THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Development of Ultra-fast Biosensors for Detection of Non-electroactive Neurotransmitters

YUANMO WANG

CHALMERS
UNIVERSITY OF TECHNOLOGY

Department of Chemistry and Chemical Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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YUANMO WANG

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Department of Chemistry and Chemical Engineering

Chalmers University of Technology

SE-412 96 Göteborg

Sweden

Telephone +46 (0) 31-772 1000

Cover image:
A graphical illustration of an enzyme-based electrochemical biosensor and the characterization of enzyme-gold nanoparticle conjugates.

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YUANMO WANG

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Chalmers University of Technology

ABSTRACT

During neuronal communication neurotransmitters are released through the process exocytosis that occurs on the time scale of sub-milliseconds to milliseconds. Neuronal activity by neurotransmitters that are non-electroactive cannot easily be monitored due to limitations in the temporal resolutions of the sensor probes for these analytes. In order to achieve a fast detection of the rapid transients derived from these non-electroactive neurotransmitters e.g. acetylcholine and glutamate, enzyme nanoparticle conjugates was carefully characterized and optimal enzymatic conditions from these studies were used to design and construct a nanostructured enzyme-based electrochemical biosensor for acetylcholine.

This sensor was constructed from a micro-sized carbon fiber (5 μm to 30 μm in diameter) sealed into a glass capillary and functionalized with gold nanoparticle (AuNP) hemispheres at the electrode surface. The nanoparticle structure at the electrode surface increases the electrode surface area and allows immobilization of larger amount of enzyme. In addition the nanoparticle provides a surface with high curvature that may prevent enzyme denaturation compared to the flat surface. The enzymes subsequently immobilized onto the AuNPs-structured surface will catalyze the non-electroactive molecules of interests and produce H_2O_2 that is electroactive and can be detected using amperometry. We have found that the key for providing high temporal resolution by these sensors is to limit the enzyme coverage at the electrode surface to a monolayer.

In **Paper I**, an acetylcholine sensor is constructed with a two-enzyme system consisting of acetylcholinesterase and choline oxidase. This sensor provided a temporal resolution that was fast enough to detect single vesicle release of acetylcholine in the millisecond time scale by an artificial cell model for exocytosis.

For the design and construction of a microelectrode glucose sensor, the characterization of the conjugation of the enzyme glucose oxidase (GOx) to the surface of AuNP was studied in **Paper II** to achieve the optimal conditions for the GOx. The work shows that the structure of GOx adsorbed on AuNP changes to much less extent than if GOx adsorbs on to a flat surface and that the enzymatic activity is maintained to the same extent as for GOx in solution.

Keywords: Biosensor, amperometry, acetylcholine, acetylcholine esterase, choline oxidase, artificial cell, exocytosis, glucose oxidase, gold nanoparticles, enzyme monolayer, adsorption, quantification, deformation, enzymatic activity, stability, nanoparticle tracking analysis, dynamic light scattering

LIST OF PUBLICATIONS

This licentiate thesis is written based on the following publication and manuscript:

I. Amperometric Detection of Single Vesicle Acetylcholine Release Events from an Artificial Cell. Jacqueline D. Keighron, Joakim Wigström, Michael E. Kurczy, Jenny Bergman, Yuanmo Wang and Ann-Sofie Cans. *ACS Chemical Neuroscience*, 2015, 6:181-88.

II. Structure Changes of Glucose Oxidase Adsorbing and Covering a Gold Nanoparticle Surface. Yuanmo Wang, Hampus Lindmark and Ann-Sofie Cans. Manuscript in preparation.

CONTRIBUTION REPORT

Pager I. I performed the sensor response time experiments together with Joakim Wigström and Jenny Bergman.

Paper II. I conceived the project together with Ann-Sofie Cans. I designed and performed all the experiments, analyzed all the data and wrote the manuscript with Ann-Sofie Cans.

LIST OF ABBREVIATIONS

AuNP	Gold nanoparticle
Acetyl CoA	Acetyl coenzyme A
ACh	Acetylcholine
AChE	Acetylcholinesterase
ATP	Adenosine triphosphate
CAT	Choline acetyltransferase
ChO	Choline oxidase
CNS	Central nervous system
CO_2	Carbon dioxide
D_{hyd}	Hydrodynamic diameter
DLS	Dynamic light scattering
EDL	Electrical double layer
FAD	Adenine dinucleotide
GABA	Gamma aminobutyric acid
GLU	Glutamate
GluOx	Glutamate Oxidase
GOx	Glucose Oxidase
HOPG	Highly oriented pyrolytic graphite
HRP	Horseradish peroxidase
NTA	Nanoparticle tracking analysis
OPD	O-Phenylenediamine
SBC	Sodium bicarbonate
TCA cycle	Citric acid cycle
UV-Vis	Ultraviolet-visible

TABLE OF CONTENTS

1. INTRODUCTION

Neuronal activities like exocytosis occur in the sub-millisecond to milliseconds time scale, which is ultra-fast. The signaling molecules released by these secretory cells can be monitored using electrochemical techniques if the neurotransmitters are electroactive. However it cannot be easily monitored if the fast transients stem from non-electroactive neurotransmitters and metabolites e.g., glucose that involved in neuronal activities in the brain. Currently the sensors for detecting these molecules give response in sub-seconds or several seconds and this detection speed is apparently not fast enough. In order to better understand the neuronal communication and the mechanism behind neurological disease (e.g., Alzheimer's disease) that involve non-electroactive neurotransmitters, there is a great need to capture these rapid transients during neuron communication in the brain.

To meet the needs of fast detection of non-electroactive neurotransmitters (e.g., acetylcholine and glutamate) and metabolites during neuronal activities, a nanoparticle structured and enzyme-based electrochemical biosensor providing high temporal and spatial resolution is designed in our group. A micro-sized carbon fiber electrode sealed with glass capillary is firstly modified with gold nanoparticles (AuNPs) to increase the surface area and surface curvature, which allows more enzyme adsorbed on sensor's surface compared to a flat sensor surface. In addition, the deformation of enzyme structure leading to denaturation may be prevented because of the highly curved surface of AuNP. The enzymes immobilized on the sensor surface will catalyze molecules of interest with high selectivity to produce H_2O_2 that can generate electric current after applying a potential on the electrode.

We have found out that the key to provide high temporal resolution for this sensor is to keep a monolayer (or thin layer) enzyme modification. In Paper I, an acetylcholine sensor is constructed by coating sequentially a thin layer of AuNPs and a two-enzyme system consisting of acetylcholinesterase (AChE) and choline oxidase (ChO) onto a carbon fiber microelectrode surface. We here showed that the sensor could detect vesicle release of the neurotransmitter acetylcholine in milliseconds. The significant improvement in temporal resolution for this acetylcholine sensor shows that the recording speed can match the time course to temporally resolve single vesicle release events during neuronal communication with this new approach in sensor design. This gives promise to bring this technology to provide fast sensing scheme of other non-electroactive neurotransmitters and metabolites that are important in neuroscience.

It is well-known that glucose is the primary energy source for the whole body including the brain for mammals. Glucose actually plays a critical role in brain function physiologically and pathologically [1]. Neuronal activities in the brain have a very high demanding of energy (e.g., adenosine triphosphate, ATP), which can be produced by glucose through glucose metabolism (e.g., the citric acid cycle, TCA cycle). Several studies have shown that the glucose level in the brain is highly related to different neurologic diseases like Alzheimer's disease [2-5]. On the way to construct a glucose sensor using the enzyme glucose oxidase (GOx), a question related to the sensor sensitivity concerns potential change in enzyme structure after adsorbing to the AuNP with highly coved surface was brought up, since it might affect the enzymatic efficiency. The structure change of GOx when adsorbed on to flat surface has been well studied, however the structure deformation of the enzyme by adsorption to highly curved surface was barely studied. Therefore characterization of the conjugates

formed by GOx adsorption to AuNP and the effect on the catalytic properties by the conjugation process was investigated in Paper II.

Glutamate is one of the major neurotransmitter in the brain affecting brain functions such as reward, cognition, and learning and memory. However, glutamate is non-electroactive molecule and a fast sensor for glutamate recording is currently lacking. For development of an enzyme-based electrochemical sensor for glutamate detection, our approach to immobilize a monolayer of the enzyme glutamate oxidase (GluOx) to the surface of AuNPs coated microelectrode is under development. Hence, to optimize the conditions for GluOx adsorption process to AuNPs and to control the enzyme coverage to a monolayer, characterization of GluOx:AuNP conjugates in bulk solution has been studied and is presented in Paper III (Appendix). The insights from this study are now being used in the construction of a fast glutamate sensor that will be introduced for glutamate recording in the brain.

2. NEUROSCIENCE

2.1 Neuronal Communication

In the nervous system, neurons communicate by electrical and chemical signals via synaptic connections. An electrical nerve signals can trigger Ca^{2+} channels to open at the presynaptic terminal and the flux of Ca^{2+} ions into the cell stimulates neurotransmitter-filled vesicles to dock and fuse with the plasma membrane in the presynaptic neuron and release neurotransmitter molecules that can reach the postsynaptic neuron via passing through the gap space between two neighboring neurons called the synaptic cleft (see Figure 1), The postsynaptic cell receive the chemical signal via specific binding of these neurotransmitters to receptors on their plasma membrane. Hence the secretory vesicle is a key organelle involved in neuronal communication. After synthesis the vesicle compartment is loaded with neurotransmitters and stored in the cytoplasm of the presynaptic neuron where vesicles are subsequently docked and primed to the active zone of the plasma membrane. This is to meet all requirements to be ready to release the vesicle neurotransmitter content into synaptic cleft when neurons are triggered to send signals in the neuronal network in a process that is called exocytosis [6].

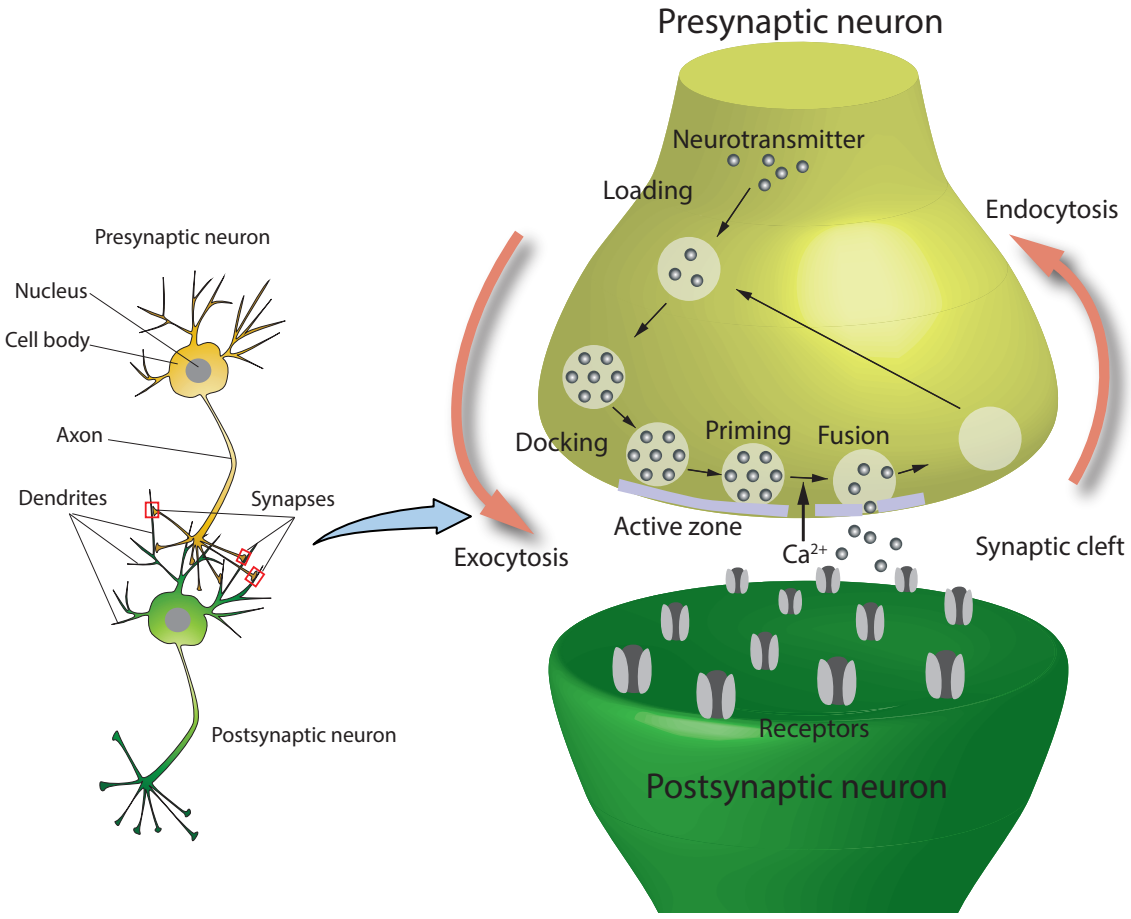

Figure 1. Schematic of neuronal communication. The left figure shows the synaptic connection between two neighboring neurons. And the right figure is the magnification of synapses from the left figure between a presynaptic neuron and postsynaptic neuron.

2.2 Neurotransmitters

The chemical messengers needed in chemical communications are called neurotransmitters. It is thought that all various kinds of neurotransmitters are still not known and therefore is hard to give an exact number of the various neurotransmitters that is used by the brain. Anyhow more than 100 chemicals have been identified [7]. Neurotransmitter can be classified in different ways such as via their size and function. Some of the most well-studied neurotransmitters that are involved in major neurochemical pathways in the brain are for instance: acetylcholine (ACh), dopamine, histamine, serotonin, glutamate, gamma aminobutyric acid (GABA).

2.2.1 Acetylcholine (ACh)

Acetylcholine (ACh) was the first discovered neurotransmitter molecule [8]. Its chemical structure is shown in Figure 2 and is composed by an ester of acetic acid and choline.

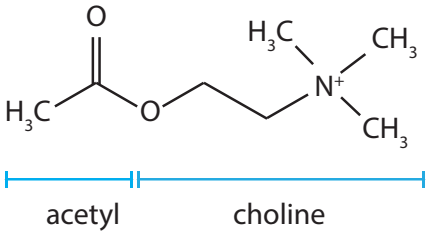

Figure 2. The chemical structure of acetylcholine.

As shown in Figure 3, ACh is initially synthesized from acetyl coenzyme A (acetyl CoA) and choline in the presynaptic nerve terminals, where choline acetyltransferase (CAT) catalyzes the reaction [7]. ACh is then released via Ca^{2+}-stimulated exocytosis into the synaptic cleft, where ACh is either bound to nicotinic and muscarinic receptors at postsynaptic neuron, or rapidly broken down into choline and acetate via the enzyme acetylcholinesterase (AChE). The choline can be transferred from the synaptic cleft via specific membrane transporter proteins back into presynaptic neuron for recycling and synthesis of new ACh.

ACh plays an important role in the human body and brain function. Specifically, this neurotransmitter has muscle-activating function, which is responsible for skeletal muscle contraction and also cause vasodilation via relaxing smooth muscle in the autonomic nervous system by binding to muscarinic receptors located at vascular endothelium. In the central nervous system (CNS), the sustaining attention and sensory perception when awake can be improved by ACh [9, 10]. It has been reported that ACh promotes rapid eye movement sleep [11]. There are also speculations that the Alzheimer's disease in CNS is associated with a decrease in ACh [12]. Myasthenia gravis, a disease of the neuromuscular junction showing as muscle weakness and fatigue, may occur when the ACh signal transmission is inhibited when ACh receptors are occupied by antibodies inappropriately produced by body [8].

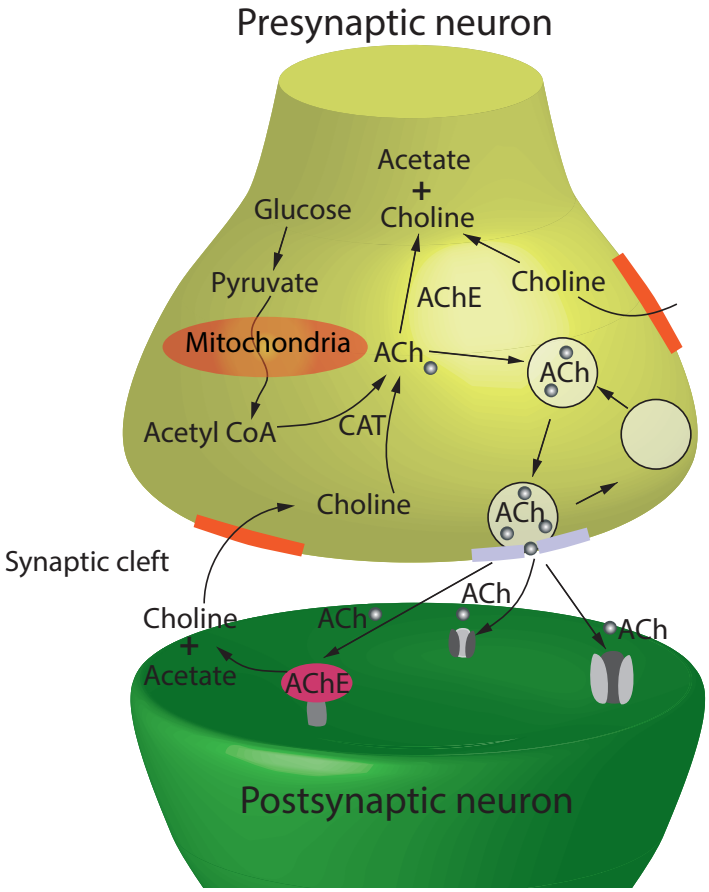

Figure 3. Synthesis and recycling of acetylcholine at the synapse.

2.2.2 Glutamate (GLU)

Glutamate (GLU) is a non-essential amino acid and the richest amino acid in the diet [7, 13, 14]. The molecule structure of glutamate is shown in Figure 4. This neurotransmitter can be found everywhere in the body in high concentration [14]. In the nervous system, GLU serves as the principle excitatory neurotransmitters in CNS in the brain and plays a critical role in learning and memory, especially for elderly people [15, 16]. It is speculated that more than half of all brain synapses release glutamate [7].

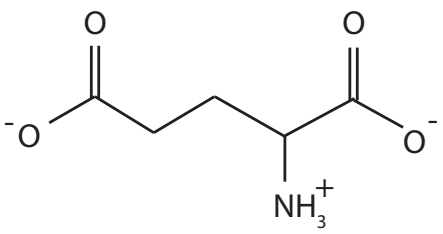

Figure 4. Structure of glutamate

As illustrated in Figure 5, GLU can be synthesized in the CNS from glutamine by the mitochondrial enzyme Glutaminase [7]. It can also be synthesized from α-Ketoglutaric acid as an intermediate in the citric acid (TCA) cycle, which is a chemical reaction series to generate energy through oxidation of acetyl-CoA into carbon dioxide (CO_2) and adenosine triphosphate (ATP) [7, 17, 18]. It means that glutamate synthesis is partially regulated by glucose metabolism. And GLU itself can also serve as a metabolic precursor for gamma-Aminobutyric acid (GABA), which is another amino acid neurotransmitter [7].

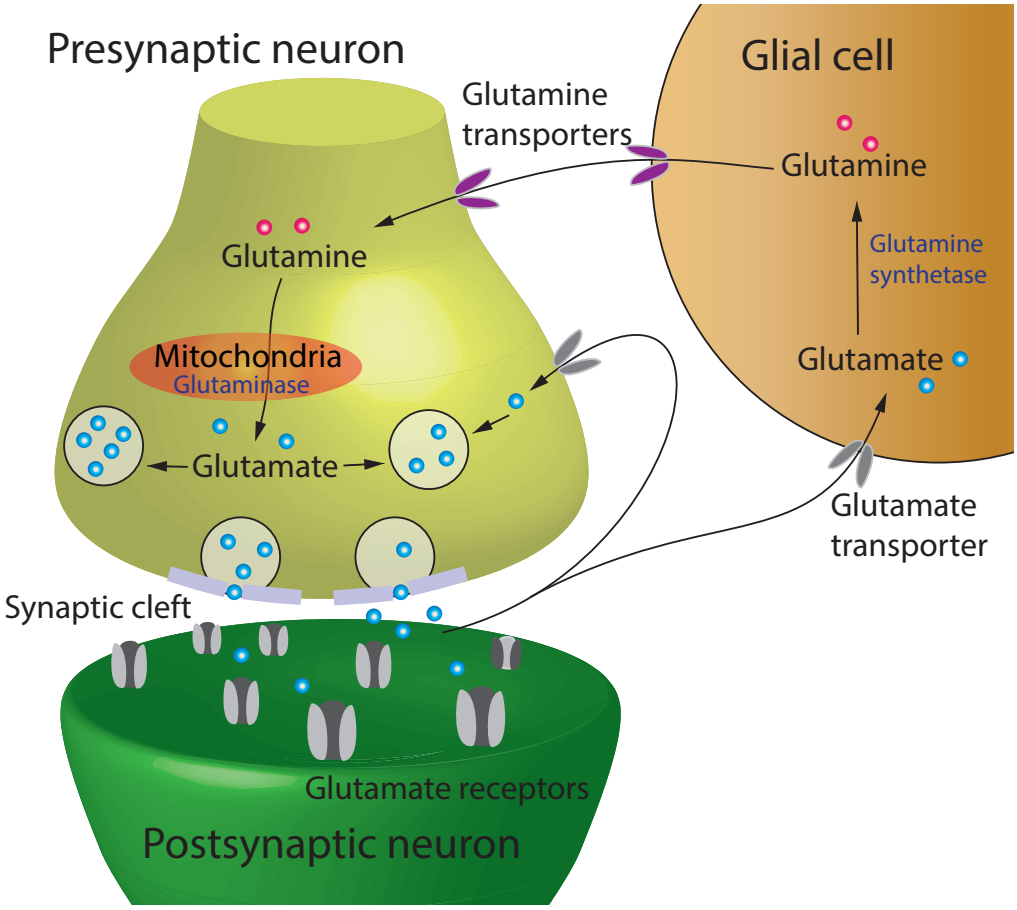

Figure 5. Glutamate synthesis and cycling between neurons and glia cells.

After release from the presynaptic terminal into the synaptic cleft, glutamate detected by the postsynaptic cell via glutamate receptors. At the same time, excess glutamate at the synaptic cleft is removed by high-affinity glutamate transporters located at surrounding glial cells and presynaptic terminals [7]. Glutamate uptake into glial cells is converted to glutamine by glutamine synthetase and is then transported back to presynaptic terminals [7]. This whole event is referred to as the glutamate-glutamine cycle. GLU also plays a crucial role in clinical neurology, since the excess of GLU accumulated in extracellular space can lead to excitotoxicity where the consequence is neural damage or even nerve cell death [7, 13].

1.3 Glucose

Glucose is well known as a sugar with molecular weight of 180.16 $g \cdot mol^{-1}$ and a chemical formula $C_6H_{12}O_6$, and is the ubiquitous energy source in the body. High blood glucose level over a long period is a sign of a group of metabolic diseases known as diabetes. The level of blood glucose for healthy human is normally approximate 5.5 mM. The concentration of glucose in some biofluids are significantly lower and may locate in the range of micro-molar, such as ~ 70 μM in saliva [19, 20], ~ 200 μM in tears [21, 22], ~ 120 μM in sweat [23, 24].

The correlation between glucose and the nerve system has also been well studied. Gold and other groups have reported that glucose does not only enhance learning and memory in healthy aged humans, but also enhance a broader range of cognitive functions in patients with Alzheimer's disease and young adults with Down syndrome [2, 3, 25-27]. Chih and Robert showed that glucose is the primary substrate for both neurons and astrocytes during neural activity [28]. In 2005, Mosconi pointed out the importance of glucose in the early diagnose of Alzheimer's disease [4].

Gold and his co-workers suggested that glucose effects human's brain cognitive functions via glucose metabolism [29, 30], since glucose produces the energy needed for brain activity, and several neurotransmitters like acetylcholine and glutamate are synthesized originally from glucose. Specifically, they reported that glucose may attenuate hippocampal acetylcholine release.

3. ENZYME AND GOLD NANOPARTICLES

3.1 Glucose Oxidase (GOx)

Glucose Oxidase (GOx; E.C. 1.1.3.4) is a flavin-dependent enzyme that oxidizes β-D-glucose into glucolactone and converts O_2 into H_2O_2. It has a dimer with a molecule weight of 160 kDa. This enzyme is able to be synthesized from several different species of fungi. GOx from *Aspergillus niger* and *Penicillium amagasakiense* are usually produced commercially, and is mostly utilized for fabrication of biosensors [31] to measure blood glucose level for diabetes patients that is a worldwide health problem. GOx from *Aspergillus niger* and *Penicillium amagasakiense* show 79% similarity and 66% identity in sequence [32], and have similar enzymatic ability. Both of these two GOx exhibit high specificity of β-D-glucose [33]. Since most of the biochemical characterization of GOx from *Aspergillus niger* has been well studied, it was chosen to use for modification on the enzyme-based sensor in this project and to be studied in regard to its structure change after adsorption on 20 nm AuNPs with highly curved surface.

A GOx monomer contains one flavin adenine dinucleotide (FAD) via tight, but non-covalent binding [33]. The substrate glucose binds to the active site close to the FAD that let the oxidation of glucose perform. For a monomer of GOx from *Aspergillus niger*, it contains 581 amino acid residues. At pH 7, part of these residues are positively charged, like lysine, arginine and histidine, and part of them are negatively charged such as aspartic and glutamic acid [34]. The relative numbers of different charged residues and their distribution lead to a negatively charged surface potential in total [35]. Although the charge of these residues will change with the variation of pH, the enzyme will keep a net negative charge on its surface when pH is above 4.2, since the isoelectric point of GOx is 4.2 [36].

3.2 Gold nanoparticles (AuNPs)

The gold nanoparticles (AuNPs) have been widely used in biosensor applications to improve the sensor stability and sensitivity due to AuNP's small size and high surface-to-volume ratio [37]. Particularly, AuNPs have been applied on to the surface of electrochemical biosensors because of their conductive property as metals [38]. Besides, a wide range of biomolecules is able to be immobilized onto AuNP because of the AuNPs' high surface energy, which may improve the sensor's selectivity.

AuNP used in the project of this thesis is bought from BBI solution (Cardiff, UK) with a size of 20 nm in diameter and a zeta potential of - 41.4 mV. Generally, the AuNPs are obtained by reducing chloroauric acid ($H[AuCl_4]$). After adding the reducing agent, Au^{3+} is reduced to Au^{+}, and then 3 Au^{+} can produce Au^{3+} and 2 Au^{0}. The initial obtained Au^{0} atoms act as the center and more new reduced Au^{0} is built up on the center to form AuNP that grows in size during the reducing reaction and can be controlled to produce AuNP with a specific size required. Turkevich method [39] is the simplest way for synthesizing AuNPs. In this method, citrate ions from sodium citrate act as both reducing agent and capping agent that stabilize AuNP surface via electrostatic force to prevent AuNPs from aggregation. The citrate capped AuNPs are negatively charged [40]. And the size of AuNP could be controlled by the ratio between gold salt and citrate added to the reaction.

3.2.1 Electrical Double Layer (EDL)

Most particles in aqueous solution have a charge (either positive or negative), and the liquid composition of counter ions affects the electrical double layer (EDL) of the particle surface. EDL consists of two layers: stern layer and diffuse layer

As shown in Figure 6, the counterions are tightly bound to the particle surface via chemical interactions in an inner region, called the Stern layer. The ions are loosely attached to the particle surface via a coulomb force, located at an outer region, called the Diffuse layer. There is a notional boundary inside of the diffuse layer where a stable entity between ions and particles is formed that is called the slipping plane, which defines the zeta potential [40-42]. The potential existing at the boundary between diffuse layer and stern layer is the Stern potential.

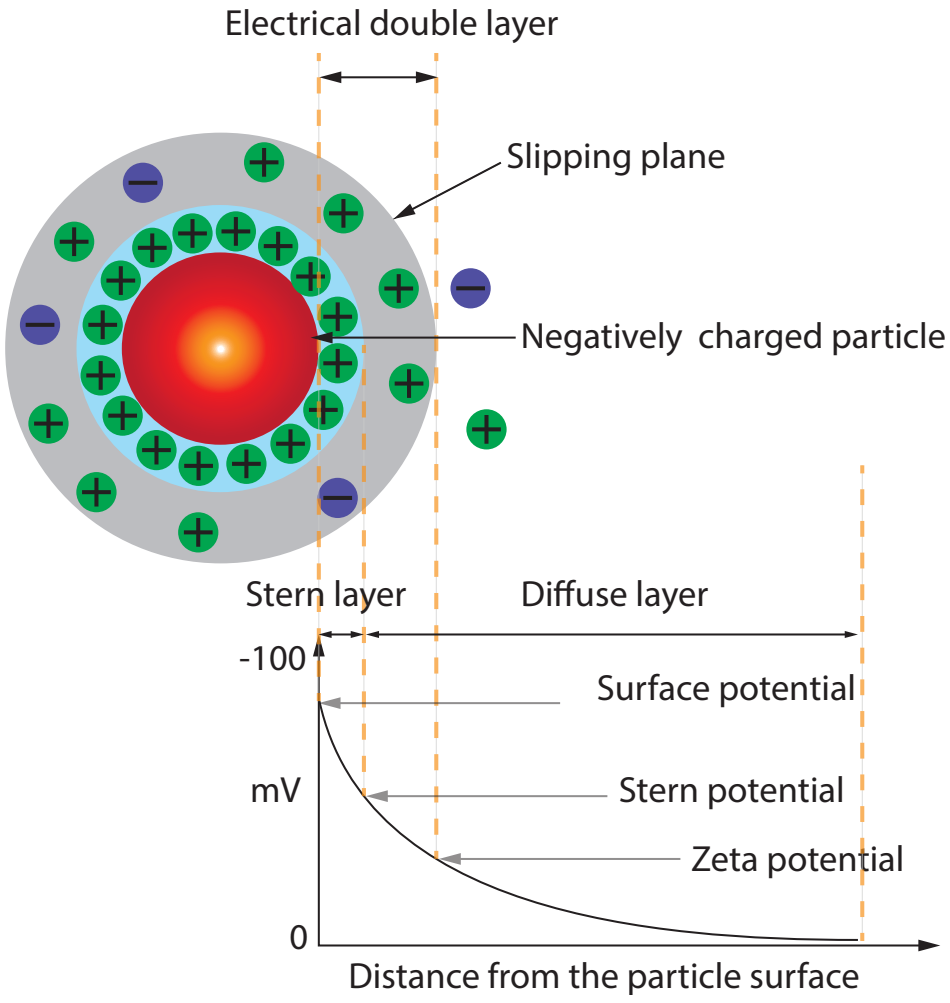

Figure 6. Schematic of the electrical double layer surrounding a particle in aqueous solution.

4. ELECTROCHEMISTRY

4.1 Electrochemical Enzyme-based Biosensor

To detect non-electroactive neurotransmitters and metabolites in the neuronal system of the brain, AuNPs structured enzyme-based carbon fiber disc microelectrodes are designed and manufactured as the electrochemical biosensor for these analytes (see Figure 7). A carbon fiber with a size ranging from 5 μm to 30 μm in diameter is sealed into a glass capillary. The carbon fiber is cut and beveled to form a disc shape microelectrode. To increase the sensor surface area and surface curvature, and to maximize the retained enzymatic activity after enzyme adsorption [45], AuNPs are deposited onto the carbon fiber microelectrode surface by electrodeposition of AuNPs at the electrode surface via a reduction of $HAuCl_4$ solution using a potential sweep of - 0.6 V for 24s at the electrode surface. A monolayer coverage of enzyme is adsorbed to the AuNP coated electrode surface by using the optimized conditions from studies of enzyme interaction with the AuNP from studies in bulk solution. This enzyme-based biosensor relies on enzymes to catalyze molecules of interests to produce electroactive compound H_2O_2. Subsequently an amperometric current can be generated from the redox reaction of H_2O_2 when applying a constant potential to the sensor surface and hence the non-electroactive analytes indirectly are detected.

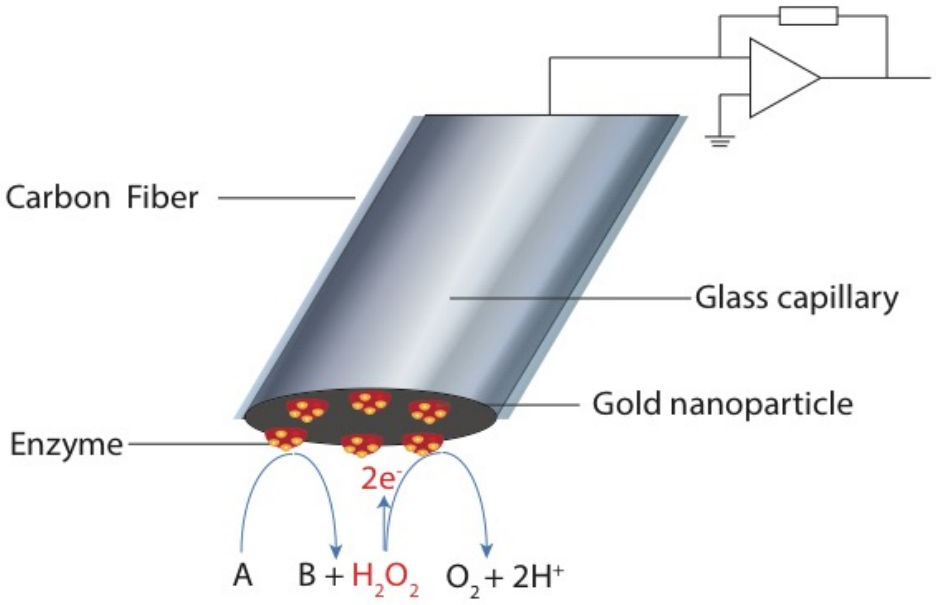

Figure 7. Illustration of the enzyme-based electrochemistry sensor. The core part of the sensor is a carbon fiber microelectrode with a modified surface using AuNPs. Enzymes are immobilized on the sensor surface to catalyze target molecules (A) and produce an electroactive product such as H_2O_2 that is detected by the sensor after applying a potential that triggers a redox reaction of H_2O_2.

Therefore, finding suitable enzymes for the catalysis of the analytes of interests is critical for the construction of an enzyme-based biosensor. For example, to detect glucose, GOx and glucose dehydrogenase are usually utilized. Glutamate oxidase is chosen in Paper III (Appendix) to oxidase glutamate and produce electroactive H_2O_2 for the indirect detection of

glutamate. In some cases, two or more enzymes are needed to work in sequence to trigger a reaction chain and to produce an electroactive specie. For instance, to detect acetylcholine, a multi-enzyme biosensor system is built up by AChE together with choline oxidase (ChO). Acetylcholine is firstly hydrolyzed by AChE to form acetate and choline. And choline is soon oxidized by ChO to produce H_2O_2 that can be detected using amperometry.

4.2 Amperometry

Amperometry is one of the most widely used electrochemical techniques in the analytical chemistry field. It can offer high temporal resolution in time scale of sub-millisecond, high sensitivity and high spatial resolution even though selectivity is not the strength of this method [46]. In amperometry, a constant potential that can trigger a redox reaction of molecule of interest (analytes) at a working electrode is applied between the working electrode and a reference electrode, and the redox reaction should be fast enough to be diffusion controlled. This can be achieved by adjusting the applied potential to the electrode [46]. In amperometric measurement the current is measured with respect to time. The changes in concentration of analytes at the working electrode surface can then be determined via fluctuations in the current recorded according to the Faraday's law [47] described in Equation 1.

$$Q = nFN \tag{1}$$

where Q is the transferred electronic charge, given in coulomb; N is the number of molecules, given in moles; F is the Faraday's constant; n is the number of transferred electrons in reaction. Since the current (i) presents the number of electrodes per second in the reaction, as Equation 2,

$$i\ (amperes) = \frac{dQ}{dt}\ (coulombs/s) \tag{2}$$

The Faraday's law can then be interpreted in Equation 3.

$$N = \frac{1}{nF} \int i\, dt \tag{3}$$

Therefore, amperometry can be used to quantify the number of molecules (e.g., neurotransmitters) released in exocytosis from single vesicle compartment by integration of the resulting current spike from each exocytosis event [46–48]. Amperometry can also provide kinetic information of vesicle release during the exocytosis process via analysis of current spike parameters like spike rise time, fall time and half width. These provide for dynamic information on for instance the vesicle fusion pore opening and closing as well as pore size and the fusion pore stability [51–53]. In Paper I, amperometry was used to show the high temporal resolution of an enzyme-based sensor for detecting the neurotransmitter acetylcholine released from vesicles in an artificial cell model [54]. Hence, the poor selectivity of amperometry for ordinary carbon fiber electrode can be improved by the design of enzyme-based sensor [46].

5. OPTICAL SPECTROSCOPY

5.1 Ultraviolet-visible (UV-Vis) Absorption Spectroscopy

Molecules absorb light emitted by a light source at a particular wavelengths. The light absorbed by samples in ultraviolet-visible (UV-Vis) absorption spectroscopy locates in the ultraviolet-visible region. Figure 8 shows the basic setup for a UV-Vis adsorption spectrophotometer. A monochromator firstly selects light with a narrow range of wavelength (approximately 200 nm to 800 nm) from a wider range of wavelength, and lets the selected light passes through sample solution. The intensity of light decreases after passing through sample solution when the light is absorbed by sample. It means the incident light intensity (I_0) is reduced compared to the transmitted intensity (I) (i.e., $I_0 \geq I$). UV-Vis absorption spectroscopy is usually used to determine the concentration of sample based on Beer-Lambert law (see Equation 4) [55]:

$$A = \log \frac{I_0}{I} = \varepsilon \cdot l \cdot c \tag{4}$$

where A is the absorbance, ε is the molar extinction coefficient of a molecule at a specific wavelength, in units of $M^{-1}cm^{-1}$, l is the optical path length passing sample, c is the concentration of sample in solution.

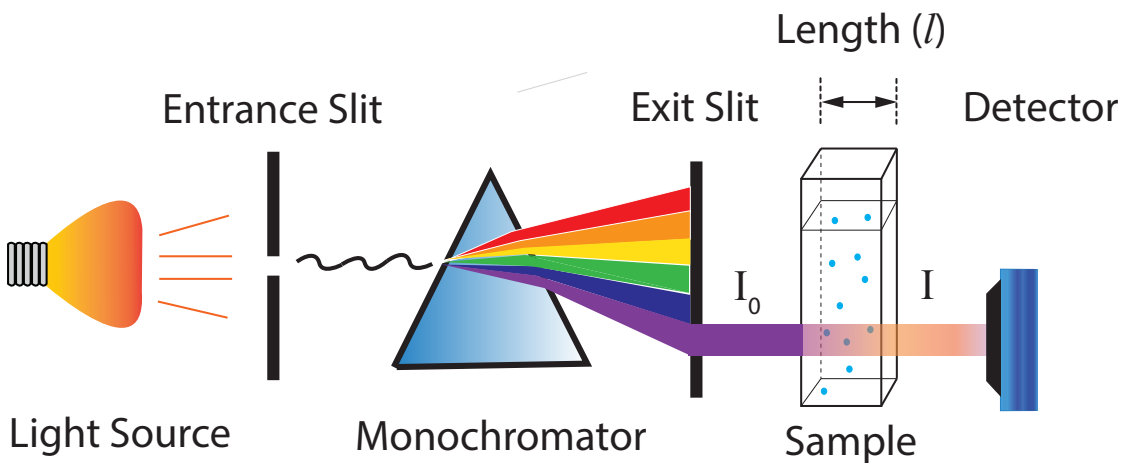

Figure 8. Schematic of the basic setup of a UV-Vis absorption spectrophotometer.

5.2 Fluorescence Spectroscopy

Fluorescence Spectroscopy typically analyzes the emitted light (i.e., fluorescence) from a sample. Figure 9 depicts the main parts of a fluorimeter. Basically, an excitation light passes through a monochromator and subjects the sample to a particular wavelength of the light that excited the analytes by the absorption of part of the incident light and results in emission of fluorescent light in all directions. The fluorescent light is captured by a detector after passing through a monochromator. Usually the detector is placed at 90° to the direction of incident light to reduce the possibility of detecting interferences from incident light. In a fluorescence

measurement, the excitation wavelength is fixed and the intensity of the emitted light is recorded from a range of wavelengths of light.

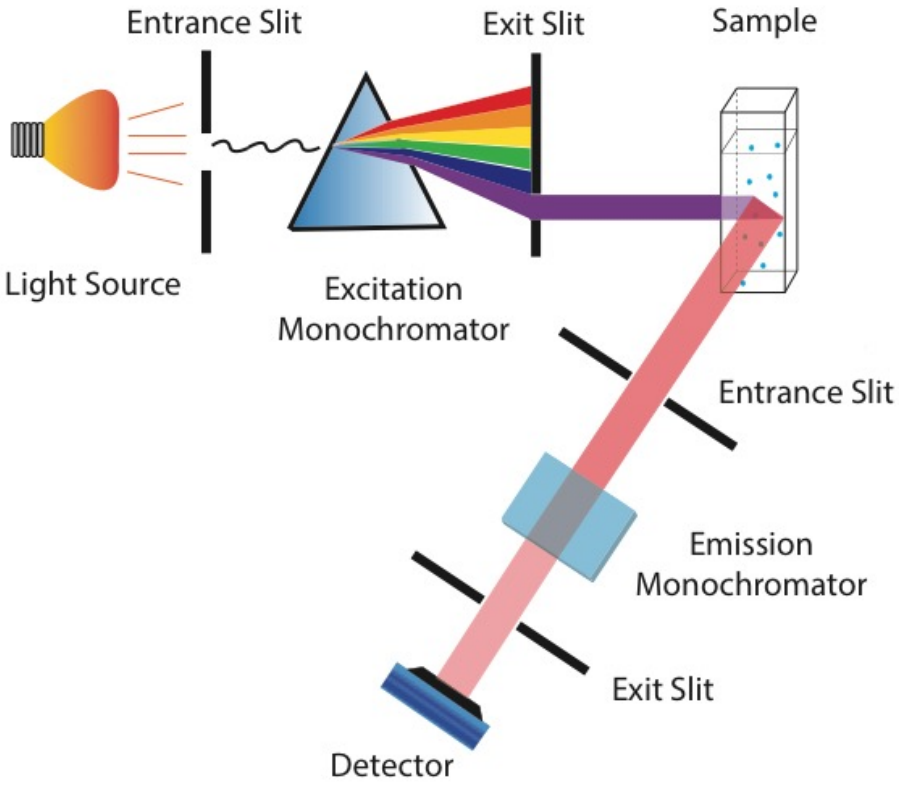

Figure 9. Illustration of the basic setup of a fluorimeter.

6. NANOPARTICLE SIZE CHARACTERIZATION

6.1 Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) is a technique used to determine the size of particles under Brownian motion. It is done via illuminating the particles by a light source such as a laser and then analyzing the intensity fluctuations from the scattered light. Brownian motion is the constant movement of particles due to their random collision with the fast-moving atoms or molecules in the gas or liquid that surrounds the particles [56]. Usually the large particles move slowly and small particles move fast. The relationship between the speed of a particle and its size can be explained by Stokes-Einstein equation (see Equation 5).

$$D = \frac{k_B T}{6\pi\eta r} \tag{5}$$

where k_B is the Boltzmann's constant, T is the absolute temperature, η is dynamic viscosity, D is diffusion coefficient of particle, $r\ is$ the hydrodynamic radius of particle.

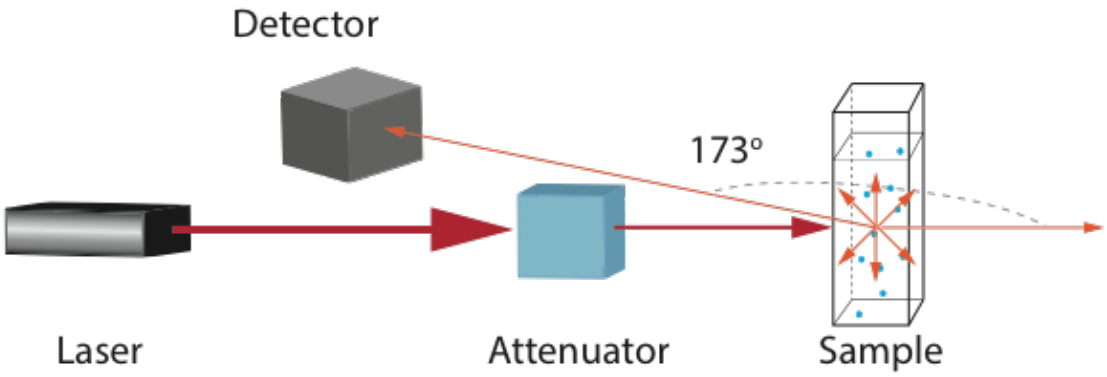

Figure 10. Schematic of the dynamic light scattering (DLS).

A typical DLS system is illustrated as Figure 10. For a successful detection, the intensity of the scattered light should be within a specific measurable range, thus attenuator is used to adjust the intensity of the laser via adjusting the sample concentration, thereby obtaining the optimal intensity of the scattered light. Theoretically, a detector is able to be placed in any position since the particles scatter light in all orientations. However, 173° backscatter detection was chosen since it can minimize the risk of multiple scattering, incident light passed through samples and side effects from contaminants.

6.2 Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) is a method to determine particle size via visualizing the particles in liquids under Brownian motion. NTA is able to profile particle size distribution in liquid suspension with diameters that range of approximate 10-1000 nm.

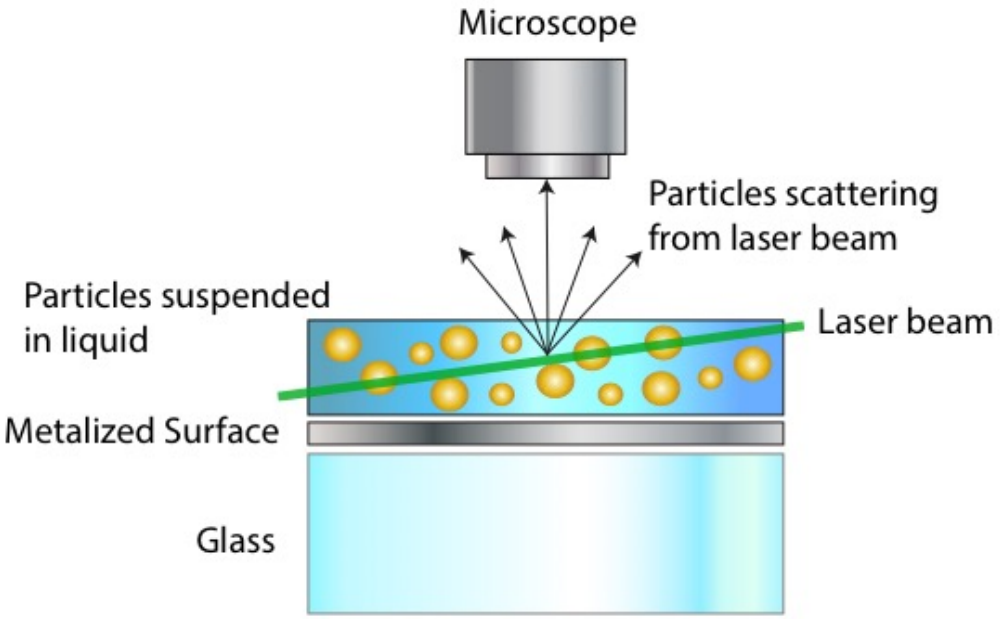

Figure 11. Illustration of nanoparticle tracking analysis (NTA).

As shown in Figure 11, a laser beam passes through the particles in suspension and illuminates the particles, the scattered light is captured and visualized by a microscope onto which is equipped with a camera. The tracking and analysis of individual particles from captured videos by a software complements DLS measurements [57]. The hydrodynamic diameter analysis is based on the Stokes-Einstein equation (Equation 5).

7. SUMMARY

The work that was performed in this thesis was focused on development of novel sensors for fast detection of non-electroactive molecules that are involved in neuronal activity in the brain. An acetylcholine sensor was manufactured in Paper I. The affect on enzyme structure by enzyme adsorption to the surface of nanoparticles that is highly correlated with catalytic efficiency of immobilized enzymes on sensor surfaces was studied in Pager II.

In Paper I, a novel enzyme-based electrochemical biosensor was designed for rapid detection of the neurotransmitter acetylcholine and displayed recordings of single vesicle release events from an artificial cell in the millisecond time scale as shown in Figure 12. Compared to the amperometric spike of electroactive dopamine (Figure 12, C), it can be seen that the detection of non-electroactive acetylcholine (Figure 12, B) is slower, but still on the similar time scale.

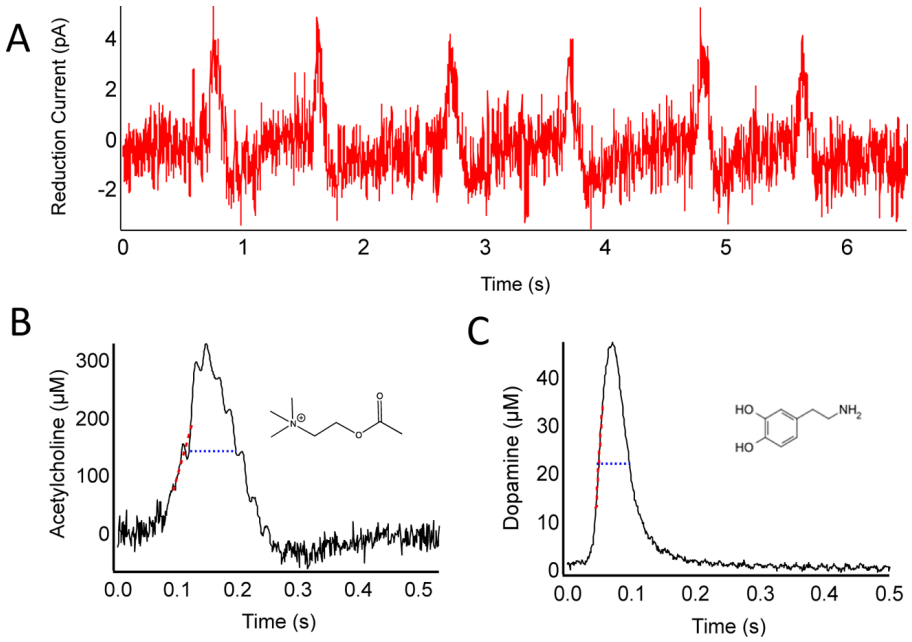

Figure 12. Time resolved detection of acetylcholine. (A) Representative trace of time resolved repeated exposure to vesicle acetylcholine release from the artificial cell model. (B) The average spike (recorded at −0.4 V vs. an Ag/AgCL reference electrode) detecting acetylcholine release (C) the average spike (recorded at +0.8 V vs. an Ag/AgCL reference electrode) detecting vesicle release of dopamine. The spikes presented in parts (B) and (C) are average spikes generated by averaging n > 10 individual vesicle release events.

The temporal resolution of these experiments were shown by using an artificial cell model to mimic single vesicle release of acetylcholine during exocytosis to temporally resolve single vesicle release event on similar time scale as detection of the electroactive neurotransmitter dopamine as shown in Figure 13. To determine the magnitude for the temporal resolution of this sensor and a home-built microfluidic flow cell system was used and the limiting size of artifacts by switching flow of solution in the chip conclusions could only be made that the temporal resolution is higher than 40 ms. The sensor's essential part is a carbon fiber microelectrode with electrodeposited AuNP hemispheres to which two sequential enzymes (i.e., AChE and ChO) were immobilized and forming a thin close to monolayer coverage.

In Paper II, Figure 13 shows the determination of GOx-AuNP conjugates size via DLS and NTA measurements. Data from both DLS and NTA measurements show that a stable monolayer of GOx covering the AuNPs. Hence, enzyme monolayer covered GOx-AuNP conjugates were obtained from using an AuNP-to-GOx ratio 1:1000 during the conjugation process. It also shows that the GOx does not completely collapse in size after adsorption to the 20 nm AuNPs. It means that the 20 nm AuNP with a highly curved surface might be able to retain enzyme structure, and is therefore able to keep the enzyme's full activity. These results imply that GOx immobilized on to the sensor surface might be able to maintain a high working efficiency.

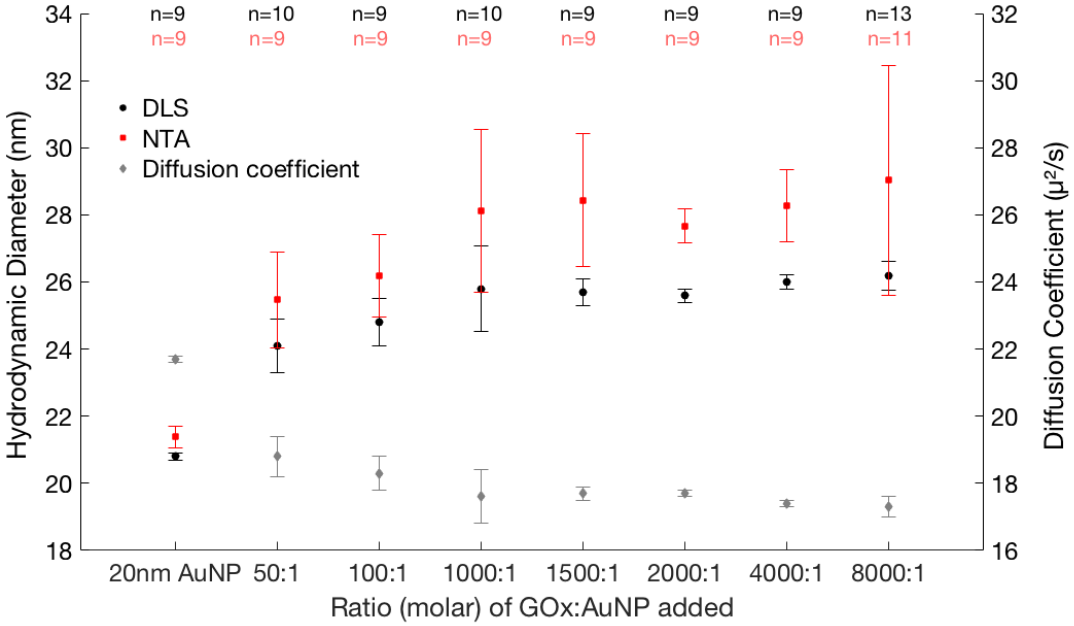

Figure 13. The hydrodynamic diameter measured of GOx-AuNP conjugates and 20 nm AuNP at room temperature 22. Red markers display the collected data from DLS measurements with attenuation 8. Black markers are diffusion coefficient of GOx-AuNP conjugates from DLS measurements. Blue markers show the data from NTA measurements. The measurements were carried out at 6 hours after the conjugation process of GOx and AuNP. Error bars are standard deviation. SBC buffer was filtered with 20 nm syringe filters.

8. FUTURE PLAN

The big goal for these projects is to design, construct and implement biosensors with ultra-fast temporal resolution for detection of non-electroactive molecules *in vivo* during neuronal activity in the brain. Based on the work that has been done, the work planned to be carried out in the near future is described as follows:

Firstly, we will finalize the characterization of the glucose and glutamate biosensors designed with monolayer of enzyme coverage at the AuNP coated microelectrode surface. The AuNP-to-enzyme ratios obtained from Paper II and Pager III (Appendix) will be used during the biosensor fabrication to control and optimize the enzyme coating for these sensors. The sensors will be characterized in terms of, sensitivity, selectivity and stability using both established analytical methods. The temporal resolution of the sensors needs to be tested. However existing methods are only applicable to other existing sensors with much slower temporal resolution and are suitable for recording speed on the second time scale. Therefore new methods that can be used for these ultra-fast biosensors needs to be developed and tested..

Secondly acetylcholine, glucose and glutamate sensors will be applied and tested on brain tissue from different animal models such as rats *in vivo* and *ex vivo*. The recorded signals will be verified with alternate analytical methods.

In addition, modifications of the sensor's surface will be designed for preventing enzyme loss during insertion of the sensor into brain tissue and be suitable for *in vivo* experiments in the brain. For example, the sensor surface will be modified with nanopores that provides a concave surface instead of convex surface of AuNPs. To encapsulate the enzymes into nanopore structure provides a physical protection for the enzymes when the sensor is inserted into tissue.

Besides, the properties of sensors with different surface modification schemes will be compared, and investigate how detection speed, stability and sensitivity of the sensor is affected.

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