THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Development of Electrochemical Biosensors for Neurochemical Applications

Jenny Bergman

UNIVERSITY OF GOTHENBURG

Department of Chemistry and Molecular Biology
University of Gothenburg
Gothenburg, Sweden
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JENNY BERGMAN

Department of Chemistry and Molecular Biology
University of Gothenburg
SE-412 96 Göteborg
Sweden

Cover illustration: Schematic of biosensor detection of vesicular release of non-electroactive neurotransmitters.

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“Life is like a box of chocolates. You never know what you are going to get.”

-Till TEAM Bergman-

ABSTRACT

The brain consists of billions of cells, including nerve cells, which have the ability of transforming an incoming electrical signal in to a chemical output by the release of neurotransmitters through a process called exocytosis. Malfunction in neuronal communication has been linked to several conditions including Parkinson's disease, schizophrenia, ADHD and autism why a better understanding of neuronal communication is of great importance contributing to increased knowledge about these conditions. For studying neuronal activity with single exocytosis events that occur on sub-millisecond to milliseconds time scale, analytical methods with high temporal resolution is the key. In my research, I have focused on developing miniaturized enzyme-based electrochemical biosensors for the detection of glucose and the neurotransmitters acetylcholine and glutamate. A biosensor is a sensor combining a biological component, here an enzyme, with a transducer part, here an electrode. In this thesis, biosensors based on a carbon fiber microelectrode modified with gold nanoparticles and enzyme have been developed with the aim to improve the temporal resolution of these probes compared to existing technology. By limiting the biosensor surface enzyme coverage close to a monolayer, millisecond time resolution was obtained. With this approach of biosensor design, we were able to detect vesicular release of acetylcholine from an artificial cell mimicking exocytosis as described in paper I, and glutamate release from mouse brain slice which is shown in paper IV. Also, a glucose biosensor able of co-detecting glucose and dopamine with millisecond time resolution has been fabricated as described in paper III. In paper II an analytical method for characterizing the interaction of the enzyme-gold nanoparticle interface was developed.

SAMMANFATTNING PÅ SVENSKA

Den mänskliga hjärnan är en av de mest komplexa strukturer som finns och består av miljarder av celler som kommunicerar med varandra. Genom denna kommunikation skapas minnen, känslor och tankar, dvs. mycket av det som kännetecknas som personlighet. I hjärnan finns bl.a. nervceller, celler som kan omvandla en inkommande elektrisk signal till en utgående kemisk signal. Den kemiska signalen utgörs av signalsubstanser som överförs mellan nervcellerna på mindre än en tusendels sekund. Många sjukdomar som drabbar hjärnan så som Parkinsons sjukdom, depression och schizofreni, men även neuropsykiatriska funktionsvarianter som autism och ADHD, förknippas med avvikelser i signalöverföringen av signalsubstanser. Att avslöja detaljerade mekanismer bakom den kemiska kommunikationen mellan hjärnans nervceller ger en bättre förståelse för hur sjukdomar uppkommer, vilket i sin tur kan leda till bättre behandlingsmetoder eller t.o.m. bot för flera av dessa sjukdomar.

För att kunna studera överföringen av signalsubstanser när nervcellerna kommunicerar behövs en metod och analytisk teknik som har tillräcklig upplösning i tid (µs) och rum (mm-µm). I min forskning har jag tillverkat biologiskt baserade sensorer med en diameter motsvarande ett hårstrå. Sensorerna består av kol, nanopartiklar av guld och ett enzym som reagerar med signalsubstansen som ska bestämmas. Med hjälp av elektrokemi, en metod baserad på molekylers olika möjlighet att ta emot eller ge ifrån sig negativa laddningar, elektroner, kan olika typer av signalsubstanser detekteras och kvantifieras i hjärnan.

I den här avhandlingen presenteras resultat från mätningar av signalsubstanserna acetylkolin och glutamat från en konstgjord cell respektive hjärnvävnad från mus med en tidsupplösning av tusendelar av en sekund. Acetylkolin och glutamat är två viktiga signalsubstanser att studera då acetylkolin skickar signalerna från hjärnan till musklerna och glutamat är hjärnans gaspedal. En analytisk metod för att karaktärisera ytan på den här typen av biosensorer samt en biosensor för att mäta glukos, hjärnans huvudsakliga energikälla presenteras också.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Amperometric Detection of Single Vesicle Acetylcholine Release Events from an Artificial Cell.

Keighron, J.D., Wigström, J., Kurczy, M.E., **Bergman, J**., Wang, Y., Cans, A-S. *ACS Chemical Neuroscience*, **2015**. **6** (1): p. 181-188.

Contribution: I was involved in finalizing the manuscript by performing control experiments for determination of the temporal resolution of the sensor, and the experiment comparing H_2O_2 detection efficiency at the surface of AuNP versus carbon surface and made the figures for this experiment.

II. Counting the Number of Enzymes Immobilized onto a Nanoparticle Coated Electrode

Bergman, J., Wang, Y., Wigström, J. and Cans, A-S. *Analytical and Bioanalytical Chemistry* Accepted.

Contribution: I designed the concept of this project together with co-authors. I designed all the experiments for the project and performed the all the electrochemical experiments and the experiments regarding enzymes by using fluorimetry. I performed all the data analysis, interpreted the data and prepared all the figures for this manuscript. I wrote the main part of the manuscript.

III. Co-detection of Dopamine and Glucose with High Temporal Resolution.

Bergman, J. Mellander, L., Wang, Y., Cans, A-S.

Catalysts Under revision after 1st review process.

Contribution: I took part in designing the concept of this project together with co-authors. I designed and performed the major part of the experiments and performed most of the data analysis, interpreted the data and prepared two of the figures for this manuscript. I wrote the main part of the manuscript.

IV. Development of a Microelectrode Biosensor for Recording of Fast Glutamate Transients in Brain Slice of the Mouse

Bergman, J*., Wang, Y*., Devesh Mishra, Keighron, J.D., Skibicka, K. and Cans, A-S.

Manuscript in preparation

Contribution: I took part in initiating the project together with co-authors. I performed the experiments for sensor characterization, performed the data analysis, interpreted the data and prepared the figures for that part. I took part in writing the manuscript together with co-authors.

CONTENT

ABBREVIATIONS

AA	Ascorbic Acid
ACh	Acetylcholine
AChE	Acetylcholine Esterase
ADHD	Attention Deficit Hyperactivity Disorder
Ag/AgCl	Silver-Silver Chloride (reference electrode)
ATP	Adenosine Tri-Phosphate
AuNP	Gold Nanoparticle
CE	Counter Electrode
CFME	Carbon Fiber Microelectrode
Ch	Choline
ChOx	Choline Oxidase
CNS	Central Nervous System
$Cu/CuSO_4$	Copper-Copper Sulfate (reference electrode)
CV	Cyclic Voltammetry
DA	Dopamine
ECF	Extra Cellular Fluid
FAD/ $FADH_2$	Flavin Adenine Dinucleotide
FSCV	Fast Scan Cyclic Voltammetry
GABA	Gamma Butyric Acid
GC	Glassy Carbon Electrode

GlutOx	Glutamate Oxidase
GOx	Glucose Oxidase
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Pressure Liquid Chromatography
IHP	Inner Helmholtz Plane
MALDI	Matrix Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
OHP	Outer Helmholtz Plane
PC12	Pheochromocytoma Cell Line
PNS	Peripheral Nervous System
RE	Reference Electrode
SCE	Saturated Calomel Electrode
SHE	Standard Hydrogen Electrode
SIMS	Secondary Ion Mass Spectrometry
SEM	Scanning Electron Microscopy
STED	Stimulated Emission Depletion Microscopy
TEM	Transmission Electron Microscopy
TIRF	Total Internal Reflection Fluorescence Microscopy
ToF	Time of Flight
UV/VIS	Ultra Violet/Visible light Spectroscopy
WE	Working Electrode

CONSTANTS

α	Transfer coefficient
A	Electrode area, cm^2
A'	Arrhenius fraction factor
C	Concentration, mole cm^{-3}
D	Diffusion coefficient, mole cm^{-2} s^{-1}
e	Electronic charge, 1.602 10^{-19} C
E	Potential, V
$E^{0'}$	Formal potential, V
F	Faraday constant, 96,485 C
ΔG	Gibbs free energy
$\Delta G^{0'}$	Standard Gibbs free energy
i	Current, A
J	Current density, A cm^{-2}
J	Diffusive flux, cm^2 s^{-1}
K	Reaction rate constant, s^{-1}
K_{eq}	Equilibrium constant
η	Overpotential, V
N	Number of electrons
N	Number of moles

N_A	Avogrado's number, 6.022 x10^{23} moles^{-1}
ρ	Density, g cm^{-3}
Q	Charge, C
R	Radii, cm^2
R	Gas constant, 8.314 J
T	Time, s
T	Temperature, K
τ	Time constant, s
ν	Scan rate, V s^{-1}

1 INTRODUCTION

Mental illnesses and neurodegenerative disorders are common health problems causing enormous human suffering and huge economic costs all over the world. Common conditions such as depression, schizophrenia, ADHD, autism, Alzheimer's disease, and Parkinson's disease are all related to malfunctions of the chemistry in the brain. Therefore, it is of great importance to study the mechanism of the chemistry in both the healthy brain as well as in the malfunctioning one. A lot of effort has been spent over several decades trying to reveal the mystery of our brain function and to find treatments and cures for brain related diseases, drug addiction and neuropsychiatric disorders. Many pieces of the puzzle have been found, generating treatments for disorders like depression, schizophrenia, Parkinson's disease and ADHD. Still there are many more pieces to be found and put together.

The human brain, probably the most complex structure there is, consists of hundreds of billions of cells in communication with each other. In chapter 2, I will introduce some functions of the brain as well as some common signaling molecules, so-called neurotransmitters.

During my Ph.D. studies, I have been working on developing new tools for studying some of the chemistry in the brain, mainly the neurotransmitters glutamate and acetylcholine, but also glucose the primary energy source of the brain. My main focus has been on developing miniaturized electrochemical biosensors with high temporal resolution with the purpose of studying neurotransmission in real time. I have also developed methods for characterizing these sensors in order to better understand the mechanism behind their function enabling further

optimization of their performance. The method I have been using is mainly electrochemistry with microelectrodes as will be described in chapter 4. The molecules studied in this thesis, glucose, acetylcholine and glutamate are all molecules important in brain chemistry and are so-called non-electroactive molecules, meaning that they cannot directly be detected at an electrode using electrochemistry. When recording neurochemical activity during neurotransmission, electrochemistry is a commonly used analytical method due to the high temporal resolution offered and the ability of miniaturization. The introduction of biosensors, where a biological component, here an enzyme, coupled to a transducer part, the electrode, has enabled electrochemical detection of non-electroactive molecules. The microelectrode is modified with an enzyme using the molecule of interest as a substrate converting it into a detectable product. The function and design of biosensors will be discussed in chapter 5, while other analytical methods commonly used for studying neurochemistry will be briefly discussed in chapter 3.

In summary, this thesis will give an introduction to brain function with the focus on the chemical communication between neurons, a review about common analytical methods for studying neurochemistry, and a more extensive discussion about analytical electrochemistry and biosensors.

2 THE BRAIN

The brain is the center of our life actions and interactions; our personality and emotions, regulation of our movements and it processes all the information received from our sensory organs e.g. vision, smell, taste and pain. The brain and the spinal cord makeup the central nervous system, CNS, and is made up of billions of cells including different types of glial cells and nerve cells. All information received by the brain is processed through neurotransmission, the communication between nerve cells through exocytosis, where an electrical signal is converted to a chemical signal by the release of neurotransmitters. In this section, neurons and one type of glial cell, astrocytes, will be introduced as well as exocytosis and brain metabolism. Also, common neurotransmitters, their role in the brain, and the connections to diseases and malfunction will be discussed.

2.1 NERVE CELLS

The nerve cell is constituted by a cell body, soma, containing the nucleus and organelles as seen in figure 2.1.1. From the cell body several outgrowths, axons and dendrites, serve as subcellular structures that connects to other nerve cells and form complex neuronal networks. These processes are in general divided into two sub groups, the axon, which is the signaling part, and the dendrites that act as the receiving part, even though other connections e.g. dendrite-soma and axon-axon are also present in the network.

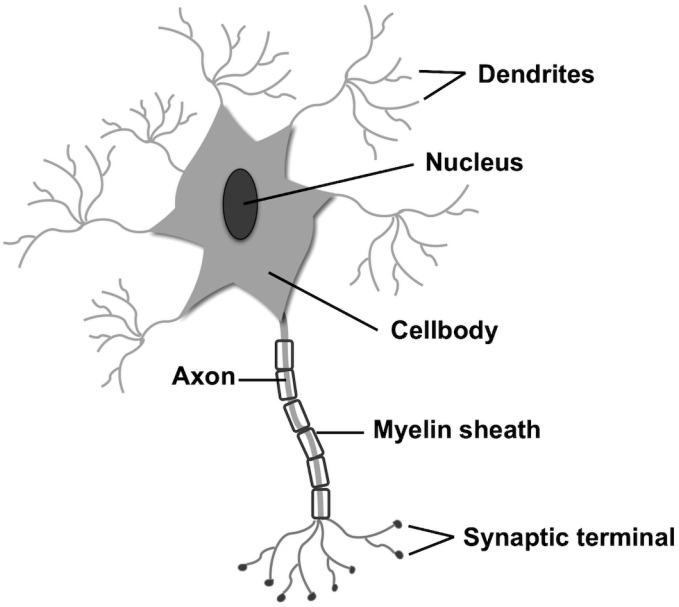

Figure 2.1.1 Schematic picture of a nerve cell with its sub-cellular structures.

The axon and dendrites form connections with other cells through a structure known as the synapse where the membranes of the cells are only separated by a nanometer sized cleft. Nerve cells communicate with each other through the release of chemical messengers, so called neurotransmitters, through a process called exocytosis. In order to trigger a nerve cell to undergo exocytosis it is first stimulated by an electrical nerve signal. In more detail, the membrane surrounding the cell consists of a lipid bilayer also containing proteins and carbohydrates. The proteins are present as membrane bound or trans-membrane proteins, e.g. receptors, ion pumps and ion channels, and also as glycoproteins. Between the intracellular and extracellular space there is a voltage difference across the cell plasma membrane. This charge difference is due to an uneven distribution of ions, with more positive charge on the outside relative to the inside, resulting in voltage across the membrane. This voltage is called the resting membrane potential and is estimated to around -70 mV. The concentration difference of ions is mainly maintained through the Na^+/K^+ pump, an ATP-mediated active transport mechanism pumping 3 Na^+ ions out of the cell for every 2 K^+ ions in, and passive diffusion of mainly K^+ through the membrane. The membrane can be depolarized by a stimulus that increases the membrane potential, often through an influx of sodium ions. This depolarization triggers voltage gated ion channels to open. When the membrane potential is positive enough it causes voltage gated sodium channels to open, thereby further depolarizing the membrane leading to even more sodium ion channels to open. This influx of ions causes a sharp rise in membrane potential, a rise referred to as the action potential. The axon is electrically shielded by myelin allowing rapid propagation of the action potential from the soma to the synaptic terminal. After firing the action potential, the cell returns

to its initial membrane potential and has a resting period, the refractive period where no action potential can take place. In the synapse, the action potential opens up voltage gated calcium channels, causing an influx of calcium ions. This causes neurotransmitter filled vesicles to fuse with the plasma membrane, releasing their contents into the synaptic cleft, a process called exocytosis. The neurotransmitters diffuse across the synaptic cleft and bind to specific receptors at the postsynaptic cell membrane. The binding of the neurotransmitter can trigger the receiving cell to fire an action potential thus passing the signal on to the next cell. After the signal has been transferred, the neurotransmitter detaches from the receptors and is removed from the synapse, partly by reuptake by the signaling cell, mainly by specific transporters but also through nearby cells, or it is degraded by enzymatic reactions.[1-2]

2.2 ASTROCYTES

Another important cell type in the brain is the astrocytes. These are a type of glial cells more than five times as abundant as neurons[3] in the CNS and they are distributed throughout the whole CNS. Although, astrocytes express ion channels such as potassium and sodium channels and can react to elevated inward currents, they cannot fire action potentials as neurons do. Astrocytes respond to stimulation by increasing intracellular calcium concentrations and this acts as a stimulus for increasing intercellular communication with mainly other astrocytes, but also with neurons. Astrocytes release neurotransmitters, e.g. glutamate and GABA, to communicate with neurons and thus play a direct role in synaptic transmission. Astrocytes have multiple interactions with blood vessels as well as neurons and they can regulate the blood flow in relation to synaptic activity. Astrocytes play an important role in the connection between blood vessels and neurons by taking up glucose from the blood and providing energy-related metabolites to neurons. The principal storage site of glycogen in the CNS occurs in astrocyte granules and the largest accumulation of astrocytic glycogen takes place in areas of high neuronal activity. The astrocyte glycogen can be broken down to lactate and transferred to neurons as an energy source.[3-4]

2.3 EXOCYTOSIS

Secretory vesicles are cellular organelles with a lipid bilayer structure that are loaded with signaling molecules, neurotransmitters. There are different types of vesicles, the smallest, clear synaptic vesicle is about 50 nm in diameter and a large vesicle containing a dense core consisting of proteins can be up to 250 nm in diameter.[1, 5] When the vesicle approaches the cell membrane, proteins in the vesicle membrane interact with proteins in the cell membrane to dock the vesicle to the cell membrane. The vesicles and cell membrane form a pore, connecting the inside of the vesicle with the exterior of the cell. The pore can expand and when large enough, the vesicle content can diffuse into the synaptic cleft, as shown in figure 2.3.1. This release can occur in several ways, the fusion pore can close again before releasing all of the content, referred to as partial release or open and closed.[6-9] The so-called full release refers to an event when the fusion pore completely collapses into the membrane releasing all of its content. There is also a third process –"kiss-and-run exocytosis" in which the cell membrane and the vesicle form an initial fusion pore that rapidly closes and thus only a very small fraction of the neurotransmitters are released.[8] This "kiss-and-run exocytosis" can further be extended to a process where the fusion pore open and closes multiple times in rapid succession, "flickering", as has been observed to occur during release from small synaptic vesicles in dopamine neurons.[10] These exocytotic events are very rapid, occurring on the sub millisecond to millisecond time scale. Exocytosis is a very important feature of the neuron and a process brain function relies on for forming memories, emotions, learning, cognition etc.

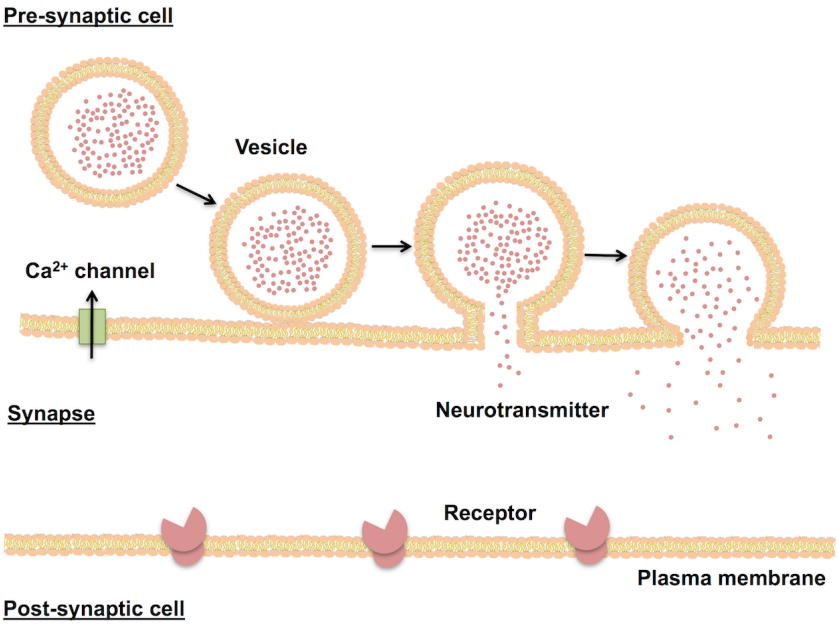

Figure 2.3.1 Overview of the exocytosis process at the synapse between two nerve cells showing how the vesicle at the axon terminal of the signaling cell docks, fuses and releases neurotransmitters through a fusion pore into the synapse, where the molecules diffuse to bind to the specific receptors of the receiving cell.

2.4 NEUROTRANSMITTERS

Neurotransmitters are the chemical messengers released from neurons during communication with other cells. Neurotransmitters can give an excitatory or inhibitory effect sending a start or stop message to receiving cells depending on the type of neurotransmitter, and also on the postsynaptic receptor that neurotransmitters bind to. The excitatory signal increases the probability of the receiving cell firing an action potential while the inhibitory signal decreases that probability. Some neurotransmitters are thought to be mainly inhibitory, e.g. GABA or mainly excitatory, e.g. glutamate. A molecule is generally considered to be a neurotransmitter if the following criteria are met. First, the molecule must be present in the neuron. Second, the molecule must be released from the presynaptic neuron as a response to a presynaptic electrical signal. Last, there must be a specific receptor for that molecule at the postsynaptic neuron. The classification of neurotransmitters varies depending on different aspects such as chemical structure, size and actions. Neurotransmitters can be classified according to size, as small molecule neurotransmitters, e.g. catecholamines and amino acids, and as large peptide neurotransmitters (Endorphins, Somatostatin) as proposed by Purves et al.[1] In table 2.4.1 some small neurotransmitters are classified according to chemical structure.

Table 2.4.1 Classification of small neurotransmitter molecules according to their chemical structure. *biogenic amines

Chemical group	Examples
Amino acids	Glutamate, GABA, Aspartate, Glycine
Purines	ATP, Adenosine
Catecholamines*	Dopamine, Norepinephrine, Epinephrine
Indoleamine*	Serotonin
Imidazoleamine*	Histamine
Others	Acetylcholine

2.4.1 DOPAMINE

Dopamine is likely the most well-known neurotransmitter. It was discovered as a neurotransmitter by Arvid Carlsson[11] in the 1950s, a discovery for which he was rewarded the Nobel Prize in year 2000. Dopamine generally functions as an inhibitory neurotransmitter and plays an important role in the brain due to its involvement in many different signaling pathways e.g. reward motivated behavior and motor control. Both excessively high levels of dopamine as well as very low levels in the brain cause serious impact to various brain functions and have been related to different brain diseases and disorders such as Parkinson's disease, schizophrenia, attention deficit hyperactivity (ADHD), and addiction. Parkinson's disease is related to degeneration of dopamine neurons leading to low levels of dopamine. This causes the characteristic uncontrollable muscle tremors in the disease. L-DOPA, a precursor of dopamine can be used to initially treat the illness by elevating the dopamine levels in the brain. The reward system is involved in development of drug abuse and addiction by increasing the levels of extracellular dopamine levels responsible for the strong feelings of joy and satisfaction.[12-14]

2.4.2 SEROTONIN

Serotonin is mainly an excitatory neurotransmitter and the molecule was isolated and characterized in 1948 by Irvine Page and Maurice Rapport.[15] Brodie and Shore proposed the role of serotonin as a neurotransmitter in 1957.[16] Most of the serotonin of the body is not located in the brain but in enterochromaffin cells in the gastrointestinal tract contributing to gastrointestinal reflexes.[17-18] The function of serotonin in the brain are very diverse and are related to the regulation of appetite, body temperature, sleep cycles and sexual behavior. It is also involved in mood and is thought to be a part of happiness and well-being. Serotonin plays a big role in psychological disorders such as depression, mania and anxiety conditions that are associated with the distribution of serotonin in the brain.[19-20] The family of drugs called selective serotonin reuptake inhibitors increases the available serotonin in the synapses by blocking the reuptake improving the symptoms of the earlier mentioned disorders.[21]

2.4.3 GABA

Gamma aminobutyric acid, GABA, has an amino acid structure and was found to act in the CNS in the 1950s.[22-24] In mammals, GABA is found in high concentrations in the brain and is the main inhibitory acting neurotransmitter decreasing the probability of neurons to fire an action potential by affecting ion channels and causing membrane hyperpolarization.[25] Disturbance in GABAergic inhibition can result in seizures and is why epilepsy treatment often is targeted against GABA activity.[26] Disorders of GABAergic function in the CNS are also related to diseases such as anxiety disorders, spasticity and schizophrenia.[27-28]

2.4.4 NOREPINEPHRINE

Norepinephrine, also known as noradrenaline is a molecule not only functioning as a neurotransmitter but also as a hormone. It belongs to the group of catecholamines, where dopamine is a direct precursor to this molecule. It was discovered as a neurotransmitter in the 1940s by Ulf Svante Euler[29] for which he was awarded a part of the Nobel prize 1970. When acting as a hormone it is released into the bloodstream from the adrenal glands. It then acts on the body's ability to respond to stress by making the body ready for action by increasing heart rate, blood pressure, blood supply to the muscles and release of glucose from energy storage. In the brain norepinephrine increases functions needed to deal with stressful situations e.g. alertness, sharper memory, excitement and anxiety.[30-32] Norepinephrine can be thought of as the gas pedal of the nervous system affecting the whole body both as a hormone and a neurotransmitter. Health problems related to chronic stress such as high blood pressure, sleeplessness and gastrointestinal problems are all related to the effect of sustained release of norepinephrine. The noradrenergic system has also been linked to depression[33] and cognitive disorders such as Alzheimer's disease.[34]

2.4.5 EPINEPHRINE

Epinephrine or adrenaline is derived from norepinephrine and is also a catecholamine acting both as a hormone and neurotransmitter. Its role as a neurotransmitter was discovered late, in the 1970's,[35] even though the molecule itself had been known of since the late 19th century.[36] It is involved in the "fight or flight response" to stressful situations with similar actions as those for norepinephrine described above. Epinephrine is responsible for the feeling of fear during the response to a stressful situation.[37] These emotionally stressful events have been found to be connected to long-term memory in humans inducing memory strength to be proportional to memory importance. It has also a major impact on heart rate, blood vessel dilation, and air passage and this is why it is extensively used as a drug to treat cardiac arrest, asthma, and anaphylactic shock.[38]

2.4.6 GLUTAMATE

Glutamate is an amino acid synthesized in neurons, this is important since it cannot cross the blood-brain barrier and thus cannot be utilized from food intake. Glutamate is often referred to as the most important neurotransmitter for normal brain function including cognition, memory and learning, and has been known as an excitatory neurotransmitter since the 1950's.[39] It has been estimated that over 50 percent of all synapses in the brain release glutamate. In the central nervous system, nearly all excitatory neurons are glutamatergic.[40] Elevated levels of extracellular glutamate in the brain are neuro-toxic and are released to a toxic level during neural injury such as cerebral ischemia (stroke) and brain trauma. The concentration of glutamate in the synaptic vesicle is estimated to be around 100 mM, and about 0.5 to 45 μM in the healthy brain extracellular fluid depending on the measurement method used, where the higher concentrations have been determined with electrochemical micro-sensors and the lower range with microdialysis.[41] The ECF concentration of glutamate has also been shown to vary between brain regions. Neurodegeneration in motor neuron diseases, Huntington's disease, Parkinson's disease, and Alzheimer's disease have all been connected to malfunction of the glutamatergic system.[40]

2.4.7 ACETYLCHOLINE

Acetylcholine, ACh, was the first neurotransmitter to be discovered and was identified in 1914 by Henry Dale.[42] Later, Otto Loewi[43] confirmed the action of ACh as a neurotransmitter and both of them were rewarded the Nobel Prize in 1936. ACh acts both in the CNS and in the PNS (Peripheral Nervous System). ACh is released in the neuromuscular junction transferring the signal from the neurons to the muscle causing the muscle to contract.[2] ACh acts as both inhibitory and excitatory depending on the target receptor. At nicotinic receptors,[44] ACh is excitatory, but it is inhibitory where the receptors are muscarinic.[45] In the CNS, ACh is involved in temperature and blood pressure regulation, learning and memory, motor coordination and controlling the stages of sleep.[46] ACh plays an important role in several illnesses such as Huntington's disease[47], Alzheimer's disease[48] and schizophrenia.[49]

2.5 BRAIN METABOLISM

There are several other molecules important for normal brain function even though they do not function as neurotransmitters. Glucose is the primary source of energy for the brain and tight regulation of glucose metabolism is crucial for normal brain function. The glucose is oxidized through glycolysis to form ATP. Oxidative phosphorylation and ATP production are tightly coupled to the rapid changes in energy demanded by functioning synapses to keep a desired level of neuronal activity. The brain is the part of the body that consumes the most glucose, approximately 20 % of all available glucose derived energy goes to the brain, around 5.6 mg glucose per 100 g brain tissue per minute corresponding to about 1 mM glucose in the ECF, even though the brain itself only makes up about 2 % of the body weight.[50] The majority of the energy demand in the brain comes from neuronal communication e.g. generation of action potentials, maintenance of ion gradients and providing energy and precursors for biosynthesis of neurotransmitters. Excitatory neurotransmission is a process with very high energy demands so the main site of ATP synthesis in the brain is in the neurons. Inhibitory neurotransmission is less energy demanding than excitatory transmission because of the differences in activity of ion channels.[51] Consistently it has been reported that the inhibitory neurotransmitter GABA uptake into astrocytes does not cause an activation of glycolysis but the uptake of the excitatory glutamate does.[52] *In vivo* studies have shown an increase in extracellular lactate accompanied by increases in oxygen and glucose consumption during excitatory neuronal activity.[53] Since neuroactive substances such as glutamate cannot cross the blood brain barrier they must be synthesized in the brain and glucose is the primary carbon source

for that as well. The astrocytes provide neurons with energy from glucose and glycogen that is stored in astrocyte granules as described previously.

Studying brain metabolism and neuronal communication is of great importance in order to understand brain function and what role it plays during abnormal brain functions due to neurodegenerative diseases[50] with the ultimate goal of enabling treatments and hopefully even cures for these malfunction and diseases in the brain. In the next chapter, some common methods for studying neurochemistry will be introduced.

3 ANALYTICAL METHODS

In this chapter, techniques for studying neurochemistry will be briefly described and their pros and cons will be discussed with regard to their possible applications. Before choosing an analytical method for an experiment, careful considerations must be taken regarding the purpose of the experiment and what parameters that are desired to analyze. For instance, analyzing the total content of neurotransmitters in the ECF of the brain requires a technique very different from what is needed for studying exocytosis from a single cell. In the first case a very complex matrix is present requiring the need of separation of analytes before detection, also the expected concentrations are very low why a method with high sensitivity is necessary. In the other case, single cells can be in a fairly simple matrix and exocytosis occurs in sub-milliseconds why a technique with high temporal resolution is required but selectivity is often not as big of an issue. The best results are often obtained when several analytical techniques are used simultaneously, either in sequence or at the same time to be complementary. Considering the first example of detecting neurotransmitters in ECF a common approach is to insert a microdialysis probe in to the brain to collect molecules from the ECF followed by separation, e.g. HPLC or capillary electrophoresis, and finally detected by, for instance, electrochemistry, fluorescence or mass spectrometry.[54-55]

3.1 *IN VIVO* MICRODIALYSIS

In vivo microdialysis has been used since the late 1960s and is a widely used technique for studying the effect of drugs in animal brain model and has the advantage of providing samples during a long time while the animal can be awoken and freely moving. By making such measurements it is possible to correlate the chemical dynamics of the surroundings of the probe to behavior, drug effect and disease progress. Microdialysis has the ability to sample the chemical environment in tissue with both high sensitivity as well as selectivity especially when coupled to separations such as HPLC and capillary electrophoresis.[56] In microdialysis, a probe with a semi-permeable dialysis membrane passing a perfusion liquid is used to recover small molecules from the extra cellular space in the brain. The microdialysis probe is inserted into the brain and an artificial extracellular solution is slowly and continuously infused through the probe until equilibrium is achieved between the inside of the probe and the extracellular space. The molecules will diffuse down their concentration gradient into the probe and after some time the perfusion solution inside the probe will contain a representation of the chemicals found in the extracellular space.[57] Microdialysis has the benefit of being able to sample larger neuroactive molecules such as proteins and peptides making it possible to, for example, study amyloid-β, a protein associated with the progression of Alzheimer's disease.[58] The microdialysis probe dimensions are usually around 200-400 μm in diameter with a 0.5-4 mm sampling length leading to both poor spatial resolution as well as a risk of causing substantial damage to the brain tissue leading to a local inflammation which itself can affect the local brain ECF chemistry.[57] One approach for minimizing the dimensions of the sampling probe is the use

of two fused silica capillaries using low-flow push-pull perfusion where one capillary "pulls" the sample and the other capillary "pushes" fluid to the sample region to maintain fluid balance in the sampling region. The micro-fabricated push-pull probe has been made as small as 85 μm wide substantially increasing the spatial resolution.[59] One drawback with the microdialysis technique is the poor temporal resolution that even with direct coupling to capillary electrophoresis for separation, the temporal information achieved will still be in a few seconds. Using push-pull probes have been increasing spatial resolution as described but at the cost of temporal resolution due to the low perfusion flow rate required leading to sampling times in the order of tens of minutes.[59] Direct coupling of the push-pull probe with nL samples to capillary electrophoresis with fluorescence detection has thus been able to push the temporal resolution down to the same time limits as conventional microdialysis. In summary, *in vivo* microdialysis is approaching the spatial resolution that can be obtained with electrochemical sensors but can still not compete with regard to temporal resolution, instead the strength of the method relies on the ability to simultaneously detect many analytes with high sensitivity and selectivity.[60]

3.2 SEPARATION TECHNIQUES

When analyzing complex matrices like blood, urine and other body fluids a separation of the present species is often needed before analysis, both for quantitative as well as for qualitative analysis. Chromatographic techniques are based on the passing of a liquid (mobile phase) where the analytes are present through a column (stationary phase). The column can consist of different materials and the separation can be based on various properties, e.g. size exclusion columns separating analytes regarding to molecular size, ion-exchange columns separating regarding electrostatic interactions with the stationary phase, but all separation basically depends on different affinity of molecules to the stationary phase in the column used. The mobile phase can often be tuned to increase separation selectivity by affecting the analyte or stationary phase properties. For instance, changing the pH of the mobile phase can change the charge of both the molecules in the mobile phase as well as in the stationary phase affecting the electrostatic interactions between the analytes and the stationary phase. The most widely used techniques in separations are high-pressure liquid chromatography, HPLC, and gas chromatography, GC, where HPLC is dominating over GC for biomolecules. HPLC uses high pressure to push the mobile phase through the stationary phase. One commonly used method is reverse-phase chromatography where the mobile phase is polar and the stationary phase is hydrophobic.[61] There are several other variants of liquid chromatography e.g. normal-phase chromatography that uses a hydrophobic mobile phase together with a polar stationary phase and HILIC based on a hydrophilic stationary phase combined with a polar mobile phase separating species regarding their polarity.[62-63] HPLC requires a column for separation and a detector for

quantification. Commonly used detection techniques for coupling to HPLC are, UV/VIS, fluorescence, mass spectrometry, MS, and electrochemistry.[64] Fluorescence and UV/VIS is based on the analytes being fluorescent or absorbing light in the ultra violet/visible light spectra respectively while electrochemistry depends on the electroactivity of the analytes and for MS the analytes mass to charge ratio is detected. MS, fluorescence and electrochemistry will be discussed later in this section.

Another method for separation is capillary electrophoresis where usually a fused-silica capillary with very small dimensions commonly in the range of a few μm to 100 μm in inner diameter without stationary phase or a pseudo-stationary phase is used and the liquid inside the capillary is driven by electrophoresis. Briefly, in capillary electrophoresis a high voltage supply is applied over the capillary connected through two buffers containing an electrolyte solution creating an electric field. The analytes will travel based on their size to charge ratio in the generated electroosmotic flow.[65] Capillary electrophoresis has the advantages over HPLC of the separation being performed over much smaller time scales and requiring very small volumes of the sample and has been used for studying biogenic amines in the fruit fly brain and even the contents of a single cell.[66-67] Also, the electroosmotic flow will create a non-laminar flow decreasing band-broadening of the peaks and thus increasing the resolution. HPLC on the other hand, is more robust and easier to handle since for example even very small changes in pH of the mobile phase will have a great impact in capillary electrophoresis and there are many factors affecting the pH e.g. temperature. In addition, the high voltage and the small dimensions used during capillary electrophoresis possess a challenge.

3.3 MASS SPECTROMETRY

Mass spectrometry, MS, is an analytical technique where the analytes are analyzed and identified by their mass to charge ratio. Analytes must be ionized via some mechanism, e.g. electrospray ionization in order to be separated by their mass to charge ratio in a mass analyzer e.g. time-of-flight (ToF) and quadrupole. The resulting mass spectrum is a plot of signal versus mass to charge ratio and the peaks obtained are characteristic for unique chemical compounds and their fragments and can be used for identification of the species analyzed. MS is often the method of choice for detection of analytes after separation with e.g. capillary electrophoresis[68] or HPLC[69] due to the excellent selectivity, sensitivity and ability to detect a large number of analytes in a complex matrix. MS is also used for high throughput analysis e.g. proteomics.[70-71] Especially when coupled to separation techniques, MS offers excellent selectivity and can detect and identify a large number of molecules in a complex matrix. MS can also be used for chemical imaging as will be discussed in imaging techniques in section 3.5.

3.4 ELECTROPHYSIOLOGY

Electrophysiology studies the electric properties of cells, tissue and whole organs. Widely used techniques in routine medicine are electrocardiography (ECG) and electroencephalography (EEG) where the electrical activity of the heart and the brain respectively can be studied. In neurochemistry when recordings at the single cell level is the aim, an electrophysiological technique called patch clamp is often used. Bert Sakmann and Erwin Neher invented the patch clamp technique in the late 1970s and early 1980s for which they were rewarded the Nobel Prize in 1991.[72] With patch clamp, electrophysiological properties of cell membranes and the current flowing through ion channels in the cell plasma membrane can be studied. Briefly, a micropipette containing a conductive electrolyte is placed in connection to the cell, here, different approaches are possible, e.g. the "cell attached" configuration where a tight seal between the pipette and the outside of the membrane is created without rupturing of the membrane. Another example is the "whole cell mode" where the membrane seal around the micropipette tip, a so-called "gigaseal" creates a direct connection with the cell through the membrane without rupturing it. For the experiment either voltage clamp or current clamp can be used depending on the cell properties of interest to study. Voltage clamp is the best choice for recording firing activity while current clamp is best-suited for recording potentials like resting membrane potential and synaptic potentials. In voltage clamp, the membrane voltage is controlled and the current, the result of ions flowing across the cell membrane through ion channels as well as the capacitive current can be measured.[73] As described earlier in the section of neurons many ion channels are sensitive to membrane potential changes and can

thus be studied by “clamping” the cell membrane at different potentials. In current clamp, the changes in voltage are measured while the current is controlled, a mode that can be used to determine if the synaptic potential is depolarizing or hyperpolarizing as well as if the depolarizing potential is excitatory or not.[74] By measuring the capacitance over time exocytotic and endocytotic process can be monitored due to the fact that the capacitance is proportional to membrane surface area. Capacitance measurements have been able to distinguish between “full-release” and “kiss-and-run” exocytosis as it is a technique with high temporal resolution.[75]

3.5 IMAGING TECHNIQUES

Optical methods for imaging biological samples are widely used in neurochemical research. Fluorescence microscopy is a very common technique due to its high selectivity and sensitivity. It is based on the fluorescent properties of the species of interest. The fluorescence of the species can be studied with several different microscopy techniques e.g. confocal microscopy, total internal reflection microscopy, TIRF,[76] and stimulated emission depletion microscopy, STED.[77] Fluorescence is emission of light occurring nanoseconds after the absorption of light. The difference between the exciting and emitting wavelengths is the critical property and what makes fluorescence such a powerful tool for studying small components with high temporal resolution such as visualizing the dynamics of exocytosis and endocytosis in real time.[78-80] Very few biomolecules are natively fluorescent why the molecules of interest or their surroundings need to be fluorescently labeled with a fluorescent molecule, a fluorophore. The fluorophores properties can be very sensitive regarding their environment and some fluorescent sensors can change their spectra when bound to certain molecules e.g. calcium- and hydrogen ions, sensitivity to electrical fields is also occurring. pH sensitive fluorophores[81-83] have been used for imaging of vesicle fusion taking advantage of the pH difference in the vesicle where pH ≈ 5.5 and the surrounding physiological pH ≈ 7.4. The strength of fluorescence microscopy lies in the relatively high temporal resolution combined with high sensitivity and selectivity while the main drawback of the technique is the need for fluorescently labeling the species of interest, where the fluorophore itself could interfere with the properties of the biological system.

Another way of imaging cellular structures is the use of electron microscopy such as scanning electron microscopy, SEM and transmission electron microscopy, TEM.[84-85] Electron microscopy is a technique based on a beam of electrons as the source of illumination instead of photons as in optical microscopy. The wavelength of electrons is in the magnitude of 100,000 times shorter than of visible light photons giving the electron microscope a resolution many times higher compared to a light microscope. A TEM microscope can achieve a resolution of 0.5 Å where the maximum resolution of a light microscope is limited by diffraction and about 200 nm. In combination with fast Cryo-fixation techniques, Cryo-TEM, it is possible to capture sub-second "snap-shots" of biological processes,[86] but it is not possible to image living organisms or cells and thus no continuous temporal information can be obtained. The main advantage of TEM is obviously its high resolution making it possible to view the structure of organelles in single cells e.g. number of vesicles present in a synapse and their size. Again, the strength of combining different methods in analytical chemistry was shown when TEM imaging was combined with super high-resolution mass spectrometry imaging, NanoSIMS, to show the distribution profile of dopamine across individual vesicles.[86] Mass spectrometry can also be used for imaging, a powerful technique for visualizing chemical species in biological samples such as tissue and single cells with high spatial resolution. Secondary ion mass spectrometry, SIMS,[87] is a technique for sensitive surface analysis that can provide chemical information with spatial resolution down to 50 nm and allow detection of intact lipids, lipid fragments, metabolites and elements. Another imaging mass spectrometry technique frequently used for biological samples is matrix-assisted laser desorption/ionization, MALDI,[88] suitable to analyze large molecules such as DNA, proteins and peptides. The images are constructed by plotting signal intensity versus

the relative position of the sample data providing high spatial resolution where the chemical distribution in the sample can be visualized, providing e.g. spatial peptide and protein profiling.[89]

3.6 ELECTROCHEMISTRY

Amperometry has been widely used in neurochemistry analysis both *in vivo* and in cell models due to its ability of very high temporal resolution. Microelectrodes have made it possible to detect single exocytotic events from single cells, e.g. PC12 cells, mast cells and chromaffin cells giving new insights in how exocytosis occurs and factors affecting it, e.g. regulation of vesicle pore formation and membrane dynamics.[90-91] In a typical single PC12 cell experiment an inverted optical microscope is used and a microelectrode is placed in close proximity to the cell surface. By stimulating the cell, the release of dopamine through exocytosis will occur. The released dopamine will hit the electrode surface and will then immediately be oxidized giving rise to an anodic current spike that can be recorded and analyzed providing information about the kinetics of the spike relating to fusion pore dynamics as well as how many molecules where released. The development of microelectrode arrays has enabled both high temporal resolution as well as spatial information of single cell exocytosis revealing individual release events originating from multiple locations at the cell.[92-94] Amperometry is also widely used as a detection method in separations e.g. HPLC and capillary electrophoresis, where a very low detection limit can be reached.[95] The main drawback of amperometry is the lack of selectivity. Everything in the solution that can be oxidized/reduced at the electrode in the potential window used will be, not only the molecule of interest. This issue is overcome by using it when less complex matrices are present or following separation of the molecules. The development of electrochemical cytometry,[6-7, 96-97] has enabled the quantification of the total neurotransmitter content in single vesicles isolated from cells. The

method is based on the adsorption of isolated vesicles from cells like chromaffin cells and PC12 cells that contain electroactive neurotransmitters. When the vesicles adsorb to the polarized electrode surface, the vesicles will rupture due to the electric field created and all of the vesicle content will rapidly be oxidized at the electrode surface creating a current spike that can be detected and analyzed. Pushing the size of microelectrodes down to nm size has enabled quantification of vesicle content inside living cells with electrochemical cytometry.[98]

The fundamentals of electrochemistry including amperometry and how to evaluate amperometric data will be discussed more in detail in the analytical electrochemistry section in this thesis.

4 ANALYTICAL ELECTROCHEMISTRY

In this chapter, the general concept of analytical electrochemistry and the techniques most relevant for this thesis are introduced. The convention used is that oxidation, anodic current, is defined as positive and reduction, cathodic current as negative. Further, increasing potential is shown as positive in the voltammograms later in this section. Throughout this section, the disk-shaped electrode is used as an example when discussing the methods and principles. There are several other electrode geometries such as cylindrical, band and spherical those are not discussed here but follow the same fundamental principles.

Analytical electrochemistry takes advantage of a molecules ability to undergo oxidation or reduction at an electrode surface. There are several different analytical electrochemical techniques but the ones treated in this thesis all rely on measuring the current when either holding the electrode at a fixed potential or when altering the potential over time. Electrochemical measurements of chemical systems can provide information about the system studied e.g. thermodynamics of the reaction, the identity of a molecule of interest or the concentration of that molecule in a sample. In electrochemical systems, transport of charge between chemical phases, e.g. a solid and a liquid, and how different factors and processes affect this charge transport is evaluated. The systems studied with the electroanalytical techniques discussed in this thesis rely on the interface between the electrode and the surrounding solution (electrolyte) and the events occurring when an electric potential is applied and current passes through the system.

4.1 ELECTROCHEMICAL KINETICS

When a molecule undergoes reduction or oxidation at an electrode surface, the molecule is either accepting or donating electrons from the electrode, respectively. This leads to charge transport through the electrode by movement of electrons, which is detected as a current. In the surrounding electrolyte, the movement of ions carries the charge. A molecule in its oxidized form may accept electrons, e^-, from an electrode and become reduced. If the reduced molecule is then oxidized again a reversible charge transfer reaction has taken place, see equation *4.1.1*

$$Ox + ne^- \underset{k_a}{\overset{k_c}{\rightleftharpoons}} Red \qquad 4.1.1$$

Ox is the oxidized and *Red* is the reduced state of a molecule; *n* is the number of electrons exchanged in the redox reaction k_C and k_A are the reaction rate constants for the reduction and oxidation process and has the unit s^{-1}. The rate, *v*, in which the reactions take place, is described as following for the two reactions always occurring simultaneously, where C_{red} and C_{ox} is the concentration of the reduced and oxidized species.

$$v_{Ox} = k_A C_{Red} \qquad 4.1.2$$

$$v_{Red} = k_C C_{Ox} \qquad 4.1.3$$

By combining these equations, (*4.1.2, 4.1.3*) the net conversion rate, v_{net}, of the oxidized species to the reduced one can be written as

$$v_{net} = k_A C_{Red} - k_C C_{Ox} \qquad 4.1.4$$

When the net flux of all molecular species (and electrons) is zero since an equal anodic current balances the cathodic current in the system, the system is at equilibrium, K_{eq}, and the concentration ratio between *Ox* and *Red* is constant yielding the following expression for K_{eq}

$$\frac{k_A}{k_C} = \frac{C_{Ox}}{C_{Red}} = K_{eq} \qquad 4.1.5$$

The reaction (*4.1.1*) has one oxidation path and one reduction path and the reaction proceeds at a rate, v_{Ox} and v_{red}, respectively (*4.1.2, 4.1.3*). Consider the reduction reaction, the reaction rate, v_{red}, is proportional to the electrode surface concentration of the oxidized specie *Ox* and by expressing the concentration of *Ox* at a distance *x*, from the electrode surface area, *A*, and at time *t* as $C_{(Ox)}\ (x,\ t)$, where the surface concentration of *Ox* is zero so $C_{(Ox)}\ (0,\ t)$, the rate constant k_c can be related to the cathodic current, i_c of the reaction (*4.1.6*).

$$v_{Red} = k_C C_{Ox}(0,t) = \frac{i_C}{nFA} \qquad 4.1.6$$

The same expression for the reaction rate, v_{ox}, is valid for the anodic component of the total current i_A (*4.1.7*)

$$v_{Ox} = k_A C_{Red}(0,t) = \frac{i_A}{nFA} \qquad 4.1.7$$

F is the *Faraday constant*, the charge of one mole of electrons, and *A* is the electrode area in cm^2. The reaction rate is also dependent on the electrode area, *A*, and in order to be able to compare processes taking place at electrodes with varying surface areas, the rate of the reaction has to be normalized for the area of the electrode, this is referred to as *current density*, *j*, current per electrode area, (A cm^{-2}).

Combining equation (*4.1.6*, *4.1.7*) an equation (*4.1.8*) describing the net current of the reaction with respect to the cathodic (i_c) and anodic (i_a) current components at the surface of the electrode is obtained and can be written as follows

$$i = i_A - i_C = nFA\lfloor k_A C_{Red}(0,t) - k_C C_{Ox}(0,t)\rfloor \qquad 4.1.8$$

In order for a non-spontaneous chemical reaction to take place energy must be added in order to decrease the energy barrier for transferring the reactant into the product. In electrochemistry, the electric potential energy drives the reaction and the energy required for an oxidation/reaction to take place is related to *the formal potential,* $E^{0\prime}$, of the species involved in the redox reaction. $E^{0\prime}$, relates to the *standard Gibbs free energy change* $\Delta G^{0\prime}$ as

$$\Delta G^{0\prime} = -nFE^{0\prime} \qquad 4.1.9$$

The relationship between the concentrations of the species *Ox* and *Red* and free energy is given in the following equation

$$\Delta G = \Delta G^{0\prime} + RTln\frac{C_{Ox}}{C_{Red}} \qquad 4.1.10$$

Where R is the ideal gas constant and T is the temperature.

The *Arrhenius equation* (*4.1.11*) correlates the rate constant k, of a reaction to Gibbs free energy, where $\Delta G^{\ddagger}$ *is the standard free energy of activation* and A' is the frequency factor.

$$k = A'e^{-\Delta G^{\ddagger}/RT} \qquad 4.1.11$$

An important general theory for describing electrode kinetics is the *transition state theory* also known as the *activated complex theory* where the assumption that the reaction proceeds through a defined transition state or activated complex before being transferred into the product, as shown in figure 4.1.1.

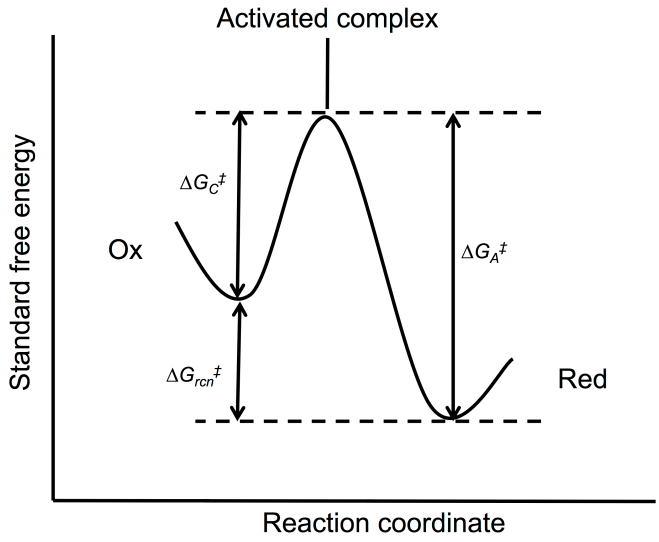

Figure 4.1.1 Free energy changes during a reaction. The activated complex is the configuration of maximum free energy during the reaction. (Redrawn)[99]

When the electrode potential is equal to the potential at equilibrium, known as the *formal potential*, $E^{0\prime}$, the anodic and cathodic activation energies $\Delta G^{\ddagger}_{0A}$ and $\Delta G^{\ddagger}_{0C}$ have the same magnitude and thus the same activation energy. By changing the potential from $E^{0\prime}$ to E the relative energy of the electrons on the electrode changes by $-F(E - E^{0\prime})$ and by

doing this for an oxidation process where E has a more positive value compared to $E^{0\prime}$ the activation barrier for oxidation $\Delta G^{\ddagger}_{A}$ will become less than $\Delta G^{\ddagger}_{0A}$ by a fraction of the total energy change as described in figure 4.1.2. This fraction is called *1-*α, where the *transfer coefficient*, α, describes the symmetry of the energy barrier of activation. In a reversible redox system, often referred to as a *Nernstian system*, the *transfer coefficient*, α, is equal to 0.5 indicating that the system is symmetric with respect to the activation barriers for the reactions. In practice, this means that the redox system is *reversible*; this will be described in the later section about cyclic voltammetry.

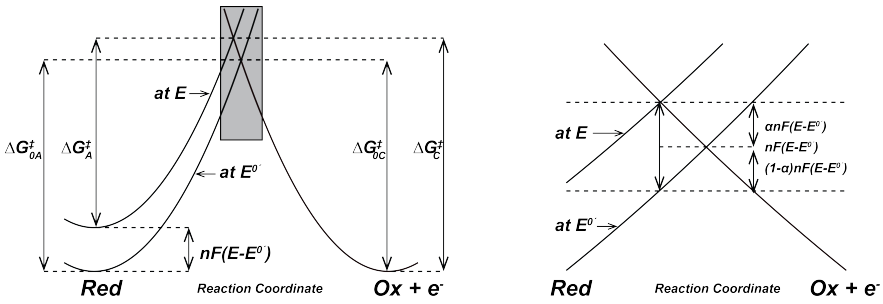

Figure 4.1.2 Effects of a potential change on the standard free energies of activation for a reversible redox system. The figure to the right is a magnification of the boxed area in the left figure. (Redrawn)[99]

The activation barrier for the anodic and cathodic reaction can then be described in terms of the transfer coefficient, the activation energy at the formal potential and the potential difference between formal potential and applied potential as follows

$$\Delta G_A^{\ddagger} = \Delta G_{0A}^{\ddagger} - (1-\alpha)nF(E - E^{0\prime}) \qquad 4.1.12$$

$$\Delta G_C^{\ddagger} = \Delta G_{0C}^{\ddagger} + \alpha nF(E - E^{0\prime}) \qquad 4.1.13$$

Inserting equation (*4.1.12*) and (*4.1.13*) into the *Arrhenius equation* (*4.1.11*) gives the rate constant for each reaction.

$$k_A = A_A e^{\left(-\Delta G_{0A}^{\ddagger}/RT\right)} e^{[(1-\alpha)f(E-E^{0\prime})]} \qquad 4.1.14$$

$$k_C = A_C e^{\left(-\Delta G_{0C}^{\ddagger}/RT\right)} e^{[-\alpha f(E-E^{0\prime})]} \qquad 4.1.15$$

(*f* is defined as *nF/RT)*

When the electrode interface and the solution is at equilibrium so $C^*_{Ox} = C^*_{Red}$, $E = E^{0\prime}$ and $k_a = k_c$ thus at $E^{0\prime}$ the anodic and cathodic rate constants have the same value, which is called the *standard rate constant* k^0. The rate constants for the anodic and cathodic reaction k_a and k_c are related to the *standard rate constant* according to the following equations

$$k_A = k^0 e^{(1-\alpha)f(E-E^{0\prime})} \qquad 4.1.16$$

$$k_C = k^0 e^{-\alpha f(E-E^{0\prime})} \qquad 4.1.17$$

Inserting the expressions for k_A (*4.1.16*) and k_C (*4.1.17*) into equation *4.1.8* gives the complete *current-potential characteristic* equation and the total current *i* of the reaction at equilibrium can be described as

$$i = FAk^0\left[C_{Ox}(0,t)e^{-\alpha f(E-E^{0\prime})} - C_{Red}(0,t)e^{(1-\alpha)f(E-E^{0\prime})}\right] \quad 4.1.18$$

Earlier the activation barriers for the redox process (*4.1.12*, *4.1.13*) and the potential difference between the *formal potential* and applied potential ($E - E^{0\prime}$) were described. This can be written in terms of *overpotential*, η, as

$$\eta = E - E_{eq} \quad 4.1.19$$

Using this expression of overpotential, η, and inserting it in to equation *4.1.18* the following expression, often referred to as the *Butler-Volmner equation* is obtained

$$i = FAk^0\left[C_{Ox}(0,t)e^{-\alpha f(\eta)} - C_{Red}(0,t)e^{(1-\alpha)f(\eta)}\right] \quad 4.1.20$$

The *Butler-Volmner equation* describes how the current depends on the applied potential considering both the reduction and oxidation processes occurring at the electrode and is very useful for dealing with electrochemical reactions in practice. As described earlier, the net current at equilibrium is zero, but there is still *Faradaic* activity often expressed as exchange current, i_0, equal in magnitude to either i_c or i_A. The exchange current, i_0, is proportional to the *standard rate constant* k^0 and when $C^*_{Ox} = C^*_{Red} = C$, the total expression for the *current-potential characteristics* can be written as

$$i_0 = FAk^0C \qquad 4.1.21$$

The total current i from the redox reaction at the electrode surface when applying an overpotential can be expressed by combining expression *4.1.20* and *4.1.21* yielding the *current-overpotential equation* below

$$i = i_0 \left[\frac{C_{Ox}(0,t)}{C^*_{Ox}} e^{-\alpha f \eta} - \frac{C_{Red}(0,t)}{C^*_{Red}} e^{(1-\alpha) f \eta} \right] \qquad 4.1.22$$

For the case when i_A is equal to i_C and thus the net current i is zero and the oxidation and reduction process has the same rates we get the following expression describing the equilibrium

$$nFAk^0C_{Ox}(0,t)e^{-\alpha f\eta} = FAk^0C_{Red}(0,t)e^{(1-\alpha)f\eta} \qquad 4.1.23$$

Since the $nFAk^0$ component in expression *4.1.23* cancel out we can simplify the equation to

$$e^{f\eta} = \frac{C_{Ox}(0,t)}{C_{Red}(0,t)} \qquad 4.1.24$$

The expression above (*4.1.24*) can be related to the *Nernst equation* (*4.1.25*) by taking the logarithm.

$$E = E^{0\prime} + \frac{RT}{nF} ln \frac{C^*_{Ox}}{C^*_{Red}} \qquad 4.1.25$$

Where *R* is the ideal gas constant *T* is the temperature, *n* is the number of electrons involved in the reaction and *F* is *Faraday's constant*. The *Nernst equation* (*4.1.25*) is also directly related to the *Gibbs free energy* (*4.1.9*, *4.1.10*) of the reaction describing that a spontaneous reaction is associated with a positive value of *E*.

In summary, electrochemical reactions follow the thermodynamic principles of general chemical reactions where the activation energy

barrier is reduced by the electric potential energy in this case. The *formal potential*, $E^{0\prime}$, defines the potential energy point above which the activation barrier of the reaction is overcome, but in reality, an overpotential, η, is used to drive the reaction during electrochemical measurements.[99]

4.2 MASS TRANSPORT AND DIFFUSION

Diffusion can be described as a movement of species in three dimensions due to random walk or concentration differences in their surrounding environment. The diffusive flux is related to the difference in concentration where the species move from high concentration regions to low concentration regions as described by *Fick's first law of diffusion* (in one dimension) as follows

$$J = -D\frac{d\varphi}{dx} \qquad 4.2.1$$

J is diffusive flux, D is the diffusion coefficient, φ is the concentration and x is position. Diffusion will cause the concentration to change over time and *Fick's second law of diffusion* (in one dimension) describes this as

$$\frac{\partial\varphi}{\partial t} = D\frac{\partial^2\varphi}{\partial x^2} \qquad 4.2.2$$

where φ here is the concentration at time t at location x and the other constants as described for *Fick's first law* (*4.2.1*). In electrochemistry, when the overpotential is large, the assumption is made that the concentration of the oxidized species during a reduction process equals zero at the electrode surface (the same is of course valid for an oxidation

process where the reduced species equals zero at sufficient positive overpotential). When molecules start to be reduced at the electrode surface due to the applied potential a concentration gradient is formed from the electrode surface to the bulk solution, where all molecules are in the oxidized phase and diffusion towards the electrode due to the chemical gradient is created. A schematic image describing a concentration gradient is shown in figure 4.2.1.

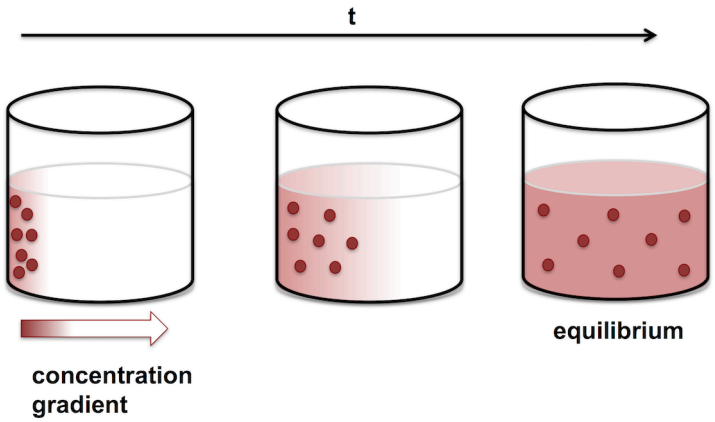

Figure 4.2.1 Molecules travel through a concentration gradient from higher concentrations to lower and with time reach equilibrium where the molecules are evenly distributed in a volume.

A potential gradient is also formed at the electrode surface, which induces the movement of charged species in solution under the influence of the electric field, called migration where both processes are involved in supplying more molecules from the bulk solution to the electrode.

Convection, natural or forced by stirring the bulk solution will influence the mass transport of molecules to the electrode surface. In order to simplify the system, the convection component can be eliminated by avoiding stirring the solution and/or minimizing vibrations in the system. By using a supporting electrolyte at a much higher concentration than the electroactive species, the migrational component can also be neglected. This leaves us with only the diffusional component for transporting species to the electrode. The species diffusion profile depends on time scale as well as the size and shape of the electrode surface. At a disk-shaped macro electrode (mm) surface the diffusion of electroactive species occurs in one dimension as linear diffusion. When the electrode dimension is small (μm), as for microelectrodes, the transport of molecules to the electrode surface by diffusion occurs in two dimensions as radial diffusion[99-100] and this is described in figure 4.2.2. This difference in diffusion profiles between macro- and microelectrodes plays an important role in electrochemistry and will be described later when different electrochemical techniques are discussed.

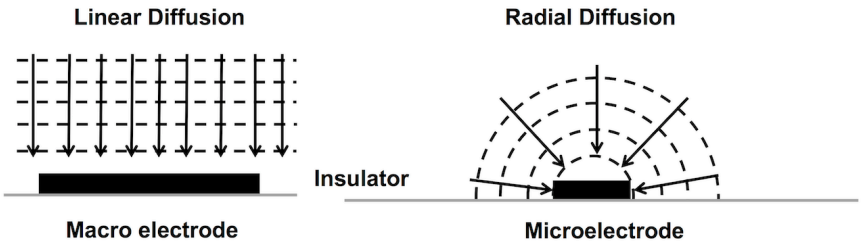

Figure 4.2.2 Illustration of different diffusion profiles between disk shaped macro- and microelectrodes where linear diffusion occurs at macro electrodes (mm) and radial diffusion at microelectrodes (μm).

4.3 ELECTRICAL DOUBLE LAYER

A surface, e.g. a platinum electrode, in contact with a solution containing ions possesses a surface charge independent of an application of a potential. Between the surface and the solution an interface called a double layer will form consisting of ions and molecules arranging themselves in a special manner towards the surface. Closest to the electrode surface molecules with the same electrical charge, co-ions, will be repelled and molecules with opposite charge, counter-ions, of the surface charge will specifically adsorb to the surface due to electrostatic interactions. The surface potential will then be dominated by the properties of the counter-ions. This layer also consists of solvent molecules in this case water, orienting themselves so that their dipole charge is attracted to the charge of the surface creating the so-called *inner Helmholtz plane, IHP*. Outside the *IHP* there are solvated ions that can approach the surface but only to a certain distance. The center of these solvated ions closest to the inner plane is called the *outer Helmholtz plane*, *OHP* and is nonspecifically adsorbed. The solution outside the center of these solvated ions and extending in to the bulk solution is referred to as the diffuse layer. This arrangement of ions and molecules creates an excess charge density in the diffuse layer with the total charge density outside the surface being the same as at the surface. The total thickness of the diffuse layer depends on the total ionic concentration of the electrolyte solution where the layer thickness decreases with increasing ionic concentration.[101] The electrical double layer plays a role in electrochemical processes due to its ability to act as a capacitor and depends on applied potential, electrolyte concentration and electrode surface material. When a potential is applied to the electrode,

reorientation of charged species in the double layer occurs and this charging of the double layer gives rise to a non-faradaic current, the so-called charging current. The rate of electrode processes may also be affected by the double layer structure. Molecules of interest to study can, unless they are specifically adsorbed at the electrode surface, only reach the *OHP* where the potential the molecule is exposed to is less than the potential between the electrode and electrolyte solution. The decrease in potential the molecule experiences compared to the electrode potential is referred to as the potential drop across the double layer. A schematic overview of the double layer is shown in figure 4.3.1.

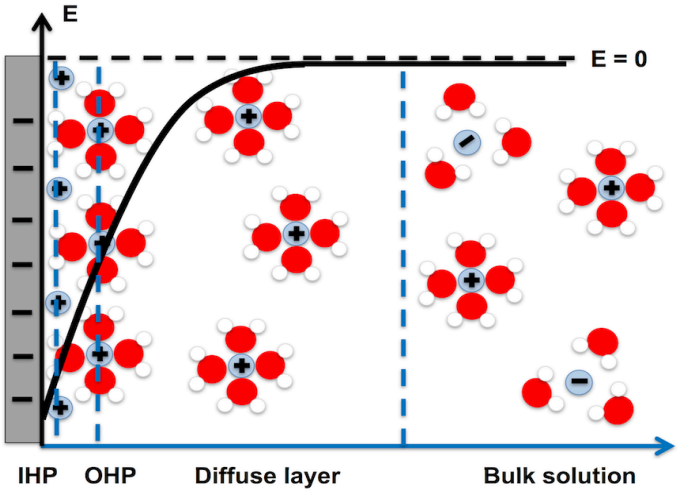

Figure 4.3.1 The electrical double layer describing the solution interface at a negatively charged electrode surface, where solvated cations at the inner Helmholtz plane (IHP) are specifically adsorbed and nonspecifically adsorbed in the outer Helmholtz plane (OHP). The potential profile across the double layer illustrates the potential drop from the distance of the electrode surface and into the bulk solution.

4.4 ELECTRODES

The working electrode, WE, can basically consist of any conductive material but should not itself take part in the reactions studied. Commonly used materials are carbon, e.g. graphite, glassy carbon, carbon nanotubes and different metals, e.g. Pt, Au, Ag and alloys. It is also common to make a WE consisting of several electrode materials as described in my papers where I used carbon as a substrate modified with gold nanoparticles. The common approach of attaching nanomaterials onto an existing electrode is a way of increasing the surface area of the electrode without actually changing the geometric area, this is especially important when using microelectrodes in vivo, where the experimental approach requires the electrode to be made as small as possible to eliminate tissue damage, whereas a larger surface area of the electrode is desirable, as it will lead to larger currents detected for the same concentration of analyte as will be described in a later section. The material of the WE affects its properties in regard to some analytes. Platinum for instance, is the most suitable material for detection of hydrogen peroxide due to the ability of adsorbing the oxygen molecules. The reference electrode, RE, sets the chemical potential against which the WE is measured and is necessary to assure that the potential applied to the working electrode is stable and of the desired magnitude. All redox potentials are reported in relation to the standard hydrogen electrode (SHE, E = 0.000 V) where the following reaction takes place at a platinum electrode (with all species at unit activity).

$$2H^+ + 2e^- \rightleftharpoons H_2 \qquad 4.4.1$$

The two most commonly used RE in practice are the saturated calomel (*4.4.2*), (SCE, E = 0.241 V vs. SHE) and the silver-silver chloride electrode (Ag/AgCl, E = 0.197 V vs. SHE in saturated KCl) (*4.4.3*) with the following reactions

$$Hg_2Cl_2 + 2e^- \rightleftharpoons 2Hg_{(s)} + 2Cl^- \qquad 4.4.2$$

$$AgCl_{(s)} + e^- \rightleftharpoons Ag_{(s)} + Cl^- \qquad 4.4.3$$

The Ag/AgCl electrode consists of a container with a chloride ion electrolyte, usually saturated potassium chloride, in which a chlorinated silver wire is inserted. The container has a membrane keeping the silver wire shielded but allowing its solution to be in contact with the solution in the electrochemical cell. The RE is a redox electrode and the *Nernst equation* (*4.1.25*) gives the electrode potential that depends on the chloride ion activity and the solubility of the metal salt keeping the activity of the metal ion stable. The electrode is thus affected by concentration of chloride ions inside as well as the temperature.

Ohm's law (*4.4.4*) tells that voltage is proportional to current multiplied with resistance.

$$E = iR \qquad 4.4.4$$

Sometimes a miniaturized RE is desired and a so-called *pseudo reference electrode* can be used. Usually a chlorinated silver wire is used in an electrolyte containing chloride ions mimicking the "real" Ag/AgCl RE. When using this pseudo RE a calibration must be performed either by measuring its potential against a known RE or by performing cyclic voltammetry with a well-defined redox couple, e.g. ferrocene methanol, comparing the measured formal potential with the formally known one. This is often used with microelectrodes that have dimensions in the μm range in a two-electrode set-up. When using a macro electrode with a large surface area, a third electrode is often introduced, the counter electrode, CE, also called the auxiliary electrode. It should consist of an inert material, such as Pt, Au or carbon and should preferentially have a surface area larger than the WE since the current will flow between the WE and the CE in order to close the electrical circuit. The WE-CE circuit allows current to flow through the system without passing through the RE. If the area of the CE is much larger than the WE area, the WE always become the limiting factor and the current observed is all due to processes at the WE.

4.5 ELECTROCHEMICAL METHODS

In this section, the experimental methods I have used will be introduced. All methods rely on measuring the resulting current when applying an either constant or with time altering potential, so called voltammetric methods. There are other electrochemical methods used for analytical electrochemistry as well, such as chronocoulometry and electrochemical impedance spectroscopy that will not be included here.

4.5.1 CHRONOAMPEROMETRY

In chronoamperometry, for the case of a reduction process, the working electrode is first held at a potential E_0, sufficiently separated from the *formal potential* $E^{0\prime}$ of the system where no electrochemical reaction occurs and all species are in their oxidized form. At a certain time, t_0, the potential is changed to E_1, an overpotential, η, as described in section 3.1, at which the reaction that occurs is so fast that the electrode surface concentration of the oxidized species is close to zero as described for the mass-transfer-limited region. The result is a sharp rise in current followed by an exponential decay gradually approaching a steady-state current with time as can be seen in figure 4.5.1. The sharp rise in current is due to the instantaneous reaction taking place at the electrode surface, reducing all molecules present at the surface resulting in a steep concentration gradient together with the charge created by the buildup of the electrical double layer close to the surface. This concentration gradient produces a continuous flux of analyte towards the electrode surface and all of these arriving molecules will immediately be reduced when reaching the electrode.

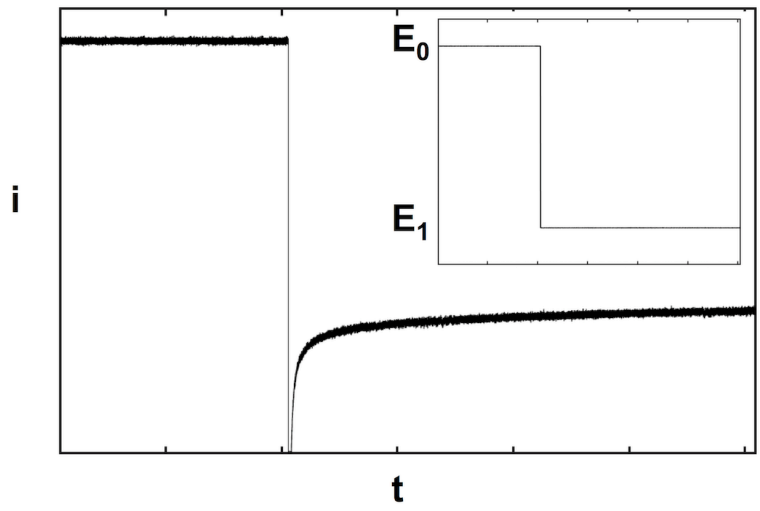

Figure 4.5.1 The cathodic current response of a reduction reaction due to a potential step from E_0 to E_1 (inset).

The flux of analyte as well as the resulting current is proportional to the concentration gradient at the surface of the electrode. The concentration gradient from the electrode to the bulk solution is caused by the consumption of analyte at the electrode surface due to the reduction process and is called the depletion layer. This continued flux of analyte towards the electrode causes the depletion layer to get thicker and the surface concentration will decline with time, seen as a time dependent declining slope of the observed faradaic current due to a reduction of mass transport of analyte towards the electrode. This faradaic current observed after the initial step is described by the *Cottrell equation* (*4.5.1*)

relating the current to the bulk concentration of the analyte as well as to the electrode surface area and the analyte diffusion coefficient.

$$i(t) = \frac{nFAD^{1/2}\,C^*}{\pi^{1/2}t^{\frac{1}{2}}} \qquad 4.5.1$$

Here, n is the number of electrons transferred per molecule reduced, F is the *Faraday constant*, A is the electrode geometric area, D is the analyte diffusion coefficient, C^* is the concentration of the oxidized species in the bulk solution and t is time after the potential step.

When using a microelectrode, mass transport occurs in two dimensions compared to the one-dimensional transport for macro electrodes, complicating the relationship between current, area, concentration and diffusion coefficient since the current density at a microelectrode is not evenly distributed over the surface but have a larger density at the outer regions of the disk referred to as the *edge effect*. In 1981 Aoki and Osteryoung[102] suggested a solution to this problem which Shoup and Szabo[103] later developed the *Shoup-Szabo equation* (*4.5.2-4.5.5*) describing the current response over all time units within an error of 0.6% by taking into account the radial diffusion profile of the microelectrode by introducing a scaled time factor τ proportional to t.

$$i = \frac{4nFADC^*}{\pi r} f(\tau) \qquad 4.5.2$$

$$f(\tau) = 0.7854 + 0.8862\tau^{-1/2} + 0.2146e^{-0.7823\tau^{-1/2}} \quad 4.5.3$$

$$\tau = \frac{4Dt}{r^2} \quad 4.5.4$$

$$i_{ss} = 4nFDC^{*}r \quad 4.5.5$$

The *Shoup-Szabo equation* describes the expansion of the depletion layer as a function of time *t* in response to a potential step. The current decay depends on the electrode radius *r* and diffusion coefficient, *D* as described in equation *4.5.2*. The equation makes it possible to obtain information in two regimes, both the initial non-steady-state and the later steady-state providing information about the same parameters as the *Cottrell equation* (*4.5.1*) determines for macro electrodes with the difference that here 2 parameters can be obtained simultaneously, *r* or *D* together with *n* or *C* in a single experiment.

In general, amperometry is the term used when a constant potential is applied between the WE and the RE and is the term that will be used for this case during this thesis. When electroactive species in the solution encounter the WE they will be oxidized/reduced and the electrons flowing give rise to a current. After the steady-state current has been reached, by varying the concentration of analyte, the related fluctuations in current can be recorded. In *Faraday's law* of electrolysis, transferred electric charge is linearly related to the number of moles of analyte as follows

$$Q = nFN \qquad 4.5.6$$

N is the number of moles of analyte detected, *n* is the number of moles of electrons transferred per mole of molecule reacted, *F* is the *Faraday constant* and Q is the charge. The definition of charge is electric current passed per time unit. From an electrochemical reaction, the resulting current passed through an electrode over a certain time at a fixed potential can be integrated and used to quantify the analyte from the reaction using *Faraday's law* (*4.5.6*). To be able to apply this experimentally, the contribution of non-faradaic current must be subtracted and the fraction of analyte not detected at the electrode must be minimized.

4.5.2 SWEEP VOLTAMMETRY

In sweep voltammetry, the electrode potential is varied linearly over time between two potentials E_1 and E_2, and the resulting current is measured. Unlike amperometry when a constant potential is applied, sweep methods can provide information about the molecule studied such as reversibility, reaction kinetics as well as concentration of the molecule in the bulk solution can be determined. Also, the molecule in the process can be identified in some cases. In cyclic voltammetry, CV, the scan starts at a potential E_1 sufficiently separated from the *formal potential* $E^{0\prime}$ of the system where no faradaic current is observed. The potential is then swept past $E^{0\prime}$ of the molecule studied to an overpotential E_2 where the faradaic current is diffusion controlled, the scan direction is then reversed and the potential is swept back to the initial value E_1 creating a triangular waveform. For a disk-shaped macro electrode, the resulting voltammogram displays a so-called "duck-shaped" current versus potential plot, as displayed in figure 4.5.2.b. When the potential is increased the current rises to a maximum peak current after which depletion starts to occur, lowering the current until it reaches a steady state. The maximum anodic peak current is caused by the oxidation of the species in the solution and the following decay is caused by depletion of the reactants in the diffusion layer due to the electrode consuming (oxidizing) the analyte in a higher reaction rate than the diffusion can supply new analyte to the electrode. If the molecule studied can undergo a reversible reaction, the backward sweep will result in a voltammogram with the same shape but in the opposite direction with a minimum peak eventually reaching the same initial current at E_1 as before the first part of the scan. The minimum cathodic peak current occurring in the reversed scan is due to oxidized species still present in the diffusion double layer at

the electrode surface. The decay of the current is due to consumption of oxidized species by the electrode reaction as well as diffusional transport away from the electrode. An ideal voltammogram for a reversible reaction with fast electron transfer at a disk-shaped macro electrode and the waveform applied are shown in figure 4.5.2.b. In an ideal reversible reaction, the anodic and cathodic peak currents are separated by a constant potential, *ΔE*. This potential is independent of scan rate and can be used as

$$\Delta E = E_p^a - E_p^c = \frac{59}{n}\, mV \qquad 4.5.7$$

where 59 mV is valid at 25 °C for an ideal reversible system. The peak separation, *ΔE*, is dependent on kinetics and can be used for determining the number of electrons transferred and also for identifying if the redox couple shows a *Nernstian behavior* as described earlier (*4.1.25*). From the positions of E_p^a and E_p^c on the potential axis, the formal potential $E^{0\prime}$ of the reversible redox process can be identified (*4.5.8*), since it is centered between the two peaks as shown in figure 4.5.2.b.

$$E^{0\prime} = \frac{E_p^a + E_p^c}{2} \qquad 4.5.8$$

The peak current i_p, is related to the scan rate, ν, where the current increases with the square root of ν, (V s^{-1}). The peak current is also directly proportional to the concentration of the electroactive species as shown in the Randles-Ševčik equation

$$i_p = 269000n^{3/2}AD^{1/2}C^*\nu^{1/2} \qquad 4.5.9$$

269,000 is a constant valid at 25 ℃. From this equation, the electrode area, diffusion coefficient or analyte concentration can be determined from the observed peak current if the other parameters are known. When a potential is changed over time, besides the faradaic current a non-faradaic current also arises, the charging current, originating from charging the electrical double layer as described in section 4.3. The magnitude of the double layer capacitance depends on the applied potential, the electrolyte concentration and is also directly proportional to the scan rate. As described earlier, in the case of microelectrodes, the diffusional flux occurs in two dimensions and thus the voltammograms from a microelectrode CV differs in shape from the macro electrode one as seen in figure 4.5.2.c. Due to the radial diffusion and the "edge-effect" described earlier, the analyte flux per electrode area is rapid enough to keep up with the consumption rate of analyte at the electrode surface. The observed current in the voltammogram is therefore a steady state current, and does not display a peak as described for the macro electrode.[100] Thus, the steady state current at a microelectrode does not depend on the scan rate, for scan rates typically below 1 V s^{-1}. The steady state current is however directly proportional to the analyte bulk concentration, the

diffusion coefficient and the electrode radii as described earlier in equation *4.5.5*.

Voltammetry can be used to study the properties of the WE such as electrode kinetics and electrode surface area by using well defined redox couples like ferrocene methanol. These molecules are so called reversible redox couples meaning they can firstly undergo oxidation on the forward potential sweep from E_1 to E_2 and then be reduced on the backward sweep from E_2 back to E_1 producing the same amount of maximum current in both directions (in the case of a macro electrode) and ideally having their *Ep* separated by $\frac{59}{n}$ mV as determined by the *Nernst equation* (*4.1.25*). In reality though, ΔEp is usually around 70 to 100 mV. These stable redox couples can also be used for studying the status of the RE by performing a CV where the $E_{1/2}$ can be evaluated towards the theoretical value. The identification of molecules with CV is based on different molecules showing different characteristic voltammograms regarding $E_{1/2}$, *Ep* separation and reversibility.

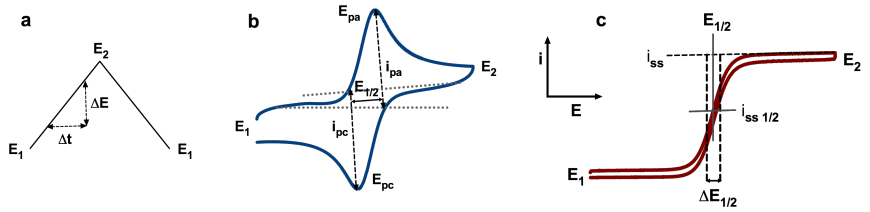

Figure 4.5.2 a) the potential waveform applied for performing cyclic voltammetry. b) the resulting voltammogram from a macro electrode. c) the resulting voltammogram from a microelectrode.

A further development of CV is a method called fast scan cyclic voltammetry, FSCV, where the potential is scanned between E_1 and E_2 with a scan rate of hundreds of volts per second compared to the conventional scan rates of 10-200 mV s^{-1} used in ordinary CV. The fast scan rate enables FSCV to combine the advantages of identification of analytes as obtained with CV with the high temporal resolution provided by amperometry. The combination of microelectrodes and FSCV have made it possible to perform dynamic measurements of neurotransmitters *in vivo* both in the brain of mammals[104] as well as in the fruit fly, *drosophila melanogaster*.[105-107] The main challenge with FSCV is the large charging currents resulting from the fast altering of the potential making background subtraction a must.

4.5.3 STRIPPING ANALYSIS

Stripping analysis is a method based on dissolving (stripping) material previously electrodeposited onto the surface of an electrode using a voltammetric technique. Usually the technique is used for metal ion analysis and is performed in the same solution by first electrodepositing the metal ions by cathodic deposition followed by a linear potential sweep referred to as anodic stripping voltammetry. This method can also be used for electrodes with previously adsorbed or deposited materials without the pre-electrolysis step and is then called adsorptive stripping voltammetry.[99] This technique was used in paper II where gold nanoparticles were deposited onto a glassy carbon electrode and then stripped off from their carbon support with the resulting charge used for quantification of the nanoparticles together with the previously determined gold surface area.

5 BIOSENSORS

A biosensor consists of a bio-recognition part and a transducer part, e.g. an enzyme immobilized on an electrode surface, respectively. There are other bio-recognition elements e.g. antibodies that can be used but this chapter will only describe enzyme-based biosensors. Clark and Lyons developed the first enzyme-based biosensor for monitoring glucose in 1962.[108] Ever since, there has been a tremendous increase and variety of enzyme based electrochemical biosensors for different applications such as the food industry, pharmacology, environmental studies, medicine and chemistry.[109-113] In this chapter, enzyme-based biosensors will be introduced including the function of enzymes, design and characterization of biosensors, as well as some history.

5.1 ENZYMES

The enzyme, the bio-recognition part of the biosensor, is a protein with catalytic function that specifically binds to one molecule, the substrate, and converts it to another molecule, the product. Enzymes increase the rate of the substrate-to-product reaction by lowering the activation energy. First, the substrate binds to the active site of the enzyme; second, an enzyme-substrate complex transition state is formed and by lowering the energy of the transition state the product is produced (5.1.1).

$$E + S \rightleftharpoons ES \rightarrow P + E \qquad 5.1.1$$

Where E is the enzyme, S is the substrate, ES is the enzyme-substrate complex and P is the product formed.

The rate of the reaction depends on the substrate concentration, where the reaction rate increases with increasing substrate concentration until a constant rate of production formation is reached, the V_{max} of the reaction. Saturation occurs at $V_{max,}$ since all the enzyme active sites are occupied with substrate forming the *ES-complex*. Hence, increasing substrate concentration cannot increase the reaction rate since all the binding sites are already occupied. The *Michaelis-Menten constant*, K_m, is the substrate concentration needed for the enzyme to react at half of its maximum reaction rate as shown in figure 5.1.1. K_m is usually specific for a certain enzyme with a given substrate and is a measurement of the substrate affinity to the enzyme; a small K_m is an indication of high affinity. The

number of substrate molecules one active site can handle per second is referred to as the turn over number, k_{cat}, a rate constant determining the reaction rate from the *ES-complex* to the product as

$$ES \xrightarrow{k_{cat}} E + P \qquad 5.1.2$$

k_{cat} is related to V_{max} as

$$V_{max} \overset{\text{def}}{=} k_{cat}[E_{tot}] \qquad 5.1.3$$

where $[E_{tot}]$ is the total enzyme concentration. The catalytic efficiency of an enzyme, how efficient an enzyme is on converting substrate to product, can be described by k_{cat} /K_m. The *Michaelis-Menten equation* (*5.1.4*) describes the rate of an enzyme reaction by relating the rate, v, to substrate concentration, $[S]$.

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad 5.1.4$$

By using the equations above (*5.1.1-5.1.4*), information about the enzymatic function of a biosensor can be evaluated with the respect to the

earlier mentioned parameters such as reaction rate, substrate saturation concentration, etc. A *Michaelis-Menten curve* relating substrate concentration to reaction rate is shown in figure 5.1.1.

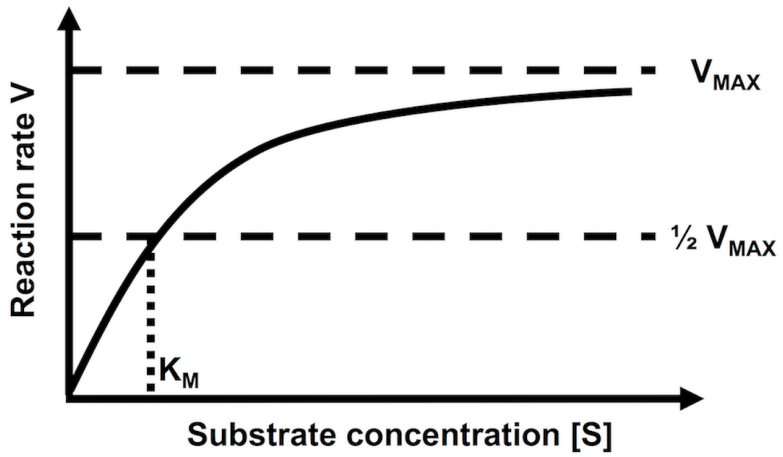

Figure 5.1.1 Michaelis-Menten saturation curve describing the reaction rate related to substrate concentration of an enzymatic reaction.

In general, enzymes are very specific, able to convert only one single substrate into product. By incorporating enzymes into sensing devices, a high selectivity for the substrate molecule is obtained. The activity of an enzyme depends strongly on its tertiary structure. The tertiary structure of a protein can be described in terms of how it folds in three-dimensions. The substrate will bind to the enzyme binding site by different interactions between the enzyme and substrate, e.g. hydrophobic/hydrophilic, electrostatic, and structural interactions, inducing the specificity for the substrate over other, very similar molecules.[114]

5.2 ELECTROCHEMICAL BIOSENSORS

Most enzyme-based biosensors use a class of enzymes called *oxidoreductase*[115] and the most frequently employed subclass is the *oxidases*. In the presence of oxygen and its substrate the oxidase enzyme produces its product together with hydrogen peroxide. Hydrogen peroxide is an electroactive molecule able to undergo oxidation or reduction at an electrode surface if a sufficient potential is applied, generating 2 electrons per molecule of hydrogen peroxide in both cases.

$$Oxidation\text{: } H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \qquad 5.2.1$$

$$Reduction\text{: } H_2O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \qquad 5.2.2$$

The glucose sensor that is based on glucose oxidase (GOx) will be used here as an example describing the action of an oxidase enzyme. When glucose binds to glucose oxidase, the first step is the production of gluconolactone (*5.2.3*) that occurs simultaneously with the reduction of the enzyme co-factor FAD to $FADH_2$. FAD is then regenerated from $FADH_2$ using dissolved oxygen as electron acceptor producing hydrogen peroxide as shown in figure 5.2.1.

$$glucose + H_2O + O_2 \rightarrow gluconolactone + H_2O_2 \qquad 5.2.3$$

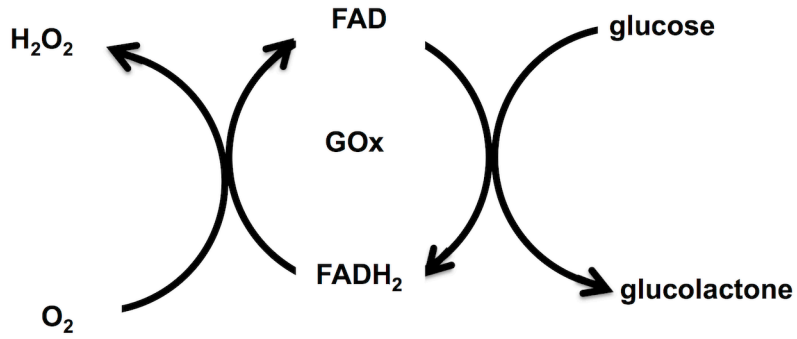

Figure 5.2.1 Schematic overview showing the glucose oxidase with co-enzyme FAD/$FADH_2$ reaction during the enzymatic production of glucolactone and hydrogen peroxide from glucose and oxygen.

Enzymatic biosensors can be divided into three different groups depending on their function[116-118] as described in figure 5.2.2.

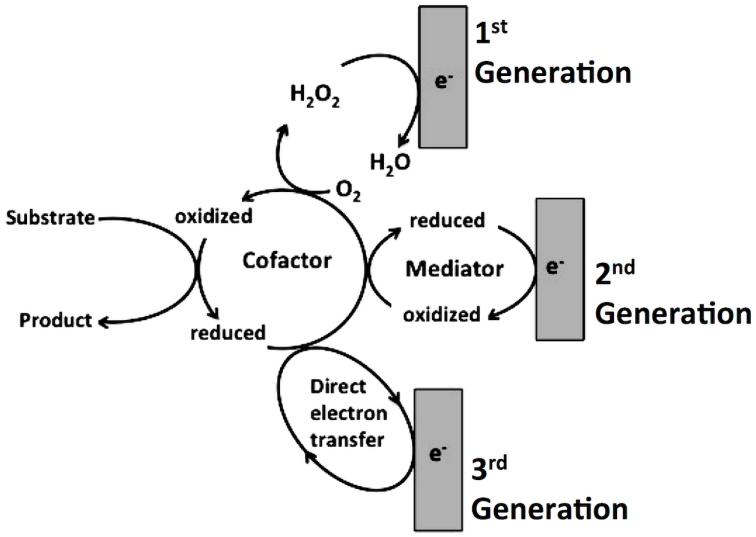

Figure 5.2.2 Schematic overview of the three generations of enzymatic biosensors. The 1st generation is based on the detection of enzymatic product, here hydrogen peroxide. The 2nd generation uses a mediator as the electron acceptor instead of the natural co-factor oxygen. The 3rd generation depends on direct electron transfer between the enzyme and electrode where the electrode itself act as the electron acceptor.

The oldest and simplest is the so-called 1st generation biosensor, which is based on the detection of the electroactive enzymatic product. For the 1st generation glucose sensor, the enzymatic product, hydrogen peroxide, can be either oxidized or reduced at the electrode surface when a high overpotential is applied generating a current that can be related to substrate concentration. The 1st generation biosensor is dependent on the dissolved oxygen concentration that is the natural electron acceptor for

the enzyme. The development of the 2nd generation biosensor was initiated to overcome the oxygen dependence by incorporating a mediator; a molecule that would act as the electron acceptor for the enzyme instead of oxygen but also to lower the overpotential needed which will reduce possible interferences. The mediator molecule acts between the enzyme and the electrode, as shown in figure 5.2.2, transferring the electrons directly to the electrode without involving oxygen and thus making the enzyme oxygen independent. The mediator chosen should react rapidly with the enzyme and it must be soluble enough to diffuse between the enzyme active site and the electrode but not so soluble that it will diffuse into the bulk solution. The mediator should also not react with oxygen, should lower the over potential needed and of course should be non-toxic to the enzyme. The so-called 3rd generation biosensors use an enzyme electron shuttle where the oxidation of the cofactor and the resulting electrons is consumed by the electrode directly instead of the natural electron acceptor, oxygen, or a mediator as in the case of the 2nd generation biosensors. When direct electron transfer can be obtained, the overpotential needed is low and the enzymatic reaction is not oxygen dependent. For direct electron transfer the function of the sensor depends strongly on enzyme orientation when immobilized onto the electrode for the ability of the electrons being favoured to transfer to the electrode.

5.3 BIOSENSOR DESIGN

All electrochemical enzymatic biosensors rely on an electrode somehow connected to an enzyme. The performance of the sensor is highly dependent of the enzyme function and in order to retain as much enzymatic activity as possible it is crucial that the enzyme is able to keep its tertiary structure. The sensor performance is also affected by the substrate access to the active site of the enzyme in order to be converted to product. Several approaches have been investigated to optimize the performance of biosensors based on enzyme conformation upon immobilization onto an electrode. When immobilizing enzymes on a flat surface they tend to flatten out losing some of their tertiary structure and thereby some of the enzymatic activity as illustrated in figure 5.3.1. High curvature surfaces can be provided by different nanostructures such as nanoparticles and nanotubes and these have been shown to be beneficial to retaining the enzymatic activity of immobilized enzymes.[119]

Figure 5.3.1 Illustration of how high curvature support enables retained enzyme tertiary structure upon immobilization.

In order to optimize enzyme activity upon immobilization, modification of electrode surfaces with different nanostructures to serve as enzyme support has become a common approach for constructing biosensors. The nanostructures can consist of different metals, e.g. gold, carbon, platinum

and alloys, and shapes such as wires, cubes and spheres. The methods for metal nanostructured modification of the electrode vary e.g. electrodeposition from a metal ionic solution, drop-casting or incorporated in polymers. Another nanostructured material that has been widely used to create biosensors is carbon nanotubes (CNT). Iijima discovered carbon nanotubes in 1991[120] and Davis et al.[121] and Balavoine et al.[122] initiated the work of incorporating proteins in to CNTs. Since this early work, CNTs have been incorporated into various electrochemical biosensors.[123-124] In addition to retaining enzyme tertiary structure and activity, incorporation of nanostructures for enzyme immobilization also increases the electrode surface area due to the high surface to volume ratio of nanoparticles allowing higher enzyme loading and thereby increased sensitivity. Nanostructures are also important for detection of the oxidase enzymatic product, hydrogen peroxide, where platinum is the most beneficial material,[125] but also CNTs and other metals are widely used. There are different approaches for immobilizing the enzyme onto the electrode surface, e.g. physical adsorption due to electrostatic interactions, cross-linking with chemicals such as glutaraldehyde and bovine serum albumin or incorporation of enzyme in a polymer film.[126-128] Desired properties of any kind of electrochemical sensor for application in biological systems are selectivity, sensitivity, stability, size, and high temporal resolution. Depending on the application of the biosensor, the most important properties may vary. When using a non-modified electrode for detection of electroactive molecules the main challenges are selectivity and stability, since an electrode surface has no selectivity itself, any electroactive molecule present within the potential window used will be oxidized/reduced and give rise to a signal. Stability can also be an issue since adsorbing species easily can foul the electrode surface, and the sensor will lose part of its sensitivity. In an in vivo measurement

when a long-time recording is desired, the most important properties of a sensor are selectivity since it is a complex matrix, sensitivity due to the low concentrations present, and stability to avoid fouling of the electrode and thereby a decrease in sensitivity. In order to minimize tissue damage when working *in vivo* a sensor should also have small dimensions. Single cell analysis provides a less complex matrix compared to *in vivo* analysis and thereby less interfering species reducing the need of selectivity. Also, the concentration of neurotransmitters in a vesicle is much higher compared to the background levels in the brain. Vesicle concentration can be more then 100 times higher compared to brain ECF, mM vs. μM, respectively.[97, 129] The release of neurotransmitters, exocytosis, occurs on the sub-millisecond time scale making high temporal resolution the key parameter for single cell analysis of vesicular neurotransmitter release as well as for real-time resolution of cell signalling *in vivo*. So, in order to be able to electrochemically probe exocytosis of non-electroactive neurotransmitters from single cells and real-time resolution of cell signalling *in vivo* a sensor with the size of tens of microns and with a temporal resolution of milliseconds is needed. With a thin coverage, preferably monolayer coverage of enzyme on an electrode modified with gold nanoparticles a biosensor fast enough to detect exocytosis from an artificial cell was constructed as described in paper I. Other biosensors for *in vivo* analysis have often put on several layers of enzyme and as well as a protective film such as *Nafion*® or a size exclusion membrane to meet the requirements needed for *in vivo* analysis.[130-132] The layers of coatings affect the temporal performance of the sensor since the diffusion of molecules towards the electrode surface is slower compared to monolayer coverage. On the other hand, more enzyme present produces more detectable product, thus an increase in sensitivity is achieved. So

basically, there is a trade-off between sensitivity and temporal resolution.[133]

All sensors constructed during my Ph.D. have been of the 1st generation biosensor type with immobilization of an oxidase enzyme, or for the case of acetylcholine detection, a combination of two enzymes, onto an AuNP coated carbon electrode where the detection has been based on the reduction of the enzymatic product hydrogen peroxide. A schematic overview of the sensor is shown in figure 5.3.2.

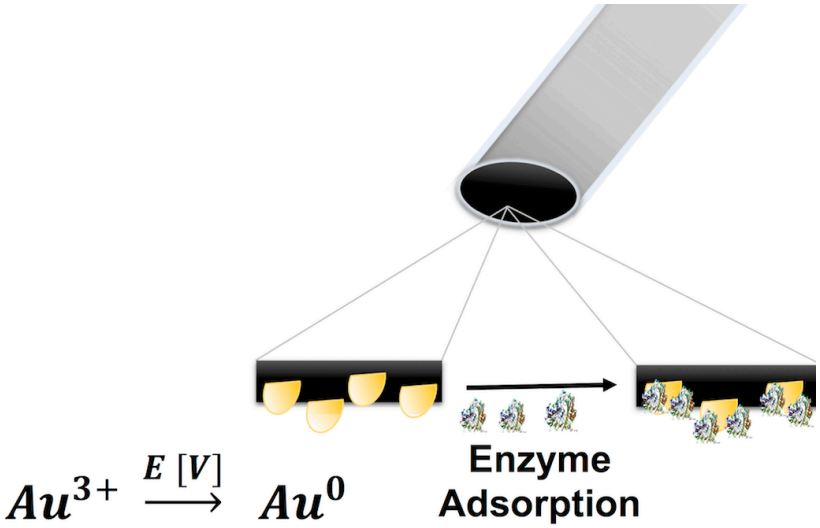

Figure 5.3.2 Schematic overview the fabrication process of a biosensor based on an electrode coated with AuNPs covered with a monolayer of enzyme.

The reduction of hydrogen peroxide for detection instead of oxidation was chosen based on possible interferences of common electroactive species present in the brain such as dopamine, ascorbic acid,

norepinephrine etc. all of them being oxidized at lower potentials than what is required for hydrogen peroxide oxidation. The main interference when reducing hydrogen peroxide is the presence of dissolved oxygen in the surrounding solution being reduced at the reduction potential used. The oxygen will give rise to a background current but the fluctuations of oxygen levels will not occur on the same rapid timescale that exocytosis do.[134] The 1st generation oxidase based biosensor is dependent on oxygen as an electron acceptor as described earlier and since the oxygen closest to the electrode will be depleted due to its reduction on the electrode surface the limiting factor of the enzyme activity might be the oxygen supply. In the biosensors constructed for glucose, glutamate, and acetylcholine all rely on the use of oxidase enzymes, glucose oxidase, glutamate oxidase, and in the case of acetylcholine co-immobilization of acetylcholine esterase and choline oxidase. The detection of these analytes is described in paper III, IV and I, respectively.

6 SUMMARY OF PAPERS

I have spent my Ph.D. education developing new electrochemical tools for studying the non-electroactive neurotransmitters glutamate and acetylcholine as well as glucose the primary energy source of the brain. My main focus has been the fabrication of miniaturized electrochemical biosensors with sufficient temporal resolution for studying real time *in vivo* neurotransmission in the brain. Hand in hand with successful sensor fabrication goes a thorough optimization of the performance of the sensor. This is done by tuning different parameters, which is why I have also developed methods for characterizing the surface interactions between enzyme and electrode for these types of sensors in order to better understand the mechanisms behind their function.

In **paper I**, the development of a micrometer sized electrochemical biosensor for detection of acetylcholine is described. This is achieved by immobilizing a sequential two-enzyme system, acetylcholine esterase and choline oxidase, on a gold nanoparticle carbon fiber electrode. The analyte of interest, acetylcholine, cannot directly be converted to an electroactive molecule by the enzyme acetylcholine esterase but the product formed, choline, can be converted in to hydrogen peroxide by choline oxidase why a two-sequential enzyme system is required. The optimal conditions for creating a AuNP structured surface on a 33 µm carbon fiber were investigated, leading to an average AuNP diameter of 20 nm and a surface coverage of about 30 %. The sensor was carefully characterized regarding the ratio between the two enzymes, revealing an optimum ratio of 1:10.[136] The biosensor was characterized regarding response towards different concentrations of analyte and possible

interferences occurring *in vivo* by using a flow analysis system. The so-called 1st generation biosensor, relying on the oxidation of the enzymatic product, hydrogen peroxide, has most commonly been constructed from platinum electrodes. Oxidation of hydrogen peroxide requires an overpotential to be applied at the electrode, typically +700 mV (vs. Ag/AgCl). At this potential naturally occurring electroactive neurotransmitters such as dopamine and other abundant species in the brain e.g. ascorbic acid will also be oxidized, giving rise to a current interfering with the signal of the enzymatically generated hydrogen peroxide. In order to reduce these interferences without additional modification of the electrode surface we chose to base the sensor response on the reduction of the enzymatic product hydrogen peroxide. The temporal resolution of the sensor was tested with an artificial cell model mimicking exocytosis from a single cell and the sensor was found to be able to detect acetylcholine and dopamine at the same sensor with millisecond response time. The key factor for high temporal resolution of the sensor turned out to be keeping the enzymes close to monolayer coverage of the nanoparticles on the electrode. Discovering that enzyme monolayer formation was the key for high temporal resolution of the biosensor lead to the idea for **paper II**.

In **paper II**, I developed a new analytical method where the number of enzymes immobilized onto a gold nanoparticle coated electrode where counted simultaneously with the determination of the number and size of the gold nanoparticles. The method is based on electrochemical adsorption anodic stripping to determine size and number of earlier electrodeposited gold nanoparticles onto a glassy carbon electrode where fluorescently labeled glucose oxidase had been immobilized. The resulting solution from the anodic stripping procedure contains

fluorescently labeled glucose oxidase that was analyzed using fluorimetry to determine the enzyme concentration. This concentration was then related to size and number of gold nanoparticles, enabling the determination of the average number of enzymes per gold nanoparticle for a single electrode. Earlier, the size and number of gold nanoparticles where determined by SEM and enzyme concentration determined by dissolving fluorescently labeled enzymes in KCN. This new method determines the same parameters but in a much faster, cheaper and non-toxic way and correlates enzyme coverage to a single electrode.

In paper III, I continued the work of developing biosensors with high temporal resolution by investigating the temporal resolution of a one-enzyme system. The enzyme of choice to study was glucose oxidase since glucose is the primary fuel source for the neurons and thus crucial for normal brain function. Furthermore, very few studies have been aimed at revealing possible very rapid fluctuations in glucose levels. The sensor also has the ability to co-detect dopamine at the same electrode surface making it possible to study local brain glucose metabolism during neuronal dopamine activity. The sensor response time was tested by applying a "puff" of solution from a micropipette placed in close approximate (30 µm) to the sensor surface. By altering the potential applied between +0.5 V and -0.5 V (vs. Ag/AgCl) when the sensor was exposed to a "puff" of solution containing both dopamine and glucose, the two analytes could be oxidized and reduced, respectively and the temporal resolution for each analyte could be compared. The sensor was characterized regarding response towards different glucose concentrations and commonly occurring interferences occurring *in vivo* by the use of chronoamperometry. The one-enzyme system sensor with glucose

oxidase immobilized on to AuNPs at a carbon fiber electrode had as fast kinetics as that of dopamine detection.

In **paper IV** a glutamate sensor was developed based on the same principle as the acetylcholine and glucose sensor with immobilization of glutamate oxidase on to AuNPs covering a carbon fiber electrode. Bioconjugate assay of immobilized glutamate oxidase onto AuNP where performed for characterization regarding what concentration of enzyme is needed during the immobilization process to achieve a thin enzyme coverage at the surface of AuNP at the electrode surface. The glutamate sensor was characterized regarding its response towards different glutamate concentrations and possible interferences with chronoamperometry revealing results well in agreement with previously published glutamate sensors regarding sensitivity and linear range of detection. The glutamate biosensor was then tested *ex vivo* in mouse brain slice and was able to detect spontaneous release of glutamate with millisecond time resolution.

7 CONCLUSIONS AND FUTURE OUTLOOK

An in depth understanding of the process of neurotransmission is vital in order to reveal the mechanisms behind brain malfunctions such as neurodegenerative diseases and neuropsychiatric disorders. In this thesis, biosensors with the ability to detect non-electroactive neurotransmitters with millisecond time resolution have been fabricated for the first time. The key for any biosensor development is the thoroughly analytical work of sensor design and fabrication followed by a careful characterization of the sensor performance. The strength of combining methods and knowledge from different scientific fields has been proven during the fabrication of the biosensors where electrochemistry and bioconjugates have been joined together. The two-sequential enzyme system (AChE and ChO) for acetylcholine detection, as used in **paper I,** was investigated to find the optimal ratio between the enzymes regarding sensor activity as well as enzyme coverage. The CFME used have been modified with AuNPs, optimized with respect to size and electrode coverage. The high temporal resolution of the biosensors was attributed to the monolayer enzyme coverage of the AuNPs. The overall goal has been to enable detection of real time neurotransmission *in vivo*. In **paper IV** we reached a break through when we were able to detect exocytotic spikes from glutamate transmission in mouse brain slice. Enabling temporal resolution of non-electroactive neurotransmitters with electrochemical biosensors will be a very valuable tool especially regarding glutamate detection that is of great interest to study due to its excitatory effect in the brain and its connection to excitotoxicity.

Even though sensors with millisecond time resolution for detection in biological tissue have been successfully fabricated here, there is a lot left to do regarding performance optimization. Since the enzyme coverage must be kept as close to monolayer as possible to achieve the high temporal resolution and the sensor dimensions must be kept small, relatively few enzymes will be incorporated into the sensor why it is of great importance to maximize enzyme activity upon immobilization. Several approaches can be used for enhancing enzyme activity upon immobilization onto the electrode. It has been shown that immobilizing the enzyme in the presence of its substrate is beneficial for maintaining enzyme tertiary structure and thus activity,[135] a simple approach well worth trying. It is not only kinetics and substrate affinity that differs between enzymes they also have different physical properties where one is the tendency of spreading out upon immobilization. Therefore, it would be valuable to investigate how AuNP curvature affects enzyme loading and activity for different enzymes as well as hydrogen peroxide detection. This can be evaluated by the analytical method introduced in **paper II** where the number of enzymes immobilized onto an AuNP covered electrode was directly related to the number and size of the AuNPs. The immobilization of enzymes has been based on physical adsorption where the interactions behind have not yet been studied. Possible interactions could be e.g. electrostatic interactions between the net negatively charged enzyme (at neutral pH) and the positively charged AuNPs and covalent thiol bonds formed between the gold and the cysteine residues of the enzyme, most probably a combination of several different interactions. This non-specific adsorption of the enzymes to the AuNPs will lead to random orientation of the enzymes when immobilized to the electrode and since orientation of enzyme can affect substrate access to the active site and thereby sensor performance it is important to control enzyme

orientation as well. Common approaches to control enzyme orientation are the introduction of cross-linkers a molecule linking the enzyme to the AuNP through covalent bonds. Tuning the electrostatic interactions between the AuNP and the enzyme can also be an approach for achieving a more favorable enzyme orientation upon immobilization. Electrostatic interactions can be tuned by changing the pH thus altering the charge distribution of the enzyme due to the different pI of different amino acids. This can also be achieved by using an applied potential during immobilization whereby the charge of the electrode can be altered. Even though the thickness of the enzyme layer should be kept as close to monolayer as possible it is still important to increase enzyme loading for enhanced sensitivity of the sensor. Increasing the amount of AuNPs while decreasing their size but avoiding the creation of a gold film may enhance enzyme loading and thus sensitivity. There are also other nanomaterials possible to use for enzyme immobilization, e.g. carbon nanotubes and other metals such as Pt and alloys yet to be investigated regarding their interactions and performance in this enzyme monolayer biosensor. With the ultimate goal of enabling the use of these sensors *in vivo* new challenges will be introduced e.g. increased recording times and thus an increased risk of sensor fouling leading to decreased sensitivity. Often, protective films and membranes have been incorporated into biosensors to protect the sensor from fouling, but since our sensor relies on the monolayer enzyme coverage, the approach of introducing a protective film must be evaluated regarding its potential influence on temporal resolution.

In conclusion, the electrochemical enzyme-based biosensors fabricated and characterized in this work are the first with sufficient temporal resolution to enable detection of single exocytotic release events of non-

electroactive neurotransmitters. This is demonstrated by detection of release from an artificial cell as well as from cells in tissue. Hopefully the development of these sensors will enable real time detection of neurotransmission from glutamate and acetylcholine neurons *in vivo* with the goal of revealing new insights regarding their function in the brain.

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