the deposition voltage and time.

Figure 2 is a summary of the procedure to gain the targeted glucose biosensors with EDP-covered CNT-networks as immobilization matrix for GOD. On both tested substrate surfaces, the platinum and the carbon one, the CNT drop/dry deposition reproducibly led to the formation of dense deposits of the nanotubes with a well-pronounced porosity on the

lower nanometer scale. The effective formation of the desired CNT network that could serve as nicely nanoporous immobilization matrix for biological recognition elements was confirmed by inspections of representative CNT layers at high resolution in a scanning electron microscope (SEM) of the University of a Collaborator at Ruhr-University in Bochum, Germany (see Figure 3).

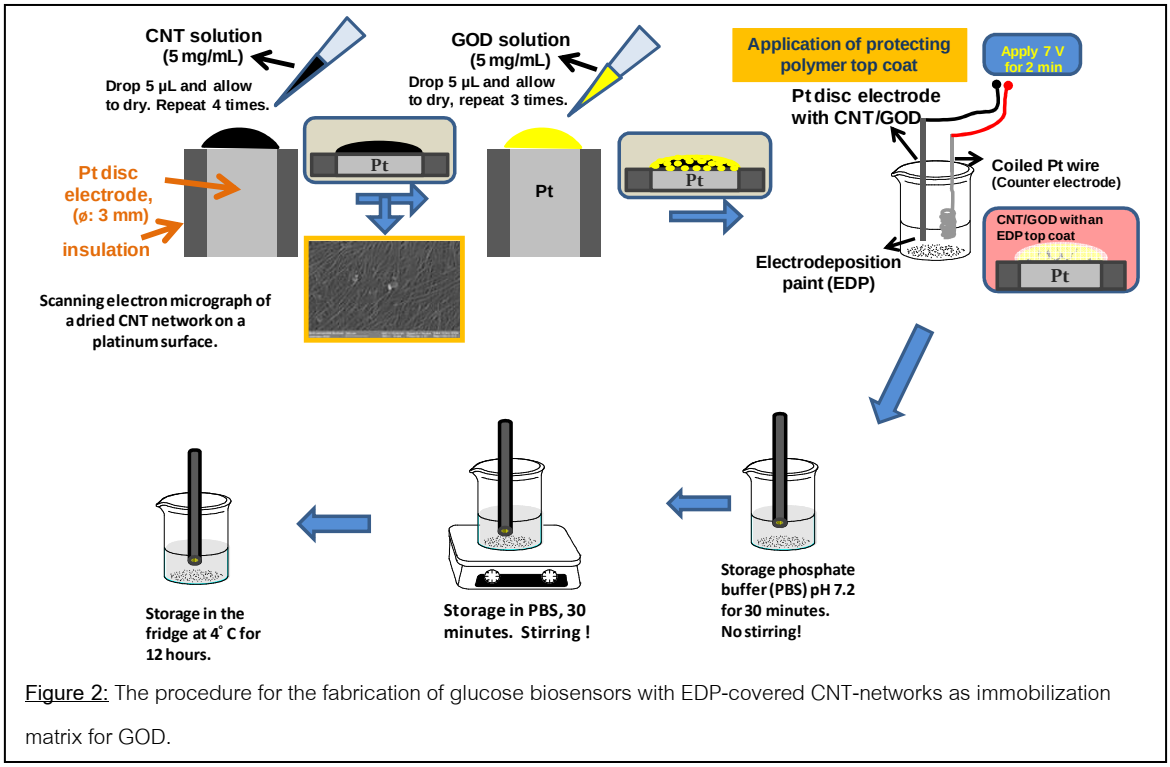

Figure 2: The procedure for the fabrication of glucose biosensors with EDP-covered CNT-networks as immobilization matrix for GOD.

Figure 4 is a representative example of the outcome of one of the many amperometric calibration measurements that have been obtained in course of trials with the CNT/GOD/EDP-based glucose biosensors of this project. Clearly visible are in the amperometric recording (current, I, vs. time, t) the expected rises in current after glucose additions. Extracted from I/t traces was the amplitude of the current steps for the individual glucose additions. The obtained values allowed construction of so-called glucose sensor calibration curves, which are actually plots of the current increments as function of the actual glucose concentration in the measuring buffer. The inset in Figure 4 is the graphical illustration of such a calibration curve. In good agreement with the common behavior of enzyme-based biosensors the current initially increased linearly with [glucose], however, at higher levels of substrate the curve flattened and finally went into saturation.

Important parameters that can be extracted from calibration measurements/curves as shown in Figure 4 are the width of the linear range, the values for I_{max}, k_m (the apparent "Michaelis-Menten" constant = concentration of glucose at $I_{max/2}$) and, via repeated measurements, hints on the long-term stability of the biosensors under trial. Sought after for analytical applications and reliable and accurate substrate concentration determination are stably responding enzyme biosensors with a reasonably wide linear range and high sensitivity (slope of the regression through points in the linear region). Figure 5 shows an example of a set of four calibration curves that were obtained for one and the same CNT/GOD/EDP-based glucose biosensor at different times after preparation. The linear range was good and extending up to about 40 mM in these cases, and did not change significantly over time, at least not in the frame of the tested about two weeks of continuous exposure to the measuring buffer, which is apparently a good sign of the firm- and gentleness of the applied immobilization matrix towards the entrapped enzyme GOD and the related long-term sensor stability.

The execution with the following set of parameters was identified for the preparation of the proposed biosensors with best performance levels:

1. 4 times drop-drying of 5 µl of 5 mg/ml CNT suspension in water
2. 3 times drop-drying of 5 µl of 5 mg/ml GOD aqueous solution
3. 2 minutes cathodic electrodeposition of paint (EDP) at 7 volts
4. (optional: 1 time 5 µl of 10% Nafion solution) FOR BLOOD SERUM MEASUREMENT (see later)

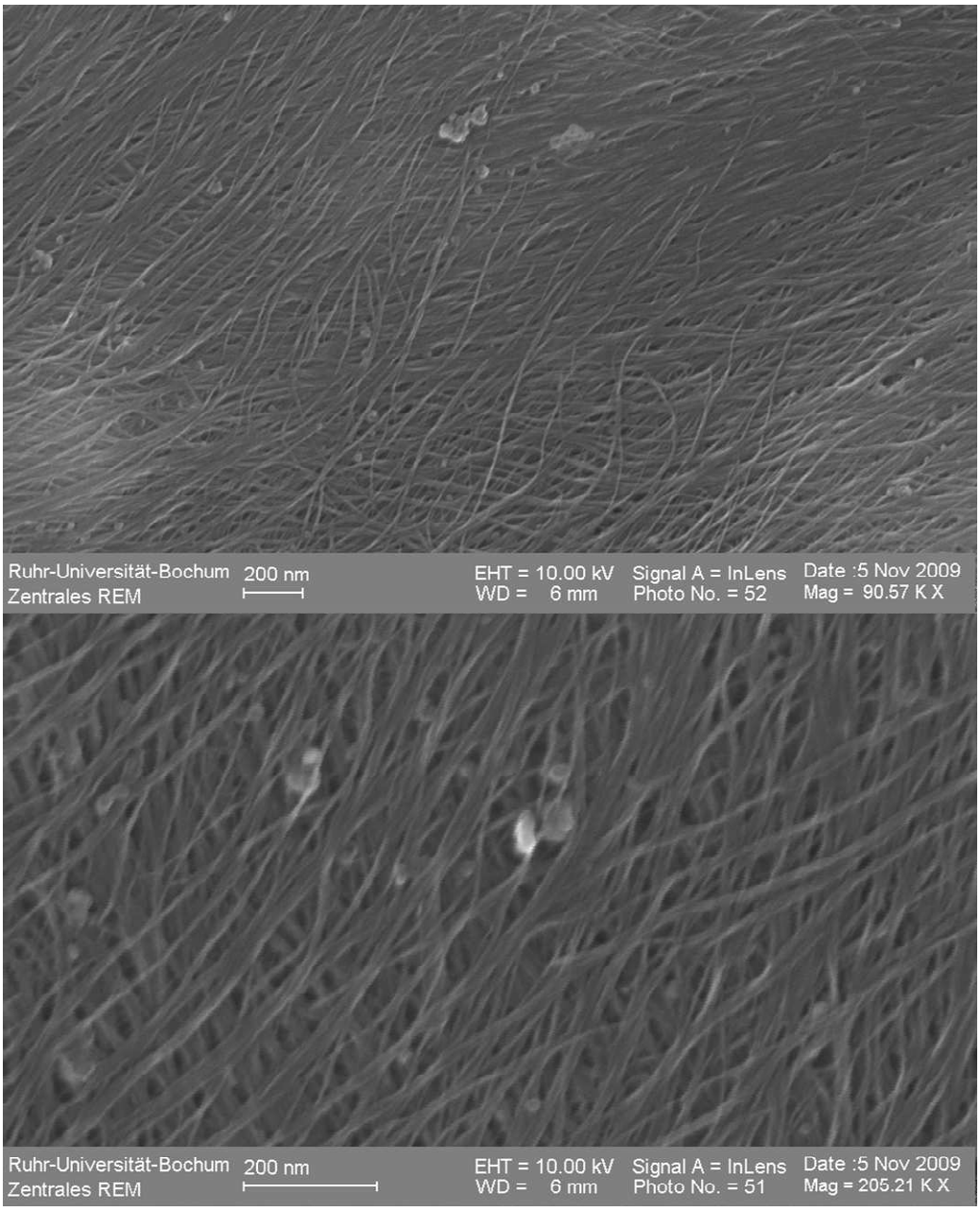

Figure 3: Scanning electron microscope images of dried carbon nanotubes coatings on a metal substrate surface. The top and bottom SEM images are of the same coating, however, at two different spatial resolutions.

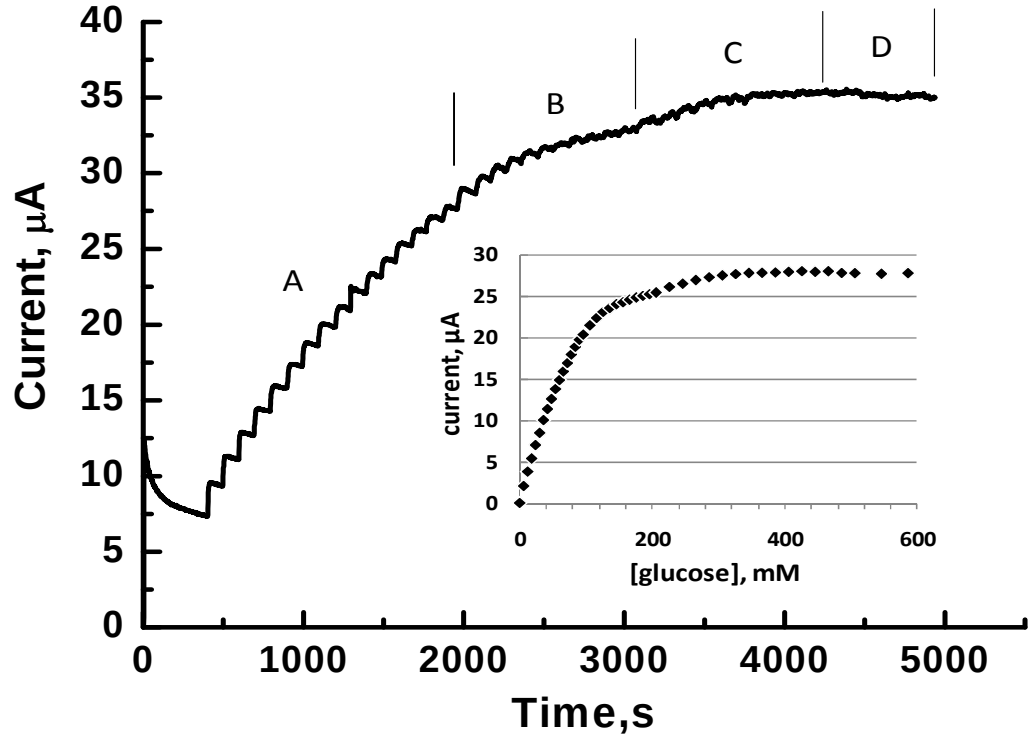

Figure 4: Amperometry response of a CNT/GOD/EDP-modified glucose biosensor to successive glucose concentration increases (6, 10, 20 and 40 mM steps in the A, B, C, and D range, respectively. +600 mV vs. Ag/AgCl was the working potential in PBS measuring buffer at pH = 7. The inset is the calibration curve that was constructed based on the data.

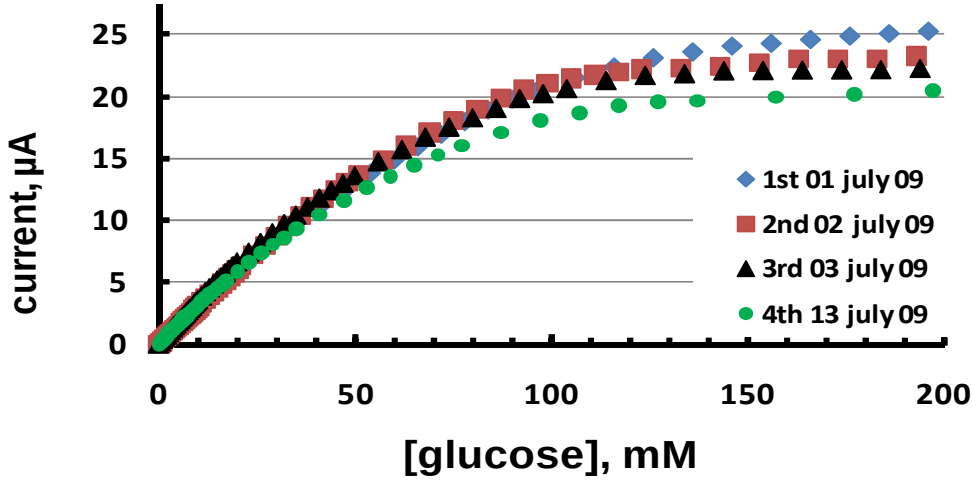

Figure 5: Long-term stability measurements for a CNT/GOD/EDP-modified glucose biosensor. Calibrations were performed with the biosensors at 20 h, 43 h, 69 h and 305 h after completion of their preparation. In between individual calibration experiments the sensors were stored in the refrigerator, but in PBS, pH 7 at 4 °C.

In the literature there are a vast number of original reports are available on the preparation and characterization of GOD-based amperometric glucose biosensors. Details on the analytical properties of the most important variants can be found in one or the other of recently published comprehensive review articles [7-10].

Usually, variations of the nature of the immobilization matrix as well as the supplementary addition of bound or freely diffusing artificial redox mediators replacing gaseous oxygen as the natural partner for the enzyme regeneration or implementation of metal/metal oxide nanoparticles as electrocatalysts for peroxide detection are among the strategy to improve general detection performance. Confirmed values for the linear range are varying from few hundreds of micro- up to several millimolars (mM). Reports on sensors, however, that calibrate linearly up to several tenths of mM are not so many and, if available, they often are associated with complicated fabrication procedures. A representative example that raised recently high attention in the sensor field is a 2009 publication from Claussen et al. in the 11.4 impact factor Amercian Chemical Society journal "*ACS Nano"* with the title "Electrochemical Biosensor of Nanocube-Augmented Carbon Nanotube Networks" (ACS Nano, 3, 2009, 37-44; see also the corresponding press releases entitled "Nano-tetherball biosensor precisely detects glucose" at http://www.physorg.com/news151854328.html and in addition in http://www.nanotech-now.com/news.cgi?story_id=31960). The work describes networks of single-walled carbon nanotubes (SWCNTs) that were decorated with Au-coated Pd (Au/Pd) nanocubes and employed as electrochemical biosensors with low glucose detection limit (~ 1.3 µM) and linear ranges spanning 10 µM to 50 mM.

The topicality of work on improvements of the sensor architecture of glucose biosensors is nicely underlined by the fact that in this year 2012 already 49 articles are listed when searching the Thomson Reuters Web of Knowledge database for the phrase "glucose AND (biosensor or biosensors)"in the title of research articles and limiting the time frame to 2012. Listing the details of all these articles is behind the scope of this report. However, the methodology that was used in this project for biosensor construction produced sensors with a linear range of up to 80 mM in best and 30 – 40 mM in the more typical case (refer to examples in Figure 5 and 6). These values are highly competitive to published alternatives. The sensitivity of CNT/GOD/EDP-based glucose biosensors was in the order of about 5 µA mM^{-1} cm^{-2} and similar to other options.

But while the well-working glucose biosensors here are the outcome of bare drop/dry procedures and a simple electrodeposition of paint published other options are often obtained via complicated multistep fabrication procedures involving e.g. microfabricated supporting templates for nanotubes growth, lithography and complex electrode bioconjugation processes or they are not as good in analytical performance. A discussion of this significant difference between own and published work will be made a major subject of the first manuscript on the type I sensors.

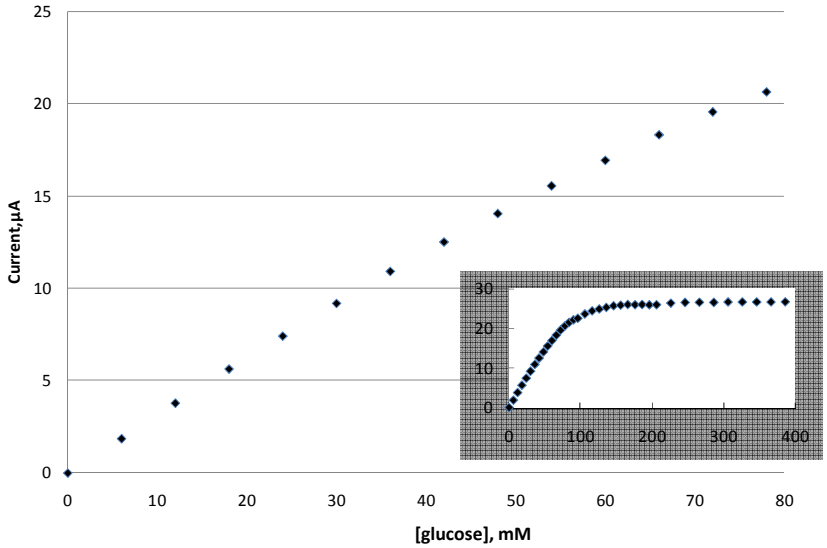

Figure 6: CNT/GOD/EDP-based glucose biosensor with an exceptionally large linear range. Conditions used for the sensor fabrication: 4 times drop-coating of the CNT suspension, 2 times drop-coating of the GOD solution and a final 2-minute EDP deposition at 7 V DC. An amperometric recording (+600 mV vs. Ag/AgCl was the working potential in PBS measuring buffer at pH = 7) revealed the current increases due to successive additions of a glucose stock solution, and allowed the construction of the calibration curve (see inset). Apparently, a linear range of 80 mM is possible.

Important other issues that had to be addressed for a further understanding of the analytical performance of the own CNT/GOD/EDP-based glucose biosensors included (1) the pH dependence of the current, (2) the dependence of the current on the sensor's working potential, (3) the question whether peroxide was detected in the CNT network on top of the faradaic reaction at the platinum surface, (4) the quality of recovery rates for measurements of model samples, (5) an assessment of the detection limit, and, last but not least, (6) the assessment of serum glucose levels in freshly drawn clinical blood samples. All these items will be handled in the order they were listed in the previous sentence.

(1) The pH dependence of the current response of CNT/GOD/EDP-based glucose biosensors:

CNT/GOD/EDP glucose biosensors were subjected to test trials in sample solutions that had a constant glucose concentration (e.g. 1 mM) but varying pH. Figure 7 is a plot of the obtained sensor signals in terms of the amperometric current for peroxide oxidation at +600 mV sensor potential as function of the pH of the measuring buffer solution. In good agreement with both the own expectation and literature, a maximum sensor performance was reproducibly obtained at a close to neutral pH of about 6.8.

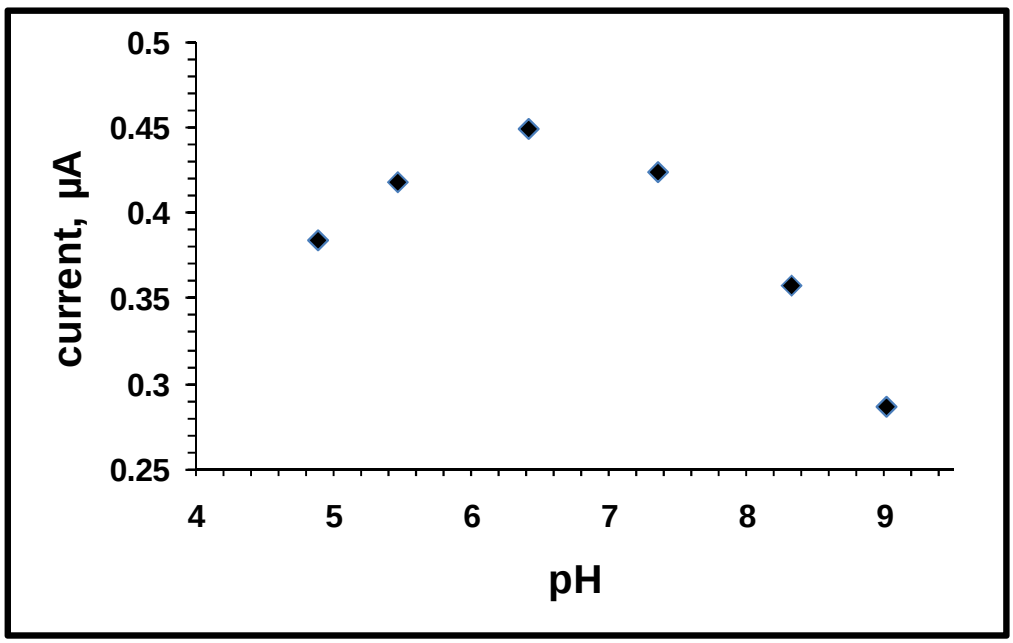

Figure 7: The signal of a glucose biosensor - prepared as shown in Figure 2 - depends on the acidity of the measuring buffer. Conditions of the sensor fabrication: 4 times drop-coating of the CNT suspension, 2 times drop-coating of GOD solution and a final EDP deposition for 2 minutes at 7 V. The current response of the biosensor to an exposure of 1 mM glucose was measured in amperometric recordings (+600 mV vs. Ag/AgCl was the working potential) in solution with a pH between 5 and 9.

(2) The dependence of CNT/GOD/EDP-based glucose biosensor currents on the sensor's working potential

The working potential of a glucose biosensor with hydrogen peroxide detection has to be suitably positive (anodic) to drive the electrochemical oxidation of the analyte at rates causing sufficient anodic current flow. On the other hand, an as low as possible working potential is desired in order to minimize background currents due to the anodic oxidation of interfering compounds that may be present in the measuring solution, at least in the case of real samples. Experiments were thus performed at a given glucose concentration and pH of the test solution but at different operational transducer (Pt disk electrode) potentials. Figure 8 summarizes the outcome of the trials in a plot of the sensor response to 1 mM glucose versus the applied working electrode potential. At the usual working electrode potential of +600 mV vs. Ag/AgCl the signal is of course highest but detectable current were observed at working electrode potentials as low as +400 mV. It should, however, be mentioned that signal generation at working electrode potentials lower than 500 mV vs. Ag/AgCl may not be well suitable for sensitive detection of glucose levels in sample solutions.

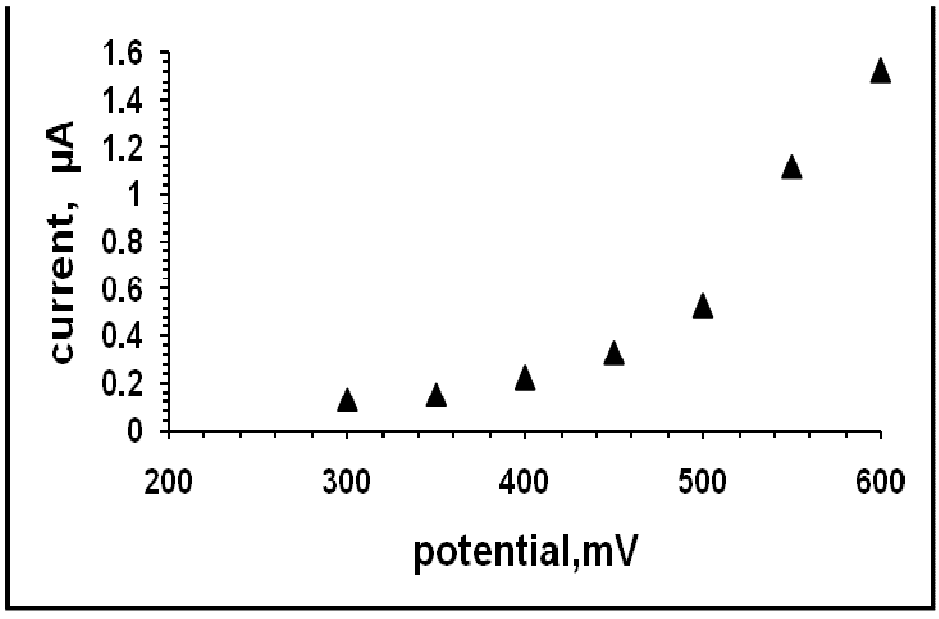

Figure 8: The signal of a glucose biosensor -- prepared as proposed in Figure 1—depends on the working potential of the substrate electrode. Conditions of the sensor fabrication: 4 times drop-coating of the CNT suspension, 2 times drop-coating of the GOD solution and a final EDP deposition. The current response of the biosensor to an exposure of 1 mM glucose was measured in amperometric recordings at working potentials between +300 and 600 mV vs. Ag/AgCl in solutions of pH 7.2.

If for special application reason lower working electrode potentials are desired to exclude the impact of interferents on the analysis of real samples, catalytically active particles (e.g. manganese oxide nanoparticles) may have to be added as additional modifier of the immobilization matrix. This aspect will be part of future work that uses the sensor design here as the basis for the preparation of advanced versions with supplementary catalytically and/or redox active sensor components.

(3) Is H_2O_2 detected at the surface of individual graphitic filaments within the CNT network on top of its faradaic reaction at the platinum surface or not?

At common carbon electrodes H_2O_2 is known to need higher overpotentials for an efficient detection than the + 600 mV vs. Ag/AgCl that are enough at catalytically active Pt electrode surfaces. To proof that a working potential of + 600 mV vs. Ag/AgCl was sufficient to allow H_2O_2 detection at the conductive graphitic filaments of the CNT network, cyclic voltammetry trials were conducted in peroxide containing and peroxide free buffer solutions with a bare carbon electrode (a polished disc electrode fabricated from commercial a pencil lead-type rod of 2 mm diameter), a CNT-modified carbon electrode of the same sort as before and a CNT-modified Pt electrode, all of disk shape. Figure 9 is displaying the obtained voltammograms. In brief, the H_2O_2 electro-oxidation was not really well established at the bare carbon electrode and the corresponding anodic currents rather small. Surface modification with CNT, on the other hand, produced a nice anodic H_2O_2 oxidation wave; currents were multiple times larger than for the untreated carbon electrode and potentials as low as about + 400 mV vs. Ag/AgCl already generated the boundary condition of a steady state anodic H_2O_2 response. When a similar CNT modification was applied to a Pt disc electrode it showed almost the same effect, however, with currents about twice as large, but this may be attributed to the fact that there is already a catalytic drive originating from the covered noble metal carrier electrode. Nevertheless, evidence is provided by the comparative cyclic voltammograms that at a working potential of + 600 mV vs. Ag/AgCl H_2O_2 detection will occur both at the surface of individual filaments within the CNT deposit and the platinum disk face of the substrate electrode of the developed CNT/GOD/EDP-based glucose biosensors.

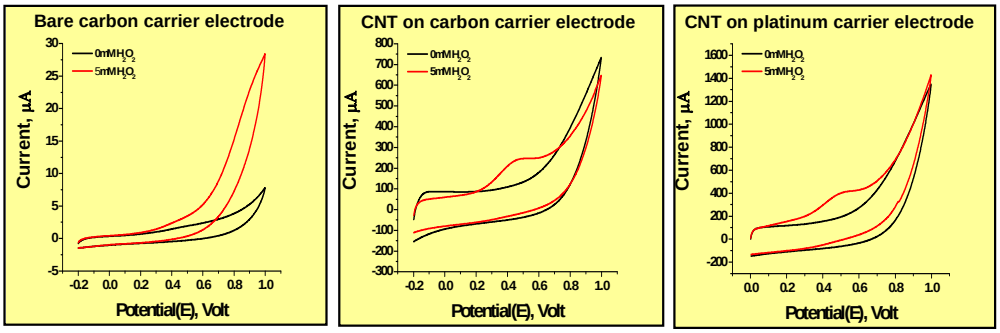

Figure 9: Comparison of the anodic hydrogen peroxide oxidation at a bare pencil lead disk electrode (left), a CNT-modified pencil lead disk electrode and a CNT-modified platinum disk electrode. Bulk level of the peroxide was either 0 or 5 mM in phosphate buffer, pH 7.2. 50 mV/s was the scan rate was for all CVs.

As illustrated in the schematics in Figure 10, this "double"-detection at the substrate electrode and its CNT coating grants a significantly enhanced current collection efficiency as less of the peroxide that is originally produced via GOD/glucose interaction is lost in course of the diffusional spread of the analyte after initial appearance at the location of the protein in the immobilization matrix. An alternative to the use of conductive CNT networks would probably be the incorporation of the biological recognition element (here: GOD) into conducting polymers that, as discussed in a review article for carbon electrodes [11], link the immobilization matrix either electronically or via ionic charge movement to the conducting carrier surface. Nevertheless, the conductivity of conducting polymers is considerably lower as the one of tubular CNTs with their superb, almost metal-like graphitic conductivity. Though not experimentally explored here, the gain of effects as described in Figure 10 is expected to be much less pronounced in the case of bare conducting polymer immobilization. However, worth trying in future work is possibly an immobilization matrix that is a merger of the CNT network with conducting polymers and may provide synergistic support of the amperometric detection of enzymatically produced hydrogen peroxide.

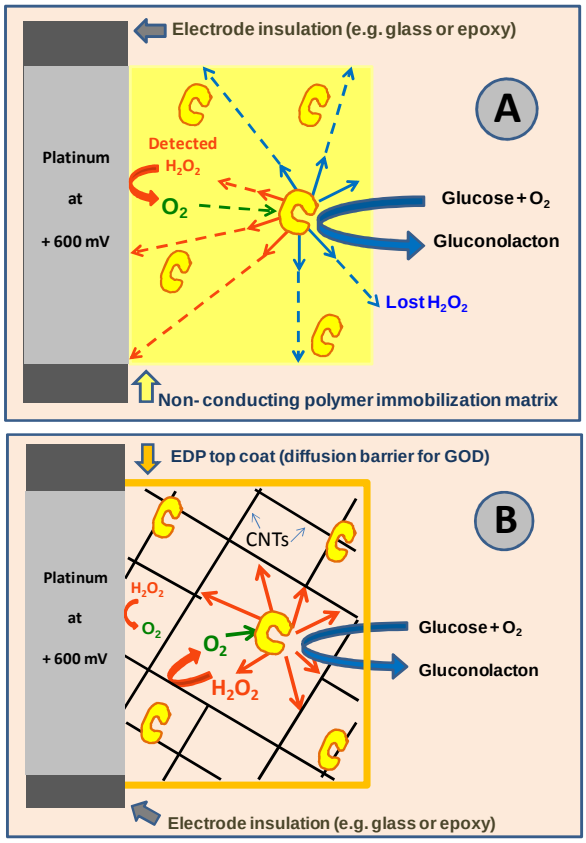

Figure 10: Comparison between the anodic hydrogen peroxide collection at a "normal" glucose biosensor with a non-conducting polymer electrode coating acting as immobilization matrix (A) and the case of an utilization of a conductive CNT deposit as immobilization matrix (B); a platinum disk electrode is assumed to be the substrate electrode. Physical contact of the CNT network with the electrode surface and among conductive individual filaments extends the electrode surface into the immobilization matrix facilitating larger collection efficiency for the anodic peroxide detection.

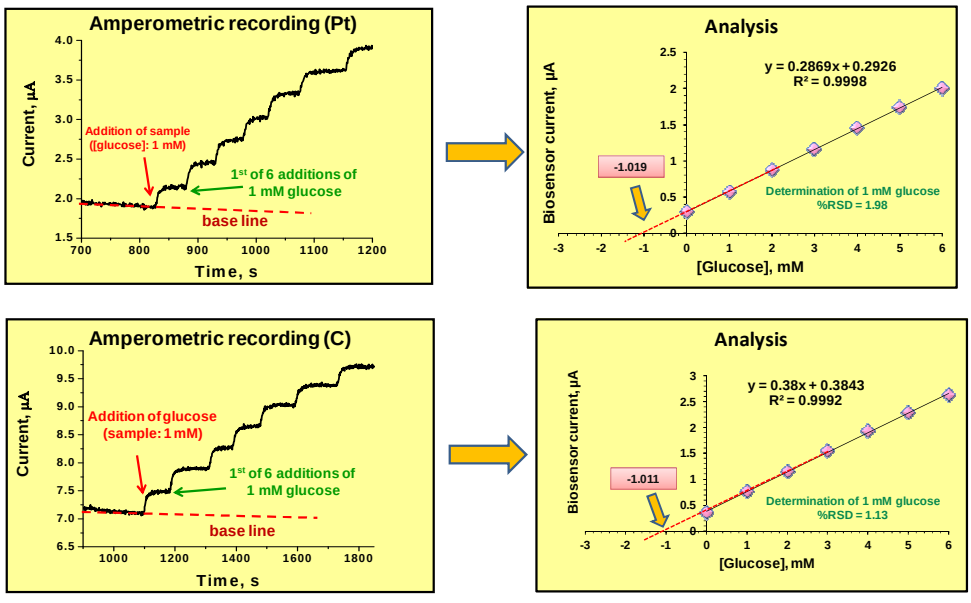

Figure 11: Recovery rate assessment for glucose quantifications in 1 mM model samples with CNT/GOD/EDP-based glucose biosensors that used either a Pt or a carbon carrier electrode. The standard addition method was employed; working electrode (biosensor) potential for the amperometric recordings was in both cases + 600 mV vs. Ag/AgCl and the measuring buffer was PBS, pH 7.2.

(4) Recovery rates for quantitative measurements of model samples with pre-adjusted glucose levels.

1 mM model samples were prepared with measuring buffer and glucose stock solution. CNT/GOD/EDP-based glucose biosensors were then used in the standard addition mode of quantification to assess recovery rates. Figure 11 is displaying two sets of such quantitative measurements obtained with glucose biosensors that had the CNT layer on a platinum or carbon disk carrier electrode, respectively. For the examples shown the experimentally determined glucose levels for the 1 mM samples were 1.019 and 1.011 mM for the Pt and carbon version of the biosensors, which corresponded to excellent recovery rates of 101.9 and 101.1 %, respectively. In the test trials the recovery rates for the two types of CNT/GOD/EDP-based glucose biosensors biosensors the measured concentrations did not deviate more than ± 10 % from the true value of 1 mM reflecting recovery rates for the determinations in the range of 90 - 110 %.

(5) The assessment of the detection limit for glucose quantifications with CNT/GOD/EDP-based glucose biosensors.

To get an idea about the lowest measurable bulk glucose level the analyte was added to the measuring solution at smaller and smaller increments and the smallest increment determined that still was able to create a visible step in the current trace. As can be seen in Figure 12 for the Pt- and pencil lead-based CNT/GOD/EDP glucose biosensors the 62.5 and 31.25 µM concentration levels were the minimum changes causing visible steps in the current recording and thus fix the limit of detection.

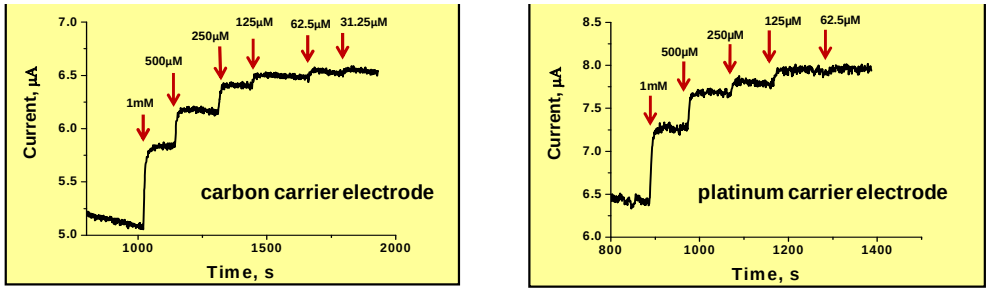

Figure 12: Detection limit assessment for glucose quantifications with CNT/GOD/EDP-based glucose biosensors that used either a Pt or a carbon carrier electrode. Working electrode (biosensor) potential for the amperometric recordings was in both cases + 600 mV vs. Ag/AgCl and the measuring buffer was PBS, pH 7.2. Glucose was added to the measuring solution at smaller and smaller increments and the smallest increment determined that still was able to create a visible step in the current trace.

(6) Quantitative measurements of the glucose levels of blood serum samples.

The most important application of glucose biosensors is the measurement of blood glucose levels [12, 13] and the sensors of this project were challenged with this task, too. Not surprisingly measurements in the normal configuration – that is a CNT/GOD/EDP coating on the transducer electrode - were not successful. As reported many times in the literature an interference of the peroxide-based current by currents from ascorbic (AA) and uric (UA) acid electrooxidation are likely as these two compounds are typically jointly present as anions in the serum as interferents and also oxidizable at the working electrode potential of the biosensor. The strategy that is recommended in literature to avoid an AA and UA signal disturbance is the application of a physical/electrostatic barrier that hinders the negative molecules to reach the buried electrode surface. Mostly widespread in use as protective covering is Nafion, a sulfonated tetrafluoroethylene based film-forming fluoropolymer-copolymer with SO_3H groups that are negatively charged and able to repel anions such as the deprotonated AA and UA. Figure 13 shows the sequence of steps that allowed the preparation of the CNT/GOD/EDP/Nafion glucose biosensors as used for the assessment of blood serum glucose levels.

As electrochemical cell for the determination of the glucose levels in serum samples served a vial of a common 24-well microtiter plate. The electrochemical cell was completed by the immersion of the Pt spiral counter, the Ag/AgCl reference and the CNT/GOD/EDP/NAFION biosensor. The well was filled with 2.6 ml of the usual PBS solution while the amperometric baseline current was recorded and this solution was then topped up with 0.4 ml of the serum to be analysed. The biosensor response in presence of the serum addition was recorded for a while, and was followed by the execution of the standard addition method with sequential supplementation of the well solution by four aliquots of a 100 mM glucose solution.

Figure 14 is a representative example of a serum glucose measurement with a biosensor of the type of this project. Typically the amperometric trace for Nafion-coated biosensors was similar than the ones without the polymer (see, for instance, Figure 11), which meant that the placement of the thin protecting polymer film did not change the mass transport of oxygen and glucose towards enzyme entrapped in the buried immobilization matrix, at least not in a disturbing way that made response times much worse. Quantification in the clinical laboratory of the hospital of the

Suranaree University of Technology (SUT) revealed for that particular sample (#1) a serum concentration of 4.72 mM (85 mg dl^{-1}). The glucose level that was determined with the CNT/GOD/EDP/NAFION biosensor and the standard addition method was 4.77 mM (86 mg dl^{-1}) and thus agreed well with the control value.

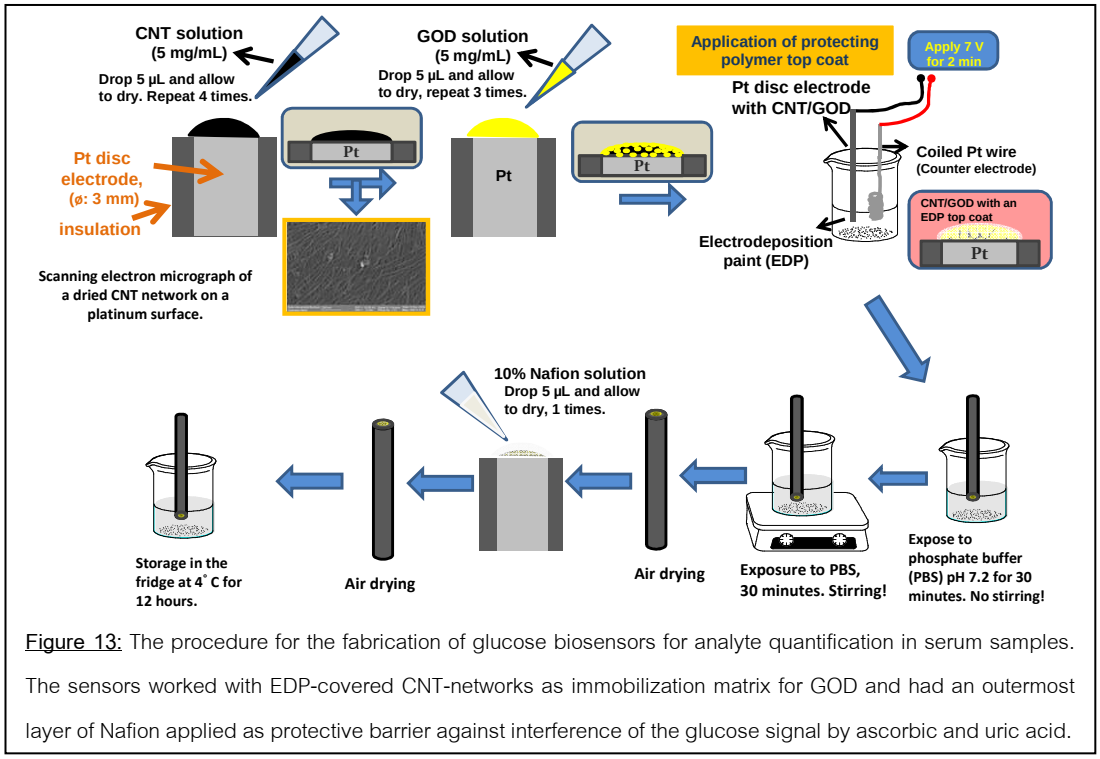

Figure 13: The procedure for the fabrication of glucose biosensors for analyte quantification in serum samples. The sensors worked with EDP-covered CNT-networks as immobilization matrix for GOD and had an outermost layer of Nafion applied as protective barrier against interference of the glucose signal by ascorbic and uric acid.

A total of four measurements was performed for sample (#1) and 5.01 ± 0.36 mM (n=4) was determined as the average serum glucose concentration. Scaled to the 4.72 mM reference value provided by the SUT clinical laboratory this corresponds to a "recovery rate" of 106.1 %, which is competitive to what other published sensor types deliver in terms of performance. For a second sample (#2, hospital reference value 4.67 mM) five repetitive measurements revealed an average serum glucose concentration and "recovery rate" were 4.88 ± 0.38 mM (n=5) and 104.5 %, respectively. With glucose serum levels of 4.72 and 4.67 mM, samples #1 and #2 were associated with normal "non-diabetic" blood. Blood with elevated glucose levels from diabetic persons was not available; however, a portion of a normal serum sample has been spiked with small aliquots of glucose stock solution to make an artificial diabetic blood. Clinical reference glucose level for the spiked blood was 11.77 mM (212 mg dl^{-1}) while the biosensor assessment revealed 12.26 mM ± 1.36 mM (n=4) (n=3). "Recovery rate" in this case was a good 104.2 %.

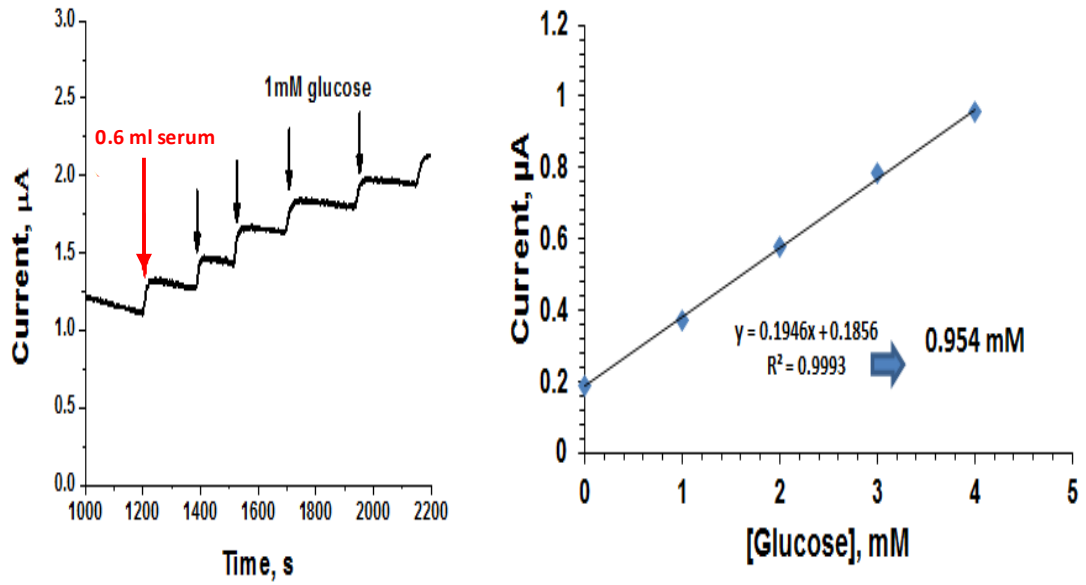

Figure 14: Determination of the glucose concentration of a serum sample. A CNT/GOD/EDP/Nafion-modified glucose biosensor was operated in phosphate buffer of pH 7 in amperometric mode a t a peroxide detection potential of +600 mV vs. Ag/AgCl and the standard addition method used for final quantitation. The dilution factor taking into account, the measured concentration (0.954 mM) refers to 4.76 mM in the original serum sample, which compares well to the 4.72 mM from a reference measurement in a hospital laboratory ("recovery rate" 100.9 %)

In a brief summary the obtained good accordance between the results from the CNT/GOD/EDP/NAFION biosensor serum test trials and the clinical reference values demonstrated that the performance of the developed electrodes for blood glucose determination was adequate and comparable to published alternatives; however, the design/construction chosen here is outstandingly simple as it does not require any special catalytically active nanoparticles or redox compounds as supplementary in the immobilization layer and uses undemanding drop/dry procedures and an easy-to-perform electrodeposition of paint for sensor making. Worth mentioning that all steps of the fabrication procedure are well compatible to mass production (solution drop coating could, for instance, be carried out by pipetting robots and the paint deposition could be automated in similar fashion as in car industry), which is an advantage when aiming on a more routine application in the various fields to which this type of sensor could apply. The design of course still demands proper levels of O_2 in the measuring buffer; however, use of soluble redox mediators or covalent redox modification of the immobilization matrix (CNTs could be carboxlyated and thus chemically adaptable) would offer practical pathways to bypass the problem coming up with an oxygen depletion in the GOD surrounding during analysis.

3.2 Carbon nanotube-chitin blends as easy-to-form but well-working enzyme biosensor immobilization matrices

A material that has been tried in a few published studies as enhancer of the biocompatibility of the enzyme immobilization matrices of sensors is chitin, which is a long-chain polymer of N-acetyl glucosamine and the structural material of, for instance, fungal cell walls, insect and crustacean exoskeletons, mollusc radula and cephalopod beaks. Commercial purified chitin is produced on a large scale by chemical treatment of the chitinous waste from seafood processing or from the silk, mushroom and honey-harvesting industries. There is a general agreement in the literature that chitin is a natural material with an outstanding biocompatibility and a successful medical application of sheaths of the marine biopolymer as beneficial covers higher-degree burns is a good sign of the capacities. Applications usually were through thin and flexible cast chitin membranes - via chitin dispersed in carbon/platinum pastes.

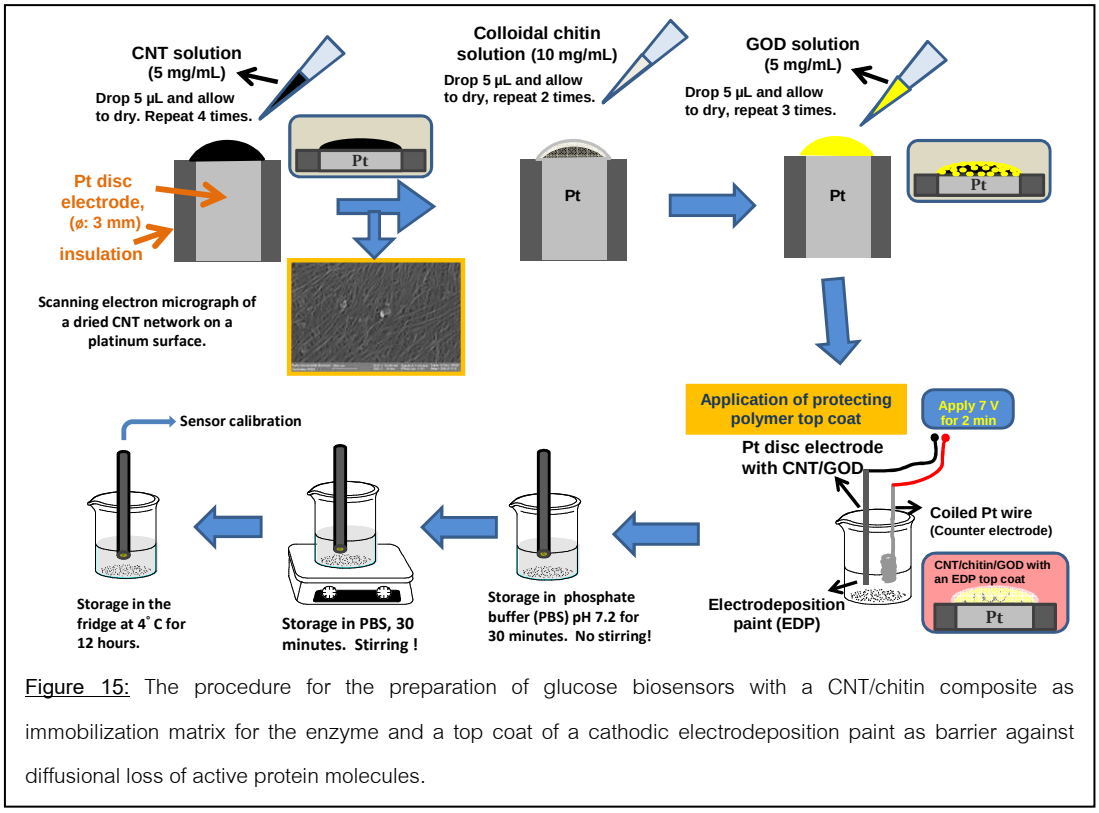

Figure 15: The procedure for the preparation of glucose biosensors with a CNT/chitin composite as immobilization matrix for the enzyme and a top coat of a cathodic electrodeposition paint as barrier against diffusional loss of active protein molecules.

Through a number of chitinase-related research projects of one of the members of the own laboratory environment, actually Associate Professor Dr. Wipa Suginta, access was there to colloidal chitin and the idea came up to test in joint efforts whether a supplementation of the CNT/GOD/EDP immobilization matrix as described under 3.1 would

positively alter the properties of the resulting glucose biosensors. A colloidal chitin/GOD-trunked carbon nanotube (CNT) network was established on the active surface of commercial 3-mm-diameter Pt disk electrodes through sequentially dropping and drying of the aqueous suspensions of CNT, colloidal chitin and a GOD solution, respectively.

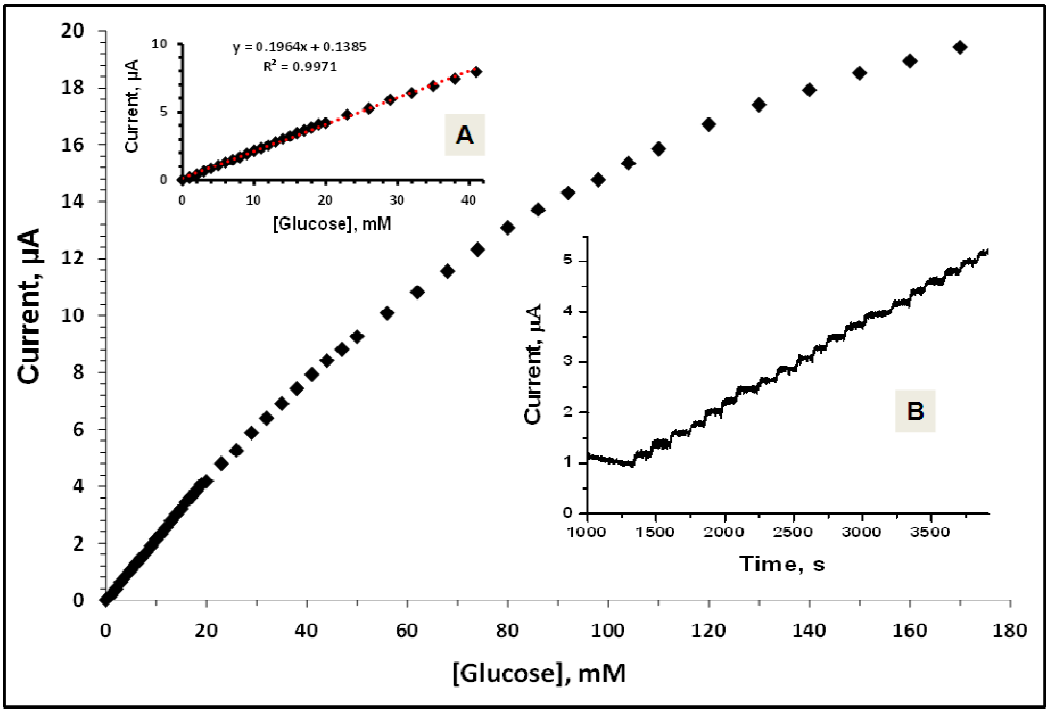

Figure 15: Calibration curve of a glucose biosensor with glucose oxidase immobilized in a drop-coated carbon nanotube/chitin network and a top-coat of cathodic electrodeposition paint to prevent leakage of the enzyme. Recording conditions were: biosensor (working electrode) potential: + 600 mV vs. Ag/AgCl; electrolyte: phosphate buffer solution at pH 7.2; sensor challenge: successive additions of small aliquots of 100 mM glucose stock solution to raise the concentration of glucose from 0 to 178 mM. Insets: (A) Zoom into the linear part of the calibration curve (B) Amperometric current trace showing the sensor response to the first 20 increases of 1 mM of glucose concentration in the electrolyte.

GOD macromolecules that penetrated the CNT/chitin matrix in the third step were prevented from subsequently leaking into the storage and measuring buffer by applying a top layer of cathodic electrodeposition paint (EDP). As before for the sensors with an untainted CNT layer the EDP coat was expected to act as a diffusion barrier after completion of the sensing CNT/Chitin/GOD configuration. Figure 15 shows the sequence of steps that allowed the preparation of the CNT/chitin/GOD/EDP glucose biosensors. Figure 16 then displays the glucose calibration curve of such a sensor together with a presentation of the raw amperometric data for the first 20 increments of 1 mM in the glucose concentration in

the measuring buffer, and the graph of the full range. With linearity extending very nicely to at least 40 mM and a sensitivity of about 3 μA cm^{-2} the chitin-based novel sensor architecture showed promising properties. But the sensor preparation remained to be very simple as, compared to the CNT/GOD/EDP option, only one extra drop/dry procedure with the colloidal chitin suspension was necessary for completion. Work is currently carried out in terms of a more detailed characterization of this type of sensor including an assessment of the long-term stability during storage under dry or wet conditions (which is expected to be improved because of the biocompatibility of the chitin supplement) and an optimization of parameters such as the amounts of CNT/chitin and their relative ratio which may lend the architecture further improved analytical properties.

บทที่ 4

บทสรุป (Conclusion/Summary)

As deliveries of the project were defined:

- An easy to do and reliable manufacture of capable amperometric glucose biosensors.
- Sensors with competitive linear range, sensitivity, detection limit and long-term stability.
- Enzyme fixation in the pores of a conductive carbon nanotube network that was supposed to contribute as H_2O_2 detecting electrode surface to glucose quantification.

It can be said at this point that the completion of the CNT/GOD/EDP-type of immobilization matrix that was established within the frame of this project certainly meets the terms of the proposed simplicity and reliability.

A sequence of easy manual drop and dry steps with bare solutions of CNT and GOD followed by an undemanding electrochemical paint deposition is behind the routine achievement of a well biofunctional surface layer that grants the carrier electrode a good responsiveness toward the captured enzyme's substrate glucose. The protection against anionic interferences as ascorbic acid - a definite requisite for blood (serum) measurements - is as easily gained in form of a negatively charged polymer outer layer via plain drop drying of a diluted Nafion solution. Evidence has been gained that the product of glucose/GOD interaction, that's hydrogen peroxide, indeed is detected at surface of individual filaments of the highly porous CNT network, which thus in addition to the carrier electrode contribute to signal generation. Though for convenience reason more exclusively tested with commercial disk-shaped Pt electrodes, the developed sensor architecture worked about the same with carbon carrier electrodes.

A realistic chance is hence seen for the transfer of the methodology to mass-produced screen printed noble metal or carbon electrodes, which have been proposed as precursors for disposable and economical electrochemical sensors [14].

The final analytical performance of glucose biosensors with any type of an immobilization matrix is a complex issue; apart from the affinity and turnover rate of the enzyme in the specific medium also the quality of the diffusional delivery of both substrate (here: glucose) and oxygen (the cofactor) to active GOD sites are determining factors for the magnitude of the linear range and sensitivity.

Details on the enzymes activity in its environment and the mass transport properties of substrate and cofactor in the particular modification layer are thus needed for clear judgments on the interplay of the parameters and an interpretation of trends in measured sensor properties but are not really known. Calibration measurements, however, made known values for linear range, sensitivity and detection limit and it turned out that the CNT/GOD/EDP- and CNT/GOD/EDP/Nafion-based variants of biosensors had a linearity width and sensitivity that was as good or than better published options with other sensor designs.

An analysis of currently published reports on enzyme (glucose) biosensors reveals a trend to very complex and hardly comprehensible and understandable multicomponent functional sensor architectures. In this context an important message of the findings of this project with more philosophical character is: A good (glucose) biosensor does not need blends of all sorts of active ingredients such as catalytic metal or metal oxide nanoparticles and/or specific redox mediators added to the immobilizing component: an untainted CNT network with a plain polymer top coat does the analytical task in as good or, compared to some instances, even better manner.

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ภาคผนวก ก Appendix 1
ผลงานตีพิมพ์และการเผยแพร่

1. ผลงานตีพิพม์ในวารสารนานาชาติ 1 ผลงาน (Output/Publication)
   Schulte A*, Khunkaewla, P, Suginta W (2013) Electrochemical Biosensor Applications of the Polysaccharides Chitin and Chitosan. *Chemical Reviews*. 113, 5458–5479. (JIF2012 = 41.3)
2. A book chapter entitled "Amperometric biosensors" which is published as part of the book "*Advances in Electrochemical Science and Engineering, Volume 13: Bioelectrochemistry*", Alkire, R. C., Kolb, D. M. and Lipkowski, J. (Eds), Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany. (2011) doi: 10.1002/9783527644117.ch1
3. A publication with the title "Untainted carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors" is in preparation and will be submitted to the either "*Biosensors and Bioelectronics"* (JIF 2011 = 5.6) or Bioelectrochemistry (JIF 2011 = 3.8).
4. A publication with the title "Carbon Nanotube-Chitin Blends as easy-to-form, biocompatible and well-working enzyme biosensor Immobilization matrices" will follow the first publication on CNT-based biosensors. Finalization of electrochemical sensor characterization is almost reached and upon completion the manuscript will be prepared and submitted to the journals listed under 3. or to *Microchimica Acta* (JIF 2011 = 3.0) or Electrochimica Acta (JIF 2011 = 3.8).

Oral presentations on international meetings:

1. KULLAWONG P., SCHULTE A. * Untainted carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors. Regional Electrochemistry Meeting of South-East Asia 2010 (REMSEA 2010); 16th – 19th November 2010, Bangkok, Thailand.
2. RERNGLIT W., KULLAWONG P., SUGINTA W., SCHULTE A* Carbon nanotube-chitin blends as easy-to-form but well-working enzyme biosensor immobilization matrices. 13th FAOBMB International Congress of Biochemistry and Molecular Biology: "Discovery of Life Processes: From Biomolecules to Systems Biology". 25 – 29 November 2012, Bangkok, Thailand.

ภาคผนวก ข Appendix 2

ประวัตินักวิจัย (Personal Resume/CV)

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Degree	Ph.D. (Chemistry), University of Muenster, Germany
Marital status	Married with one child
Current Position	Associate Professor in Analytical Chemistry

Research Interest

1. Electrochemical biosensing
   Enzyme biosensors, Immunosensors
2. Automated electroanalysis in microtiter plates
   Food analysis, Drug analysis, Environmental analysis
3. Electroanalysis in ultrasmall volumes
4. Electrochemical scanning probe microscopy
   Scanning electrochemical microscopy, Electrochemical scanning tunneling microscopy

List of publications (2007-2013)

1. SUGINTA W., KHUNKAEWLA P., SCHULTE A.* Chem. Rev. Biosensor Applications of the Polysaccharides Chitin and Chitosan. Chemical Reviews. 113 (2013) 5458–5479. (JIF2012 = 41.3)
2. SUGINTA W., CHUMJAN W., MAHENDRAN K. R., JANNING P., SCHULTE A., WINTERHALTER M. Molecular Uptake of Chitooligosaccharides through chitoporin from the marine bacterium Vibrio harveyi. PLoS ONE 8/1 (2013): e55126. (JIF2012 = 3.7)
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1. KULLAWONG P., SCHULTE A. * Untainted carbon nanotube networks as competitive immobilization matrix for amperometric enzyme biosensors. Regional Electrochemistry Meeting of South-East Asia 2010 (REMSEA 2010); 16th – 19th November 2010, Bangkok, Thailand.
2. SCHULTE A. * Electrochemistry and Nanoscience: The world of ultrasmall electrodes, nanoscale sensor modifiers, and electrochemical microscopy schemes. (Invited). German-Thai Symposium on Nanoscience and Nanotechnology (GTSNN2011); 13th – 16th September, 2011, Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand.
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