Chapter 1

General Introduction
1.1 Research on biosensors

Definition of biosensors

A biosensor is defined as a sensing device, which consists of a biological recognition element and a suitable transducer (Thévenot et al., 2001; Schumann, 2002). Often the biological recognition element is an enzyme or antibody, but it also can be a protein (e.g. a peptide or oligonucleotide), a nucleic acid, a microorganism (e.g. a bacterium), or even tissue-material. The transducer is the device on which the biological recognition element is attached and which converts the biological recognition reaction into a quantifiable signal. When the dimensions of the transducer are minimized to a micrometer-scale, biosensors are referred to as microsensors, microbiosensors or microelectrode biosensors. The detection at the transducer can occur via different principles, for example optically, calometrically, acoustically, or electrochemically. The latter can be subdivided in amperometric, conductometric, potentiometric, or impedance monitoring (Habermüller et al., 2000; Palmisano et al., 2000; Pearson et al., 2000; Chaubey and Malhotra, 2002). Although in theory many different types of biosensors can be constructed, by far the most frequently used type is the enzymatic amperometric biosensor. The next paragraphs focus on this type of biosensor.

History, application and commercialization of biosensors

The modern concept of the biosensor evolved in 1962 when Clark and Lyons proposed that enzymes could be immobilized at an electrochemical detector to form an enzyme electrode. A few years later Updike and Hicks (1967) presented the fabrication of a functional enzyme electrode for glucose. After a long time of relative silence, the last decade research on biosensors has received more attention. In addition, the application of biosensors is not only restricted to biomedical purposes, but they are also applied in a wider range of fields, such as in environmental monitoring and in the monitoring of food quality (Hanrahan et al., 2004; Amine et al., 2005).

When compared to traditional analytical methods, biosensors display a few distinct advantages. They are often easy to handle and are prone to miniaturization. Moreover, they display short response times and the detection of the analyte of interest can occur without prior separation (Castillo et al., 2004). The first commercialized biosensor, which was applied for the monitoring of glucose, was launched in 1975 by Yellow Springs Instruments. Soon, several companies followed. A comprehensive overview of commercially available biosensors for biomedical applications is given by Castillo et al. (2004). In general, enzyme-based amperometric sensors are applied in three different ways. First, as “off line” detector: samples are obtained from the investigated subject and administered to the biosensor. A typical example is the commercially available glucose sensor, used by diabetic patients. A droplet of blood from the fingertip is placed on a strip bearing the electrode system. The strip is placed in a handheld device and in turn the
blood glucose levels are displayed. Examples are the Accu Chek, OneTouch, Glucocard and Freestyle (respectively exploited by the companies Roche, LifeScan Inc, Menarini diagnostics and Abbott laboratories). Secondly, as "on line" detector: the biosensor is integrated into a flow line of a sampling device and this device is placed in the body or in biological material. Most often, the experimental design consists of an implanted microdialysis probe and a flow through detector with an integrated biosensor (Schoonen et al., 1990; Obrenovitch and Zilkha, 2001; Rhemrev et al., 2001; Gáspár et al., 2004). Third, as an "implantable device": the biosensor is implanted in the body and the concentration of the analyte of interest is continuously monitored. The latter is the most elegant design, but until now analytical difficulties have restricted this approach to the experimental stage (Heller, 1999; Feldman et al., 2003). At present, the company Therasense, recently bought by Abbott Laboratories, is developing an implantable glucose biosensor ("Navigator") for diabetic patients based on a wired enzyme redox hydrogel technology. The glutamate microsensor presented in this thesis is based on the same technology.

Classification of amperometric biosensors

The greatest challenge in constructing a proper functioning biosensor is to realize a fast and selective electron transfer (ET) between the prosthetic group of the enzyme and the electrode surface. Enzymes are generally very selective, but often the biosensor loses this enzyme-induced selectivity due to biological interferences in the electron transfer cascade.

Enzyme-based biosensors most frequently use an enzyme of the class of oxidoreductases. In particular, oxygen-dependent oxidases are applied, but NAD⁺-dependent dehydrogenases, PQQ-dependent dehydrogenases, peroxidases and multi-cofactor enzymes are also used. The substrate of interest is oxidized by these enzymes and in turn the prosthetic group of the enzyme (e.g. FAD, NAD⁺, FMN, PQQ, heme, transition metals) is reduced. Next, the prosthetic group needs to be re-oxidized in order to regenerate the enzyme and to prepare it for further substrate recognition and conversion reactions.

The biosensor has to participate in this process in order to mediate a fast electron transfer between the enzyme and the electrode surface. However, often the redox enzymes are designed by nature to protect the integrated cofactor against unwanted redox processes. This implies that an efficient regeneration of the active site of the enzyme is of key importance and that a proper functioning of the biosensor depends largely on the kinetics of this ET process (Habermüller et al., 2000; Schumann, 2002). This ET process is influenced to a great extent by the specific properties of the used enzyme, e.g. the physical chemical properties of the prosthetic group, the accessibility and distance of the prosthetic group from the protein surface, the nature of the redox cofactor, the intrinsic protein stability and the possibility of immobilizing the enzyme to the electrode surface.
At first sight the easiest ET mechanism would be the electrochemical recycling of the enzyme's prosthetic group directly at the electrode surface involving an electron tunnelling mechanism (referred to as “third generation type of biosensors”, as will be discussed next). However, the ET kinetics between two redox species are determined by the driving force (i.e. the potential difference), the reorganization energy (which is qualitatively reflected by the structural rigidity of the redox species) and the distance between the two redox centers (Marcus and Sutin, 1985; Marcus, 1993). Because prosthetic groups are often well insulated and deeply buried within the active site of an enzyme, direct ET with a high rate is difficult to achieve (Habermüller et al., 2000; Schumann, 2002). This implies that the ET transfer between the enzyme's prosthetic group and the electrode surface has to be arranged otherwise, which has resulted in the development of many different types of biosensors. Based on their ET mechanisms, biosensors can be divided into three “generations” (Fig. 1).

![Figure 1: Different generations of biosensors. A) First generation biosensor: the detection is based on monitoring freely diffusing redox species, i.e. either the oxidation of H$_2$O$_2$ or the reduction of O$_2$. B) Second generation biosensor: a redox mediator communicates with the prosthetic group of the enzyme and the electrode surface. C) Third generation biosensor: the prosthetic group of the enzyme is reoxidized by the electrode surface via direct electron transfer (DET). D) Redox hydrogel based biosensor: this is a specific subtype of the second generation type of biosensors. The prosthetic group of the enzyme communicates with a nearby osmium redox mediator, which in turn communicates with another nearby mediator and a sequence of self-exchange reactions between the redox mediators bridges the electron transfer to the electrode surface (“electron hopping mechanism”).](image-url)
Each generation has its own specific architecture with typical analytical advantages and disadvantages. Next, a brief summary of the different generations of biosensors will be given, directed to biosensors constructed with oxygen-dependent oxidases, as those are the most frequently used (Wilson and Gifford, 2005).

**First generation of amperometric biosensors**

The architecture of the first generation type of biosensor consists of an enzyme attached to an electrode surface (Fig. 1A). The prosthetic group of the enzyme is recycled by freely diffusing oxygen \( (O_2) \), and either the decrease of \( O_2 \) or the increase of the reaction product, i.e. hydrogen peroxide \( (H_2O_2) \), is monitored. This detection, which is based on freely diffusing redox species, is referred to as a “shuttle mechanism”. The first biosensors (Clark and Lyons, 1962; Updike and Hicks, 1967) also belonged to this class: glucose oxidase was immobilized on top of a Clark oxygen electrode and the consumption of \( O_2 \) was detected by monitoring changes in the reduction of \( O_2 \). Since then many biosensors based on this principle have been constructed. Examples of glutamate microsensors based on this principle that were used in CNS research were developed by Lowry et al. (1998) and by the research group of Dr. G Gerhardt (Gerhardt and Hoffman, 2001; Burmeister et al., 2000, 2001).

Although the architecture of the first generation type of biosensor is relatively simple, it does have certain disadvantages. First, a high reduction or oxidation potential is required to respectively reduce \( O_2 \) or oxidize \( H_2O_2 \). Consequently, many other electrochemically active compounds are reduced or oxidized at this working potential, which decreases the selectivity of the sensor. Several attempts have been made to prevent this interference and to increase the selectivity of the biosensor. For example, the sensor is covered with an additional membrane that excludes interfering molecules on the basis of their charge (Vaidya and Wilkens, 1994; Moussey et al., 1994b), or size (Sternberg et al., 1989; Bindra et al., 1991). In addition, active compounds such as enzymes, redox reagents or other ligands are incorporated in the outer layer of the sensor (Maiden and Heller, 1991; Hu et al., 1994; Anzai et al., 1998), or discriminating layers are applied between the electrode surface and the enzymatic layer (Ryan et al., 1997; Burmeister et al., 2002; Chen et al., 2002).

Second, the enzyme needs to be immobilized on the electrode surface, which requires additional immobilization methods. However, often this complicates the architecture of the biosensor and, in turn, its construction. Throughout the years several immobilization methods have been developed, e.g. entrapment behind or in a membrane (Gough et al., 1985), entrapment in different sorts of gels (Updike and Hicks, 1967), mixing into carbon paste (Wang and Lu, 1998), cross-linking with glutaraldehyde or other cross-linkers (Migneault et al., 2004), electrochemically aided adsorption (Strike et al., 1995), controlled deposition based on avidin/biotin recognition (Hoshi et al., 1995), the use of photopolymerizable materials (Jiménez et al., 1995) and...
electrochemical formation of polymers in the presence of the enzyme (Bartlett and Cooper, 1993; Cosnier, 1999; Kurzawa et al., 2002). An additional advantage is that most of these immobilization methods possibly may improve the stability of the enzyme.

Third, only the enzymes that are immobilized close to the electrode surface contribute to the output of the sensor. This limits the sensitivity of the sensor. Besides, the $\text{H}_2\text{O}_2$ that is produced by enzymes that are remote from the electrode surface can leak into the surrounding tissue and in turn may cause tissue damage (Chaubey and Malhotra, 2002; Wilson and Gifford, 2000). In addition, high concentrations of $\text{H}_2\text{O}_2$ may also degrade the enzymes (Habermüller et al., 2000; Palmisano et al., 2000).

Fourth, in many cases the performance of the biosensor appears to be limited by oxygen deprivation, in particular when substrates are monitored at high concentrations, for example glucose or lactate (Wilson and Gifford, 2005). To prevent this problem, the sensor can be covered with membranes that limit the diffusion of substrate to the electrode surface. Consequently, the diffusion of the small oxygen molecules will be facilitated over the substrate of interest (Clark et al., 1988; Zhang and Wilson, 1993). Another option is to incorporate oxygen-donating materials in the architecture of the sensor, such as poly (dimethylsiloxane) oils or perfluorocarbon oils (Wang and Lu, 1998; Wang et al., 2002).

Second generation of amperometric biosensors

The disadvantages of the first generation type of biosensors have stimulated the development of the second generation type of biosensors (Fig. 1B). In this approach artificial redox mediators are used instead of $\text{O}_2$. Second generation biosensors involve a two-step procedure in which the substrate is converted by the enzyme in the first redox reaction, whereas in the second part the prosthetic group of the enzyme is reoxidized by the redox mediator. In this way, so-called “reagentless” biosensors are created: all components that are required for the electrochemical reaction are immobilized on the electrode surface and only the concentration of the substrate of interest is a variable parameter. For a proper functioning of the biosensor, the redox mediator has to meet certain criteria. Ideally it has to display stable oxidized and reduced forms, a fast reaction rate with both the enzyme and the electrode surface, and the possibility of a tight attachment into the architecture of the biosensor (Chaubey and Malhotra, 2002; Castillo 2004). Mainly soluble low-molecular weight metal complexes are used for this purpose, e.g. ferrocene derivatives, $\text{K}_4[\text{Fe(CN)}_6]$, ruthenium- or osmium-complexes, but other materials are also used, e.g. quinones, tetrathiialfulvalene (TTF), tetracyanoquinodimethane (TCNQ), conducting salts (e.g. TTF-TCNQ, $N$-methylphenazin (NMP)-TCNQ, etc) and organic dyes (e.g. methylene blue, prussian blue, phenazines, methyl violet, Alizarin yellow, thionin, toluidine blue, azure A and C, etc) (Habermüller et al., 2000; Palmisano et al., 2000; Chaubey and Malhotra, 2002). An additional advantage of this approach is that also enzymes can be used which cannot be directly regenerated at the electrode
surface at a reasonable potential. For example, this allows the use of dehydrogenases in amperometric biosensors (Marko-Varga et al., 1986).

Nevertheless, a clear disadvantage of the initial second generation biosensors was the leakage of diffusible mediators, which induced sample contamination, unpredictable deterioration and instability of the biosensor (Schumann et al., 1990). This has led to the development of a design that tightly retained the redox mediators at the sensor surface, for example by co-entrapment of the mediator and enzyme (Foulds and Lowe, 1988), entrapment of the mediator and enzyme in different layers (Jönsson et al., 1989), or electrically wiring enzymes to a redox polymer (Gregg and Heller, 1990). The latter is referred to as the redox hydrogel technology. This is also used for the construction of the hydrogel-coated glutamate microsensor and will be discussed in more detail in the next paragraph.

The biosensors of the second generation also encountered some other disadvantages. For example, oxygen can interfere in the electrochemical detection of the sensor. Not because of oxygen deprivation, as is the case with the first generation type of biosensors, but now due to the presence of oxygen. Oxygen may compete with the redox mediators and in turn interrupt the redox cascade to the electrode surface (Ohara et al., 1993a; Csöregi et al., 1995). In addition, reducing agents, e.g. ascorbic acid (AA), can also interfere by reducing the redox mediators in the redox cascade. Both types of interference suppress the sensitivity of the biosensor. Finally, the fact that the redox mediator needs to be present in its oxidative state is another disadvantage. This requires a working potential that is considerably higher than the own potential (E°) of the redox mediator, which allows easily oxidizable components to interfere.

**Biosensors based on redox hydrogels**

Biosensors based on redox hydrogels form a specific subtype of the second generation type of biosensors. Redox hydrogels consist of a combination of a redox polymer and an enzyme, which are wired to each other by a cross-linker. Redox polymers consist of a polymer backbone with covalently bound redox mediators. Examples of redox polymers are poly (vinyl pyridine) (Gregg and Heller, 1990), poly (vinyl imidazole) (Ohara et al., 1994), poly (acyrylic acid) (de Lumley-Woodyer et al., 1995), or poly (allyl amine) (Danilowics et al., 1998) backbones connected with osmium (Gregg and Heller 1990, 1991a, 1991b) or ruthenium (Ryabov et al., 2001) complexes. Another approach is a poly (siloxyane) backbone connected with ferrocene units (Inagaki et al, 1989). The advantage of osmium-complexes is that they have a rather low redox potential and easily can be loaded onto a polymer backbone. Consequently, the osmium based redox polymers with a poly (vinyl pyridine) or poly (vinyl imidazole) backbone are by far the most frequently used. The next paragraphs focus in particular on this type of polymer, as it is also used for the hydrogel-coated glutamate microsensor.
The redox hydrogel is a three-dimensional polymer network, which is created by applying a solution containing the osmium redox polymer, the enzyme and the cross-linker on an electrode surface. Subsequent evaporation of the solvent enables the cross-linking process and in turn the formation of the hydrogel on the electrode surface. The enzymes are entrapped in this network. The ET in the hydrogel occurs as follows (Fig. 1D): the enzyme converts the substrate of interest, in turn the prosthetic group of the enzyme communicates with the closest nearby osmium mediator. This redox mediator communicates with another nearby osmium mediator and a sequence of self-exchange reactions between adjacent mediator molecules occurs to bridge the ET transfer to the electrode surface. This process is also referred to as “electron hopping”. In this way, the overall ET process is divided into a sequence of electron-hopping reactions between the different redox mediators (Jernigan et al., 1985; Degani and Heller, 1989; Dalton et al., 1990). The electrochemical reactions of the ET in the hydrogel can be described as follows:

\[ \text{Glu-ox} \rightarrow \text{Glu-ox} + \alpha\text{-ketoglutarate} + \text{NH}_3 \]
\[ \text{Glu-ox} + 2 \text{Os}^{3+} \rightarrow \text{Glu-ox} + 2 \text{Os}^{2+} + 2 \text{H}^+ \]
\[ \text{Os}^{2+} + \text{Os}^{3+} \rightarrow \text{Os}^{3+} + \text{Os}^{2+} \]

Glu-ox represents the oxidized form of the flavin adenine dinucleotide center (FAD), which is bound to the active site of glutamate oxidase and Glu-ox represents its reduced form. Glu-ox is reoxidized by two Os\(^{3+}\) centers of the osmium redox polymer (or by two sequential oxidations by a single Os\(^{3+}\) center) with the corresponding reduction of the redox polymer centers and the release of two protons. Electrons of the Os\(^{2+}\) center “diffuse” through the hydrogel by “hopping” between adjacent osmium centers.

Biosensors based on redox hydrogels have several specific advantages. For example, the hydrogel is hydrophilic and swells when applied in an aqueous environment. This increases the flexibility of the polymer backbone and generates a rapid electron self exchange in the redox polymer, a facile electrical communication with the active site of the enzyme and a fast diffusion of the counter anions. The latter is important for fast charge-transport reactions through the hydrogel. In turn, this “open structure” within the polymer network also permits rapid diffusion of substrates and reaction products to and from the enzymes, which improves the temporal resolution of the biosensor. In addition, the stability of the enzymes at the electrode surface is also improved and therefore enzymes that display a rather low stability at an electrode surface can be used as well (Kenausis et al., 1997; Habermüller et al., 2000). Because the enzymes communicate via the redox polymer, it occurs that enzymes that are localized remote from the electrode surface can also contribute to the current output of the biosensor. This improves the sensitivity of the biosensor and lowers its detection limit. Finally, enzymes and redox mediators are tightly wired to the electrode surface, which prevents leakage of the components, as well as biosensor
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deterioration and tissue fouling (Gregg and Heller, 1991b; Rajagopalan et al., 1996; Chen et al., 2000; Schuhmann, 2002). Due to these advantages, a large number of papers based on this technology have been published since the first pioneering work of Heller and co-workers (Degani and Heller, 1987, 1988; Gregg and Heller, 1990, 1991a, 1991b). In addition, glucose biosensors based on this technology are in development for commercial purposes (Therasense, 2000; Feldman et al., 2003).

As hydrogel-based biosensors belong to the second generation type of biosensors, distinct disadvantages are to be expected. Throughout the years, much effort has been given to deal with these limitations. For example, the communication of the osmium redox mediator with the active center of the enzyme requires that the osmium complex is in its oxidative state (i.e. Os$^{5+}$). As a result, a relatively high working potential (initially ~ 400 mV) needs to be applied, which allows easily oxidizable components to interfere. Attempts to lower the $E^{\circ}$ of the osmium redox polymer has therefore received much attention (Gregg and Heller, 1991a; Ohara et al., 1993a; Mao et al., 2003). At present, this has resulted in an osmium redox polymer with an $E^{\circ}$ of -195 mV, which can be operated at approximately –100 mV (Mao et al., 2003). Second, the construction of redox hydrogel based biosensors relies on manual dropping or dipping procedures. These procedures are difficult to standardize and to control (Schuhmann 2002, Mikeladze et al., 2002; Fei et al., 2003). For that reason, approaches have been developed to electrodeposit the redox polymer onto the electrode surface, while simultaneously entrapping the enzyme (Gao et al., 2002; Fei et al., 2003). Third, not each enzyme is suitable to be applied in this particular concept. By far the most studied enzyme is glucose oxidase, but other enzymes, such as horseradish peroxidase and lactate oxidase are also used. On the contrary, enzymes such as alcohol oxidase, D-amino acid oxidase, choline oxidase, but also glutamate oxidase are less suitable (Ohara et al., 1993b; Heller 2006; Michael, 2006). (Note, only for reasons of consistency glutamate oxidase was used as an example to demonstrate the principle of the redox-hydrogel based biosensor). The reaction centers of these enzymes most likely cannot be approached sufficiently by the osmium complexes, e.g. due to a low accessibility of the enzyme’s prosthetic group or due to a too large distance between the redox centers. A slower electron exchange between the prosthetic group of the enzyme and the osmium complex will occur and, consequently, compounds such as oxygen and AA will interferere by competing with the osmium center. This will interrupt the electrochemical cascade, as described above.

Third generation of amperometric biosensors

The third generation of biosensors implies a direct electrochemical communication between the prosthetic group of the enzyme and the electrode surface (Fig. 1C), as discussed previously. Theoretically, this has many advantages, as direct ET (DET) avoids intermediate ET steps via self-exchange reactions, which are prone to interference. However, a clear disadvantage is that
DET is only applicable to those enzymes that are immobilized in the first monolayer on the electrode surface, which limits the sensitivity and the detection limit of the sensor. This implies that DET is only possible for relatively small enzymes with an easily accessible prosthetic group. Additional anisotropic and oriented immobilization of these enzymes is also required for a proper functioning of the biosensor (Gorton et al., 1999; Zimmerman et al., 2000). Until now, the most intensively studied and best characterized enzymes showing DET properties belong to the group of peroxidases, for example horseradish peroxidase (Ruzgas et al., 1996), lactoperoxidase (Csöregi et al., 1993), or microperoxidase (Razumas et al., 1992).

These disadvantages have stimulated the development of other DET approaches, which are referred to as “electroenzymes”. In these approaches, the enzyme itself is modified with covalently bound redox-mediators or the enzyme is connected to a conducting polymer. Both methods are supposed to shorten the ET distance between the active site of the enzyme and the electrode surface. In the first approach, “electron hopping” via enzyme bound artificial mediators occurs and in the latter, the electron transfer is mediated via electrical conduction by a polymer. Connection with the enzyme occurs at the outer protein shell of the enzyme (Schuhmann et al., 1991; Ryabova et al., 1999), at the inner surface of the enzyme in close proximity to its active center (Bartlett et al., 1996; Degani and Heller, 1987), or directly at the prosthetic group of the enzyme (Katz et al., 1999; Xiao et al., 2003). With the latter approach, the redox center of the enzyme is “plugged” directly to an “electrical cable”. The connection between the enzyme and the electrode surface is mediated via conducting polymers (Koopal et al., 1994; Bartlett et al., 1996; Cosnier, 1999), conducting polymers modified with redox mediators (Schuhmann, 1998; Willner and Katz, 2000), or entrapment of enzymes in conducting materials, such as sol-gel composites with graphite particles (Coche-Querente et al., 1997). Polymers based on osmium complexes are often used for this purpose (Schuhmann, 1998; Habermüller et al., 2000; Rusling and Forster, 2003; Rusling, 2004).

The conducting materials can be seen as means to increase the “virtual” electrode surface, because they also allow enzymes that are immobilized relatively far away from the electrode surface to take part in the DET reactions. This is important for microsensors in particular, as they possess only a small electrode surface. Note, due to the use of redox mediators, the third and second generation type of biosensors show some architectural overlap and discrimination is not always clear.
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1.2 Glutamate microsensors

Microsensors as an alternative for microdialysis: motivation for the set-up

Although the first biosensors were already developed in the 1960s, it is the construction of microsensors that is of special interest for application in brain tissue. Microsensors for this purpose have been developed over the past two decades. They offer several analytical advantages when compared to the techniques that are currently applied on a routine base.

At present, microdialysis is the most frequently used analytical technique to monitor extracellular glutamate levels in vivo. Other techniques are push-pull perfusion (Gaddum, 1961), or direct sampling of extracellular fluid by fused silica tubing followed by capillary electrophoresis (Bowser and Kennedy, 2001; Kennedy et al., 2002; Sauvinet et al., 2003). With the microdialysis technique, a concentric probe is implanted into the brain. The probe consists of a semipermeable membrane that surrounds two tiny canullae, through which fluid flows in and out the probe. The extracellular compounds reach the perfusion fluid by diffusion (Ungerstedt, 1991). Microdialysis is applied for the detection of many extracellular compounds, such as neurotransmitters and metabolites. However, the neuronal origin of glutamate in dialysate has been questioned (Westerink et al., 1987; Waldmeier et al., 1992; Herrera-Marschitz et al., 1996; Miele et al., 1996b; Timmerman and Westerink, 1997; Westerink and Timmerman, 1999; Baker et al., 2002; Drew et al., 2004). It has repeatedly been shown that glutamate detected by microdialysis does not fulfill the classical release criteria for exocytotic release, as the sampled glutamate does not show calcium dependency or response to the sodium-channel blocker tetrodotoxin (TTX). Consequently, it was concluded that basal glutamate levels in microdialysis, in the absence of pharmacological or neuronal stimulation, reflected a non-neuronal pool.

What might be the reason that microdialysis does not detect glutamate from neuronal origin? A first explanation may be found in the fact that the “spill-over” of synaptically released glutamate is limited because a highly active glutamate reuptake mechanism is present in the brain, as will be discussed in the next paragraph. This implies that glutamate will diffuse only very briefly into the sampling domain of the microdialysis probe. Consequently, the temporal resolution of the microdialysis technique is of crucial importance. Previously, it was demonstrated that the temporal resolution of the microdialysis technique could be improved by combining it with different on-line detection techniques, such as capillary electrophoresis (Lada et al., 1997; Rossel et al., 2003), chemiluminescence detectors (Wang et al., 2001), or online enzymatic assays (Obrenovitch and Zilkha et al., 2001; Gáspár et al., 2004). These approaches indeed improved the monitoring of physiologically derived glutamate.

Secondly, it is known that the large dimensions (200 - 500 μm) of the microdialysis probe cause traumatic brain injury. Close examination of the tissue near the implantation site of the
microdialysis probe has produced evidence for disturbances in microcirculation, edema, changes in the rate of glucose utilization, a loss of integrity of the blood brain barrier, production of cytokines and eventual proliferation of glial cells (Benveniste et al., 1987; Zhou et al., 2001; Khan and Michael, 2003; Borland et al., 2005). Moreover, the induced tissue trauma might present a barrier to diffusion that even further separates the dialysis membrane from the synapse (Clapp-Lilly et al., 1999). The sum of these processes may inflict the physiology of neurotransmission largely.

It is hypothesized that a detection technique that causes less brain damage and which has a higher spatial- and temporal resolution might provide an alternative method for the detection of neuronally derived glutamate (Drew et al., 2004). Glutamate microsensors provide a promising solution to this requirement, as it was reported that the hydrogel-coated glutamate microsensor presented by Kulagina et al. (1999) was able to detect TTX-sensitive glutamate. This was the final motivation for introducing this microsensor technique into our laboratory. It is important to note that for a long time this was the only paper that has reported the detection of TTX-sensitive extracellular glutamate. Recently, Day et al. (2006) also have reported similar observations with a microsensor. This sensor is a so-called ceramic based microsensor and is developed by the research group of Dr. G Gerhardt. This specific microsensor is discussed in the next paragraph.

What is the origin of glutamate detected by microdialysis? As microdialysis most likely does not detect neuronally derived glutamate, this question automatically raises. At present, it is not known to what extent the detected glutamate is derived from neurons, astrocytes, other physiological or pathophysiological origin, or from an artificial source. A hypothesis that has received much attention by the research group of Dr. PW Kalivas the last couple of years is that microdialysis detects glutamate that is derived from a cystine-glutamate exchanger (Baker et al., 2002, 2003). However, currently it is not known if this exchanger contributes to extracellular glutamate release at normal brain functioning. In the next paragraphs more attention is paid to this exchanger and to other possible sources of glutamate.

Various glutamate microsensors

It is observed that although many studies deal with the construction and in situ evaluation of microsensors, the number of studies that successfully have applied glutamate microsensors in vivo or in vitro is rather limited. There are several aspects that limit their use and for that reason the practical application of a microsensor is still in its infancy. For example, it appears that the reliable production of thin, robust and highly active biosensing layers on small electrodes of μm-dimensions is difficult to realise (Niwa et al., 1996; Dale et al., 2005; Wilson and Gifford, 2005), and has only be demonstrated for a limited number of sensors. Moreover, when a microsensor is applied to detect glutamate in brain tissue, e.g. in the living brain (in vivo) or in brain slices (in vitro), a large number of analytical properties are of crucial importance. Critical properties of a
microsensor are its sensitivity, detection limit, linear range, selectivity, temporal and spatial resolution, biocompatibility, stability, etc (Turner, 1987, 1996; Abel and Woedtke, 2002; Wilson and Gifford, 2005). Many glutamate microsensors fail at this point. An overview of the various types of glutamate sensors that have been applied in brain tissue is given in Table 1.

<table>
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<tr>
<th>Architecture</th>
<th>Generation</th>
<th>Characteristics</th>
<th>Application</th>
<th>Reference</th>
<th>Remarks</th>
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Table 1: Different type of glutamate microsensors that have been applied in vivo or in vitro. Remarks: 1) Several papers based on the same technology have been published for peroxide, choline and acetylcholine (Garguilo et al., 1993; Garguilo and Michael, 1993-1996; Kulagina et al., 2003). 2) Triangle shape: from a 1 mm basis to a 2 μm tip. Abbreviations: AA-ox = ascorbate oxidase, BSA = Bovine serum albumin, CP = conducting polymer, Diam. = diameter of the sensor, Elec = type of electrode, Gen = generation of the biosensor, Glu-ox = Glutamate oxidase, HRP = horseradish peroxidase, PEA = phosphatidylethanolamine (type II-S), PEDGE = poly (ethylene glycol) diglycidyl ether (see also Fig. 2B), POs-EA = osmium redox polymer see Fig. 2D, Pot = operating potential, PPD = poly(o-phenylenediamine), Pt = platinum, Pts / Ir = platinum / iridium, PVI-dmeOs = osmium redox polymer based on a polyvinylimidazole backbone, Tr = response time.
Each specific sensor has its own advantages and disadvantages, of which the most characteristic ones are listed. In general, the first generation microsensors have a superior temporal resolution, but often are poorly selective, as a high operating potential is necessary to oxidize $\text{H}_2\text{O}_2$. Several other compounds may be easily oxidized at this potential, generating non-specific currents. The research group of Dr. J Lowry and Dr. RD O’Neill (Ryan et al., 1997; Lowry et al., 1998) even has retrospectively questioned the performance of their microsensor (Lowry et al., 2001) due to such a limited selectivity. The opposite is valid for the second generation type of biosensors, which in general display a higher selectivity and sensitivity, but a slower response time. In addition, their construction is often more complex due to the fact that several individual compounds have to be cross-linked with each other to form the sensing layer.

It should be noted that often promising sensor concepts are presented (e.g. Hu et al, 1994; Kulagina et al., 1999; Mikeladze et al., 2002), but that no sequel to this research is published. This of course questions the validity of such research. Interesting to mention is that recently the sensor presented by Hu et al. (1994) is commercially available and is exploited by the company Pinnacle (see the reference at chapter 9). At present, the only research group consistently publishing papers with the same microsensor concept is the group of Dr. G Gerhardt. This group uses a first generation type of sensor. Different platinum electrode sites of 50 x 150 μm$^2$ are applied on a ceramic-based sensor. These sites are coated with a Nafion layer, followed by application of a glutamate-oxidase-based sensing layer. A second sensor site, which is localised close to the glutamate sensor one, functions as a blank or reference sensor and therefore this sensor is referred to as self-referencing. This technology is not only applied for the construction of glutamate sensors (Burmeister et al., 2001, 2002; Pomerleau et al., 2003; Nickell et al., 2005; Day et al., 2006), but also for choline (Burmeister et al., 2003; Parikh et al., 2004), acetylcholine (Bruno et al, 2006), GABA (Pomerleau et al., 2006) and oxygen sensors (Burmeister et al., 2006). Recently, biosensors based on this technology are commercially available.

In addition, biosensors also have been combined with other methods. Examples are combinations of a biosensor and a microdialysis probe (Zilkha et al., 1995; Niwa et al., 1996; Yao et al., 2001; Shi et al., 2003; Zhang et al., 2004a), and the use of two-dimensional imaging biosensors. The latter approach is applied for in vitro research, in which slices are placed or grown on top of an electrode surface (Kasai et al., 2001; Hirano et al., 2003; Qhobosheana et al, 2004; Castillo et al., 2005). No further attention will be paid to these types of sensors, as it is beyond the scope of this chapter.

The hydrogel-coated glutamate microsensor

The microsensor presented in this thesis is referred to as hydrogel-coated glutamate microsensor. It is a specific subtype of the second generation type of biosensors based on redox hydrogels. The initial concept of this sensor was developed by the research group of Dr. A Heller (Gregg and...
Heller, 1991a,b; Maiden and Heller, 1992), while the application to carbon fiber electrodes for the detection of glucose, choline, glutamate and peroxide with a microsensor was further developed by the research group of Dr. AC Michael (Garguilo et al., 1993; Garguilo and Michael, 1993-1996; Kulagina et al., 1999, 2003). The detection of glutamate with this microsensor was reported for the first time by Kulagina et al. in 1999.

Figure 2: Different aspects of the hydrogel-coated glutamate microsensor. A) Schematic diagram of the carbon fiber electrode (CFE): 1. Carbon fiber (CF; 10 μm diameter; 300-500 μm length); 2. Glass-capillary tube (1 mm diameter). Note: the tube is pulled to a tip, from which the CF protrudes; 3. Silver wire (250 μm diameter). The Teflon coating (40 μm thick), which insulates the wire, is removed at both ends to mediate electrical contact; 4. Silver paint. This mediates the electrical contact between the CF and the silver wire; 5. Spun-epoxy glue. This attaches the silver wire in the glass capillary and offers mechanical stability. B) Poly (ethylene glycol) diglycidylether (PEDGE), which is the cross-linker that connects the enzymes to the osmium redox polymer. C) General cross-link reaction by which the enzymes are connected to the osmium redox polymer. D) The osmium redox polymer, abbreviated as POs-EA: a poly (vinylpyridine) backbone (y), complexed with osmium (bipyridine)chloride groups (Os(bpy)_xCl; x), and quarternized with ethylamine groups (z). The ratio of side groups (x : y : z) is respectively 1.0 : 4.0 : 1.2. E) Chemical structure of Nafion.
The sensor consists of a carbon fiber electrode (CFE) that is coated with a redox-hydrogel. The CFE consists of a carbon fiber (CF), which protrudes out of a pulled glass capillary (Fig. 2A). The CF has a diameter of 10 μm and a length of 300 – 500 μm. The hydrogel contains the enzymes glutamate oxidase (Glu-ox), horseradish peroxidase (HRP) and ascorbate oxidase (AA-ox), which are wired via the cross-linker poly(ethylene glycol) diglycidylether (PEDGE) (Fig. 2B) to an osmium redox polymer. PEDGE wires the enzymes by creating a three dimensional network in the hydrogel in which the enzymes are entrapped. In addition, PEDGE also covalently binds the enzymes to the redox polymer via a ring-opening reaction at primary and secondary amines (Fig. 2C). The osmium redox polymer (Fig. 2D), which is abbreviated as POs-EA, is a poly(vinylpyridine) backbone complexed with osmium (bipyridine)chloride (Os(bpy)$_2$Cl) groups and partially quarternized with ethylamine groups. The ratio of unmodified pyridines to osmium-complexed ones to ethylamine-quarternized ones is 4.0 : 1.0 : 1.2 (Gregg and Heller, 1991a).

The hydrogel is applied on the CFE via dipcoating. This is performed by dipping the CFE in an aqueous solution containing the five individual components. The solution adheres to the CFE and subsequent evaporation of the solvent enables the cross-linking procedure. In chapter 2 this dipcoating procedure is further examined. A thin Nafion coating (Fig. 2E) around the hydrogel completes the construction of the microsensor. Nafion is a commercially available perfluorosulfonic acid that rejects negatively charged compounds (e.g. AA, UA, but also glutamate). For that reason, Nafion is frequently used as a discriminator within biosensors (Garguilo and Michael, 1993, 1994; Hu et al., 1994; Karyakin et al., 2000; Burmeister et al., 2002). Due to its specific properties, Nafion is also used to improve the biocompatibility of biosensors by protecting against biofouling (Garguilo and Michael, 1996; Wisniewski et al., 2000; Wisniewski and Reichert, 2000).

In Fig. 3A a few scanning electron microscopic micrographs (SEMMs) of a CFE are shown. In Fig. 3B and C SEMMs of two different glutamate microsensors are shown. Clearly visible are the CF, the hydrogel and the Nafion layer. The sensors show some variation in the thickness and constitution of the separate layers. Several cracks in the outer layer of the microsensor are also visible. This is probably induced by the vacuum conditions of the electron microscope (Garguilo et al., 1994).
Compared to several other redox hydrogel biosensors, the concept of this hydrogel-coated glutamate microsensor is more complex, as it is based on the action of three enzymes. Figure 4A shows the enzymatic detection of glutamate by the microsensor. The detection is performed according the following reactions:
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I. Glu-oxFAD + glutamate $\rightarrow$ Glu-oxFADH$_2$ + α-ketoglutarate + NH$_3$

Glu-oxFADH$_2$ + O$_2$ $\rightarrow$ Glu-oxFAD + H$_2$O$_2$

II. HRP-native + H$_2$O$_2$ + 2 H$^+$ $\rightarrow$ HRP-Compound-I + 2 H$_2$O

HRP-Compound-I + Os$_3^{3+}$ $\rightarrow$ HRP-Compound-II + Os$_5^{3+}$

HRP-Compound-II + Os$_5^{3+}$ $\rightarrow$ Native-HRP + Os$_3^{3+}$

III. 2 Os$_3^{3+}$ + 2 e$^-$ $\rightarrow$ 2 Os$_2^{2+}$

IV. Ascorbic acid + AA-oxCu$_3^{1+}$ $\rightarrow$ Dehydroascorbic acid + AA-oxCu$_{2+}$

AA-oxCu$_{2+}$ + O$_2$ + 2 H$^+$ $\rightarrow$ AA-oxCu$_{3+}$ + H$_2$O

Figure 4: Electrochemical detection of l-glutamate on the hydrogel-coated glutamate microsensor. A) Enzymatic detection. B) Schematic drawing of the polymerized hydrogel on the CFE.
First (I), glutamate oxidase (Glu-ox) converts glutamate to H$_2$O$_2$, α-ketoglutarate and NH$_3$. In this process the FAD center of Glu-ox is reduced to FADH$_2$ and is reoxidized by oxygen. Next (II), H$_2$O$_2$ is converted by horseradish peroxidase (HRP), which in turn communicates with the POs-EA. In this regard, the osmium complexes of POs-EA react with the heme center (Fe$^{2+}$) of HRP in a two step reaction. Finally (III), the POs-EA mediates the ET to the CFE surface, in which the osmium complexes communicate with other nearby osmium mediators and a sequence of self-exchange reactions between adjacent osmium mediators occurs to bridge the ET transfer to the electrode surface, according the “electron hopping mechanism” (as explained in Fig. 1D).

Ascorbate oxidase (AA-ox) is incorporated to prevent the interference by ascorbic acid (AA). (IV) AA-ox converts AA to H$_2$O, and not to H$_2$O$_2$, in which the copper centers of AA-ox are respectively reduced by AA and reoxidized again by O$_2$. The fact that H$_2$O$_2$ is not produced makes AA-ox suitable to be used in this approach.

AA is present at high concentrations in the CNS (200-500 μM; Grünewald, 1993; Lai et al., 2000; Rice, 2000; Rebec et al., 2005) and interferes by reducing the intermediate steps in the reaction cascade that are in an oxidized state, respectively Os$^{3+}$, H$_2$O$_2$ and HRP$_{ox}$ (Maiden and Heller, 1992; Garguilo et al., 1993; Doherty et al., 1995). This interrupts the electrochemical cascade and suppresses the initial glutamate signal. In the first chapters (chapters 2-4) attention is paid to this issue.

In fact, the hydrogel is a polymerized mixture of five individual components on the CFE, as presented in Fig. 4B. The conditions during the dipcoating procedure and the physical chemical properties of the individual constituents are critical and determine their incorporation into the hydrogel. In chapters 3 and 4 the influence of the individual hydrogel components on the final performance of the microsensor is investigated in detail.

The previously mentioned benefits of redox hydrogels are also valid for the glutamate microsensor, i.e. a high sensitivity, selectivity and stability, a fast diffusion of substrates and products to and from the enzymes, a rapid electrical communication within the hydrogel and a low sensor deterioration and tissue fouling. Moreover, this specific microsensor has a few additional advantages. For example, the applied working potential can be decreased to a negative value, because the osmium mediators need to be present in the reduced state (i.e. Os$^{2+}$). This negative potential prevents side oxidation reactions by easily oxidizable compounds. Another advantage are the small physical dimensions of the microsensor, which offer a high spatial resolution.

It is obvious that the presented glutamate microsensor concept also has some disadvantages. First, the interference by reducing agents, predominantly by AA. Incorporation of a relatively large amount of AA-ox is necessary to prevent this interference. Secondly, as the microsensor uses two oxygen consuming enzymes, it relies heavily on the availability of dissolved oxygen levels. Third, it appears that application of the hydrogel to the CFE is a difficult procedure to standardize and to control. In chapters 2-5 attention is paid to these specific disadvantages.
1.3 Glutamate as a neurotransmitter

History of glutamate as a neurotransmitter
Approximately 70 years ago it was already noticed that the amino acid l-glutamate (Fig. 5) played a central metabolic role in the physiology of the brain. For example, in the 1930s and 1940s it was observed that brain tissue possessed a very high glutamate uptake activity and that glutamate played an important role in patients with mental disorders (Krebs, 1935; Stern et al., 1949). In 1954 it was hypothesized for the first time that glutamate could act as a synaptic neurotransmitter in the brain (Hayashi et al., 1954). Despite, full acceptance of glutamate as a neurotransmitter was first established at the end of the 1970s. In the following years the knowledge about the role of glutamate in the CNS increased rapidly. In particular, due to progresses in the field of molecular biology, which revealed the structure and function of multiple glutamate receptors throughout the 1990s. A comprehensive historical overview on the role of glutamate as a neurotransmitter was written by Watkins (2000).

Multifunctionality of glutamate
The reason that it took so long before glutamate was fully recognized as a neurotransmitter was due to the fact that glutamate serves a multifunctional role in the CNS. Beside its role as a neurotransmitter, glutamate also plays an important role in the energy metabolism of the CNS, in the detoxification of ammonia and it is an important building block in the synthesis of proteins and peptides, including glutathione. Furthermore, it serves as a precursor for the inhibitory neurotransmitter γ-aminobutyric acid (GABA). For years, it was difficult to dissociate the role glutamate played in neuronal metabolism from its possible role as a neurotransmitter (Conti and Weinberg 1999; Watkins, 2000).

Glutamate belongs to the class of amino acid neurotransmitters. In the CNS approximately 90 % of all neurons use a neurotransmitter of this class, while the well studied transmitters, e.g. acetylcholine, noradrenaline, dopamine, histamine and 5-hydroxytryptamine only account for neurotransmission at a small percentage of central synaptic sites. On the basis of
neurophysiological studies, amino acid neurotransmitter can be separated into two general classes: excitatory and inhibitory amino acids. Excitatory amino acids generally depolarize neurons and the most important representatives of this class are glutamate and aspartate. Other members are homocysteic acid, quinolinic acid and N-acetyl-L-aspartyl-L-glutamic acid (Cooper et al., 1996, Orlowska-Madjak, 2004). On the contrary, inhibitory amino acids generally hyperpolarize neurons and examples of this class are GABA, glycine and taurine (Cooper et al., 1996; Vitten and Isaacson, 2001). However, when studied in more detail, the different amino acids have a more refined role, e.g. inhibitory amino acids can also induce excitatory effects and vice versa.

L-Glutamate is recognized as the primary excitatory neurotransmitter in the CNS. It is generally agreed that approximately 50-60 % of all terminals in the CNS use glutamate as a neurotransmitter (Coyle and Puttfarcken, 1993; Javitt, 2004) and that more than 90 % of all neurons display sensitivity for glutamate. In addition, recently it was demonstrated that glutamate can be co-released in addition to monoaminergic neurotransmission (Trudeau, 2004). Therefore, glutamate is involved in most aspects of normal brain functioning, including cognitive processes and the formation of memory. It also plays a major role in the development and plasticity of the CNS, including the migration, differentiation and death of cells, and in the induction and elimination of synapses. Consequently, glutamate is involved in the pathophysiology of many neurological, neurodegenerative and psychiatric disorders, such as, epilepsy, Huntington’s chorea, Parkinson’s disease, Alzheimer’s disease, amyotrophic lateral sclerosis, depression, stroke and schizophrenia (Nicholls, 1993; Cooper et al., 1996; Danbolt, 2001; Palucha and Pilc, 2005).

In addition to its role in the CNS, glutamate plays a signalling role in peripheral organs as well. It is present at high concentrations (30 - 80 μM) in the blood and tissue fluids, whereas glutamate receptors have been identified in many organs, such as the heart, spleen, testis, ovary, kidney, bone, bone marrow, pancreatic β-cells, intestine, oesophagus, hepatocytes, lung and keratinocytes. This suggests an involvement in many physiological and metabolic functions. Although many of these functions have not been established, the role of glutamate in insulin release from pancreatic β-cells, in bone metabolism and in the proliferation of several tumour cells has already been demonstrated (Danbolt, 2001; Nedergaard et al., 2002).

The concentration of glutamate in the extracellular fluid

The brain contains large quantities of glutamate. The intracellular concentrations are in the millimolar range, in which the cytosolic concentration is about 5 to 10 mM and the vesicular concentration is approximately 0.1 M. It appears that the intracellular concentration in neurons is higher than in astrocytes, due to conversion of glutamate to glutamine by glutamine synthetase in astrocytes, as discussed below. Despite these high intracellular concentrations, only a small
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Fraction is present in the extracellular fluid (ECF). Note, the ECF represents 13-22% of the brain tissue volume. Interestingly, the real concentration of glutamate in the ECF is still a matter of debate, mainly due to limitations of the available recording techniques. Studies with quantitative microdialysis have consistently reported ECF glutamate levels in the range of 1-5 μM (Miele et al., 1996; Kennedy et al., 2002; Zhang et al., 2005). Although at present this is the most reliable and most frequently used technique to estimate absolute glutamate concentrations in the brain, it is possible that quantitative microdialysis does not provide a reliable answer (Peters et al., 2000). As mentioned previously, microdialysis has some serious drawbacks concerning its temporal and spatial resolution. On one hand it has been hypothesized that microdialysis overestimates the extracellular glutamate concentration as the probe mainly samples from damaged brain tissue. Glutamate may accumulate in this damaged tissue as it lacks access to the high-affinity membrane transporters, which in turn have the capacity to reduce glutamate concentrations to the low nanomolar range (Nedergaard et al., 2002; Cavelier et al., 2005). Other possible causes of overestimating the extracellular glutamate levels are microbial contamination (Zhou et al., 2002) and misinterpretation of glutamate in the micodialysate samples due to insufficient chromatographic conditions (Rea et al., 2005), or by detecting a mixture of glutamate enantiomers (Quan and Liu, 2003). On the other hand it has been claimed that microdialysis underestimates the extracellular glutamate concentrations due to damage of the release processes (Khan et al., 2003), or due to the composition of the dialysis perfusion medium (Lai et al., 2000; Rebec et al., 2005).

In this respect, glutamate microsensors may give a better estimation of the extracellular glutamate concentration due to their superior temporal and spatial resolution. Interestingly, several microsensor studies have reported much higher basal glutamate concentrations than observed with microdialysis (Kulagina et al., 1999; Oldenziel et al., 2005 (chapter 5); Oldenziel et al., 2006 (chapter 7) and observations by the research group of Dr. G Gerhardt: Hascup et al., 2006; Nickell et al., 2006; Rutherford et al., 2006; Stephens et al., 2006). This subject is discussed further in chapters 5, 7 and 8.

Glutamatergic neurotransmission

In brief, glutamatergic neurotransmission is arranged as follows (Fig. 6A). After depolarization of the neuron, the vesicles fuse with the presynaptic membrane and glutamate is released in the synaptic cleft. This is a calcium and ATP dependent process. The quantity of glutamate that is released depends on three important parameters: 1) The concentration of glutamate in the synaptic vesicles (approximately 60 mM, but higher concentrations, up to 210 mM, have been reported), 2) The volume of the vesicle (25-45 nm), and 3) the number of synaptic vesicles available per time constant for exocytosis (roughly two vesicles per μm² per sec). It is estimated that between 400 and 5000 molecules of glutamate are released in the small volume of the
synaptic cleft (which has a volume of approximately 2 attoliters). As a result, peak concentrations of several millimolar are reached in the synaptic cleft. These high concentrations are required to activate the postsynaptic glutamate receptors (Bergles et al., 1999; Danbolt, 2001). It is estimated that complete emptying of a transmitter from a small synaptic vesicle requires less than 100 μs and that the time glutamate is present in the synaptic cleft is approximately 1 millisecond. The real time that glutamate remains elevated directly affects the quantity of occupied receptors. For example, a difference between a concentration decay time constant of 0.5 and 1.5 ms can make the difference between a low receptor occupancy and nearly saturated receptors (Bergles et al., 1999; Conti and Weinberg, 1999; Syková, 2004). The decay of the glutamate transient in the synaptic cleft is dependent on diffusion and uptake by membrane-bound high-affinity transporters, as will be discussed below.

The synaptic cleft is in continuity with the ECF and it appears that in certain brain areas (e.g. hippocampus, cerebellar cortex) part of the glutamate may diffuse out of the synaptic cleft. This process is referred to as ‘spillout’. This escape provides a mechanism for activation of glutamate receptors that are located extrasynaptically.

Figure 6: Physiology of glutamergic transmission. A) Vesicle release of glutamate. B) Glutamate-glutamine cycle. C) Different receptors and channels on astrocytes that contribute to the physiology of glutamatergic neurotransmission. Abbreviations: mGluR = metabotropic glutamate receptor; iGluR = ionotropic glutamate receptor; EAAT = excitatory-amino-acid transporter; dCT = different channels and transporters that contribute to release of glutamate; [Ca^{2+}]_i = intracellular calcium concentration; TCA = tricarboxylic acid cycle; GS = glutamine synthetase; PAG = phosphate activated glutaminase.
In addition, this 'spillout' of glutamate may lead to 'spillover', which is defined as the activation of glutamate receptors on neighbouring neurons, as well as in neighbouring synaptic clefts. The latter is referred to as "synaptic crosstalk". The extent of these phenomena depends on the amount and rate of escape, the distance between adjacent synapses, and the location and abundance of glutamate transporters (Bergles et al., 1999; Sem'yanov, 2005).

The transport of glutamate from blood to the brain only plays a minor role in the regulation of brain glutamate levels. The blood-brain-barrier prevents the entry of glutamate into the CNS and glutamate needs to be synthesized from glucose within the CNS, which is referred to as "de novo synthesis". Consequently, the glutamate-glutamine cycle is of crucial importance for glutamatergic neurotransmission (Fig. 6B). Briefly, after its release into the synaptic cleft, glutamate is taken up by the high-affinity uptake process into astrocytes and (to a much lesser extent) into neurons. Only in astrocytes glutamate is converted by the enzyme glutamine synthetase to glutamine. In turn, glutamine is transported via a low-affinity process from astrocytes into the neighbouring nerve terminals and the enzyme glutaminase converts glutamine back to glutamate, which is finally stored again in the vesicles. Previously it was thought that glutamate in the nerve terminal was synthesized from two sources. First, from glutamine, derived from the glutamine-glutamate cycle, and secondly, from glucose, derived from the tricarboxylic acid (TCA) cycle. However, recent evidence has revealed that neurons, in contrast to astrocytes, lack the enzyme pyruvate decarboxylase and therefore cannot perform the net synthesis of glutamate from glucose (Hertz and Zielke, 2004). This implies that neurons depend heavily on astrocytes for their supply of glutamate.

Reuptake of glutamate
To assure a high signal-to-noise ratio for glutamatergic transmission and to avoid excitotoxic actions of glutamate, the extracellular concentration of glutamate should be kept low. The only rapid way to remove glutamate from the extracellular fluid is by cellular uptake. The excitatory-amino-acid transporters (EAATs) are crucial for the clearance of released glutamate (Danbolt, 2001; Gallo and Chittajallu, 2001). Astrocytes and neurons express a total of five isoforms of the high-affinity sodium dependent excitatory-amino-acid transporter (EEAT): GLAST (EAAT1), GLT1 (EAAT2), EAAC1 (EAAT3), EAAT4 and EAAT5. The five cloned glutamate transporter subtypes share 50 to 65 % of similarity in their amino acid sequence. The majority of the transporters are expressed on astrocytes and only little is found on neurons (Danbolt, 2001). The uptake of glutamate by EAATs is coupled to cotransport of sodium and potassium. The stoichiometry of this process is such that the inward movement of one molecule glutamate, three sodium ions and one proton is coupled to the outward transport of one potassium ion. In turn, the binding of glutamate to the transporter activates a chloride conductance. At present, it is not known whether the...
transporter itself can act as a channel, or whether a separate chloride channel is linked to it (Amara and Fontana, 2002).

The transporters appear to have sophisticated functions in the modulation of glutamatergic neurotransmission. They may modify the time course of synaptic events, as well as the extent and pattern of activation and desensitization of receptors outside the synaptic cleft and at neighbouring synapses. A variety of soluble compounds, e.g. glutamate itself, cytokines and growth factors, can influence the expression and activity of the glutamate transporters (Danbolt, 2001; Gegelashvili et al, 2001; Amara and Fontana, 2002). The uptake of glutamate can also be regulated by changing the distance between the astrocytic processes and the synaptic cleft, a process referred to as "astrocytic wrapping". The extent to which astrocytic processes wrap around synapses is dynamically changed by the swelling of astrocytes. This mechanism, which occurs in a calcium dependent fashion, allows a time dependent modification of the efficacy of glutamate uptake, which in turn can shape extrasynaptic glutamergic transmission (Oliet et al., 2001; Nedergaard et al., 2002; Hirrlinger et al., 2004; Benediktsson et al., 2005).

Classification of glutamate receptors

Glutamate receptors can be divided into two major classes: ionotropic- and metabotropic receptors. The ionotropic receptors (iGluRs) form a large family, which can be subdivided into three groups: NMDA, AMPA and kainate. Although, there is supporting evidence that the delta receptor maybe represents a fourth member of the iGluR family. All the iGluRs share a common conserved transmembrane topology and stoichiometry. In addition, they all contain integrated, ligand-gated, cationic ion channels and are assembled from several subunits (Hansson et al., 2000; Madden, 2002; Mayer, 2005).

The NMDA (N-methyl-D-aspartic acid) receptor was the first glutamate receptor to be discovered. Each NMDA receptor is constructed from 4 or 5 subunits. Currently, seven different types of subunits have been characterized: NR1, NR2 (NR2A-NR2D) and NR3 (NR3A and NR3B). NMDA receptors exhibit a relatively high affinity for glutamate (an EC$_{50}$ of 0.2 to 1.7 μM) and generally they exert a more modulatory function, i.e. they take longer to open (> 2 ms) and remain open for longer when compared to the other iGluR family members. The NMDA receptor is blocked by Mg$^{2+}$ at resting membrane potentials and carries only significant current when the membrane is depolarized. In this way fine-tuning of neuronal processes can be achieved. For example, the NMDA receptor can act as an "AND" gate in neuronal processing as it requires activity of nearby receptors (for example AMPA) to be functional. The NMDA receptor is unique in its requirement of two obligatory co-agonists. Beside glutamate, that binds to the NR2 subunit, co-agonism by glycine is required, which has to bind to the NR1 subunit. Other co-factors can also act at allosteric sites of the NMDA receptor. For example, the NR2 subunit possesses a binding site for D-serine and polyamines. Substances as Zn$^{2+}$, H$^+$ and opioid peptides can also act at the
NMDA receptor. Currently, it is thought that the NMDA receptor plays an important role in long-term potentiation (LTP) and long-term depression (LTD), and consequently in the process of learning and memory (Lamprecht and LeDoux, 2002; Cavalier et al; 2005; Millan, 2005).

The AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor forms a second subclass of the ionotropic receptors. For years this class was referred to as the Quisqualate receptor, but after additional research it was renamed to AMPA (Watkins, 2000). Four homologous proteins termed GluR1-GluR4, assemble in various combinations of 4 to 5 subunits to form the AMPA receptor. In addition, all the AMPA receptor subunits exist as two splice variants: ‘flip’ and ‘flop’. The difference is a slight conformational change in the constitution of the receptor (i.e. an alternative splicing in the extracellular S2 domain). Although the difference between the two splice variants is a modification of only a few amino acids, it induces large changes in desensitisation kinetics. AMPA receptors exhibit a relatively low affinity for glutamate with an EC\(_{50}\) between 3 and 30 μM. They open quickly, but briefly (<1 ms), while mediating fast synaptic transmission in the CNS.

The kainate receptor forms the third member of the iGluR family. The role of kainate receptors in synaptic transmission has been discerned only recently. For years it was thought that the AMPA and kainate receptors belonged to the same subclass, as they shared several agonists and antagonist. Consequently, this class was referred to as the non-NMDA receptor. However, ongoing research concerning the cloning of glutamate receptor subunits has legitimized the pharmacological classification of the kainate receptor. The kainate receptor is constituted from five different subunits, which are related to two families: GluR5, GluR6, GluR7 belong to one family and KA1 and KA2 to the other. Like the AMPA receptor, kainate receptors can mediate excitatory synaptic signals. In addition, they are also involved in modulating the presynaptic release of neurotransmitters and therefore in regulating the strength of synaptic connections. Kainate receptors exhibit a relatively low affinity for glutamate with an EC\(_{50}\) between 6 and 23 μM. The kainate receptors are considered as potential therapeutic targets in the treatment of epilepsy and pain. However, their physiological role has been explored very limited until now, mainly due to the lack of selective pharmacological tools (Huetttner, 2003; Lemna, 2003).

The most recently discovered member of the class of ionotropic receptors are the delta receptors. The delta receptor consists of two subunits, δ1 and δ2. Based on their amino acid profile the delta receptors are classified as iGluRs. However, due to their apparent lack of ion channel activity, they are often referred to as orphan receptors. Additional research is required to clarify their functional role in the CNS (Lomeli et al., 1993; Mayat et al., 1995; Yuzaki, 2003).

In the mid 1980s a new class of glutamate receptors was recognized. These receptors appeared to be coupled to G-proteins and second messenger systems and are referred to as metabotropic glutamate receptors (mGluRs). mGluRs are member of the G-protein-coupled receptor family (GPCR), which also include the GABA\(_B\) and Ca\(^{2+}\)-sensing receptors. Today, eight
different mGluRs are subdivided into three groups (I – III) according to their amino-acid similarity, pharmacological profile and signal transduction mechanism. Group I consists of mGluR1 and 5, group II of mGluR 2 and 3 and group III of mGluR 4 and 6-8. With exception of mGluR 6, which is confined to the retina, all members of the mGluR family are expressed in the CNS and display an EC\textsubscript{50} between 4 and 30 μM. It appears that the group I mGluRs are predominantly positively coupled to phospholipase C (PLC), whereas the group II and II receptors are negatively coupled to the inhibitory action of adenylyl cyclase. Although the mGluR receptors mediate synaptic transmission via activation of slow excitatory postsynaptic potentials, they generally exert a more modulatory role. It is thought that they play an important role in regulating neuronal excitability, in synaptic transmission and in processes concerning plasticity (Hansson et al., 2000; Kew and Kemp, 2005; Sem'yanov, 2005).

Neuron-astrocyte interaction

For a long time it was thought that astrocytes were passive cells with only an intermediate function between blood vessels and neurons. However, our understanding of the crucial role that glia cells play in glutameric neurotransmission is rapidly growing. Nowadays, it is recognized that astroglia and neurons are intimate partners in glutameric synaptic transmission, in which astrocytes appear to be dynamic signalling components with many functions in the CNS (Fig. 6C). In this respect, one should not overlook the crucial contribution of astrocytes when studying glutameric neurotransmission (Araque et al., 2001; Haydon, 2001; Nedergaard et al., 2002). The synergy between neurons and astrocytes can be expressed as follows (citation from Haydon; 2001): “Consider the brain to be like a theatrical production. Although the actors (neurons) take centre stages, the play is nothing without the stagehands, the director and so on (glial cells). The more technically sophisticated the performance, the greater the number of backstage people needed. Perhaps it is no coincidence that the ratio of glia to neurons increases through phylogeny”.

Although many functions of astrocytes have not been revealed, they appear to be an important source of extracellular glutamate in the brain. In this regard it is likely that part of the glutamate that is detected by the microsensor is derived from astrocytes. Therefore, the role of astrocytes is discussed next.

Astrocytes

Astrocytes belong to the family of glia cells. There are three types of glia cells, which can be divided into two categories: microglia and macroglia. Microglia have a macrophage like function. They respond to neuronal impulse activity and mediate neuroimmune interactions, e.g. in chronic pain conditions (Watkins et al., 2001). Macroglia consists of Schwann cells, oligodendrocytes and astrocytes. Schwann cells and oligodendrocytes are the myelin-forming cells of, respectively, the peripheral nervous system and the CNS. They wrap layers of myelin membrane around axons to
insulate them for impulse conduction (Araque et al., 2001; Fields and Stevens-Graham, 2001). On the contrary, the role of astrocytes in the brain was unclear for a long time. Initially they were considered as “brain glue” and thought to fulfill an intermediate function between blood capillaries and neurons. However, the past few years previously unrecognized and surprising functions have been revealed for astrocytes. Nowadays, astrocytes are considered as highly polyvalent cells that are implied in almost all processes that occur in the CNS. Although many functions of astrocytes still need to be revealed, it is evident that they play a key role in the glutamate-glutamine cycle (Nedergaard et al., 2002; Hertz and Zielke, 2004), in the regulation of the cerebral blood flow (Zonta et al., 2003; Mulligan and MacVicar, 2004), in the regulation (Fields and Stevens-Graham, 2001; Oliet et al., 2001), modulation (Hintlinger et al., 2004; Benediktsson et al., 2005; Sem’yanov, 2005) and synchronization of neuronal transmission (Angulo et al., 2004; Fellin et al., 2004; Perea and Araque, 2005), in both neurogenesis (Seri et al., 2001; Song et al., 2002) and synaptogenesis (Ullian and Sapperstein, 2001; Hama et al., 2004), in learning and memory (Araque et al., 2001; Miller, 2003), and in pathological processes in the brain (Volterra and Meldolesi, 2005).

One of the reasons that the active properties of astrocytes have remained in the dark for so long is related to the difference between the excitation mechanisms of astrocytes and those of neurons. Until recently, the electrical language of neurons was thought to be the only form of communication in the brain. As astrocytes show only few responses to electrophysiological recording techniques, the absence of electrical activity was interpreted as functional inactivity (Nedergaard et al., 2002; Volterra et al., 2005). The recognition that astrocytes communicate chemically has triggered the explorative research on their functional role.

Communication by astrocytes: gliotransmission

Astrocytes communicate by delivering specific messages to neighbouring cells. This activity is referred to as “gliotransmission”. Gliotransmitters are chemicals that act on adjacent neurons, glial cells and blood vessels, and are released after excitation of astrocytes. Astrocytic excitation can be revealed by monitoring assays of intracellular Ca$^{2+}$ ([Ca$^{2+}$]), which are activated by elevations of the second messenger inositol-1,4,5-triphosphate (Ins(1,4,5)P$_3$). This messenger is generated as a consequence of the activity of phospholipase C, which in turn is activated by certain G-protein-coupled receptors.

Two main forms of astrocyte excitation are well documented: neuron-dependent excitation and spontaneous excitation. Neuron-dependent excitation represents the transfer of information from neurons to glia. The release of various transmitters and factors such as glutamate, GABA, acetylcholine, noradrenaline, dopamine, histamine, ATP, nitric oxide and brain-derived neurotrophic factor (BDNF) have been reported to “spillout” and affect extrasynaptic receptors on astrocytes (Araque et al., 2001; Bezzi and Volterra, 2001; Haydon, 2001; Syková, 2005; Volterra and Meldolesi, 2005).
Spontaneous excitation is an unexpected property of astrocytes and occurs independently of neuronal input. Spontaneous [Ca\(^{2+}\)] oscillations are generated when Ins(1,4,5)P\(_3\) receptors are activated by the influx of extracellular Ca\(^{2+}\), possibly through voltage-gated channels. Importantly, spontaneous excitation of astrocytes may result in the excitation of neighbouring astrocytes and neurons. This finding overturns the common idea that information is generated by neurons and travels through neuronal circuits before reaching the glia (Haydon, 2001).

Many questions that concern the excitation of astrocytes still need to be answered. For example, the range of propagation of [Ca\(^{2+}\)] signals under physiological conditions remains undefined. It is not known to what extent these responses represent physiological, pathological or artificial processes. Furthermore, it is not exactly known how Ca\(^{2+}\) signals travel from one astrocyte to another, although there is strong evidence that this is mediated by two prominent routes (Araque et al, 2001; Fields and Stevens-Graham, 2001; Haydon, 2001). First, an intracellular route: representing cell-to-cell diffusion of Ins(1,4,5)P\(_3\) between astrocytes through GAP-junctions to stimulate the release of [Ca\(^{2+}\)] of neighbouring cells and to give rise to intercellular waves of Ca\(^{2+}\): Secondly, an extracellular route: waves of elevated Ca\(^{2+}\) can jump the gap between two groups of astrocytes that are separated by cell-free lanes, as long as the gap between them does not exceed approximately 120 μm (Enkvist and McCarthy,1994; Hassinger et al.,1996). In addition, extracellular messages probably play an important role in these Ca\(^{2+}\) waves, in which ATP is also thought to play a key role.

Gliotransmission was first revealed in 1994 when increases in [Ca\(^{2+}\)] were shown to induce glutamate release followed by neuronal activation (Parpura et al., 1994). Over the years the number of proposed gliotransmitters has increased. Despite, a precise definition is missing. Recently, the following criteria for a gliotransmitter were proposed (Volterra and Meldolesi, 2005):
1) Synthesis and/or storage of the gliotransmitter in the astrocyte.
2) Regulated release triggered by physiological stimuli.
3) Activation of rapid (milliseconds to seconds) responses in neighbouring cells.
4) A role in physiological processes.

Compounds which meet these criteria are: glutamate, ATP, adenosine, D-serine, prostaglandin, 20-hydroxyicosatetraenoic acid (HETE), tumor necrosis factor-α (TNFa), and certain proteins and peptides, e.g. acetylcholine binding protein (AchBP) and atrial natriuretic peptide (ANP). Several other compounds also have been proposed, e.g. homocysteic acid, taurine and secretogranin II, but the evidence is still inconclusive.

It is concluded that in many respects astrocytes are similar to neurons: they respond to transmitters, integrate inputs and have long-range-signalling systems that can lead to the release of transmitters at a distance. However, a profound difference between the two cell types is the timescale: glial Ca\(^{2+}\) signals propagate at rates of micrometers per second, whereas action potentials propagate at rates of meters per second (Haydon et al., 2001). In addition, the
biochemical events associated with excitation of astrocytes and the release of gliotransmitters is probably more complex than is currently understood and is likely not a simple consequence of increases in $[\text{Ca}^{2+}]_i$. Several distinct stimuli that affect $[\text{Ca}^{2+}]_i$ responses evoke release of different gliotransmitters, which in turn activate distinct receptors with ensuing positive or negative cooperativity. It is likely that this induces heterogeneity in the response of astrocytes.

**Release of glutamate from astrocytes**

It is obvious that the release of extracellular glutamate by astrocytes is of particular interest for the interpretation of the microsensor experiments. It appears that astrocytes release glutamate via two main mechanisms: exocytotic and non-exocytotic release.

It has been unclear for a long time whether astrocytes could release glutamate via exocytotic processes (Nedergaard, 2002). However, recently a clear synaptic-like microvesicle (SLMV) compartment, that is equipped for the uptake, storage and release of glutamate, has been identified in certain brain regions and $\text{Ca}^{2+}$ dependent exocytotic glutamate release has been documented. Astrocytic SLMVs resemble glutamergic synaptic vesicles from a morphological point of view, but they have a much less dense and orderly arrangement. Besides, astrocytic exocytosis seems to be slower and the $\text{Ca}^{2+}$ affinity of the release machinery is significantly higher than of synaptic exocytosis (Bezzi et al., 2004; Kreft et al., 2004; Zhang et al., 2004b).

Non-exocytotic release is represented by transport of glutamate across the plasma membrane through specialized proteins, such as channels and transporters. Several types of such membrane channels and transporters are described (Cavalier and Attwell, 2005; Volterra and Meldolesi, 2005). However, their actual contribution to the release of glutamate is often not fully known and needs further clarification. Some examples of non-exocytotic glutamate release are:

1) Release of glutamate controlled by volume-sensitive organic anion channels (VSOACs), also referred to as VRACs (volume-regulated anion channels). These channels are activated as part of the volume regulation ("astrocytic swelling") and they allow the efflux of aspartate, taurine, glutamate, chloride and other anions in a calcium independent manner. Activation of metabotropic glutamate receptors is probably also associated with these processes (Hansson et al., 2000; Kimelberg et al., 2004).

2) Release of glutamate by GAP-junction hemichannels. These channels are formed by hexamers of connexion 43 (CX43) and assemble in astrocytes where they function as autonomous permeation pathways. They release both ATP and glutamate, and their opening probability is controlled by changes in $\text{Ca}^{2+}$ concentration (Hansson et al., 2000; Parpura et al., 2004; Takano et al., 2004; Volterra and Meldolesi, 2005).

3) The purinergic P2X$_7$ receptor. This receptor is gated by ATP and has been proposed to be involved in the release of glutamate and D-aspartate. It shares some properties with the GAP-
junction hemichannels, including the increased opening probability at low Ca\(^{2+}\) concentration, but their pharmacology is different (Duan et al., 2000; Sperlagh et al., 2002; Wang et al., 2004).

4) Sodium-dependent high affinity heteroexchange mechanisms for glutamate and ascorbate (Wilson et al., 2000; Song et al., 2002), for glutamate and GABA and for glutamate and glycine have been reported, but are poorly characterized (Bonanno and Raiteri, 1994; Raiteri et al., 2002).

5) Release of glutamate by a prostaglandin-dependent mechanism (Bezzi et al., 1998).

6) Release of glutamate regulated by a cystine-glutamate exchanger. This is a sodium-dependent glutamate transporter that can operate in the reverse mode, i.e. cystine is exchanged for glutamate by transporting one molecule of cystine intracellular for one molecule of glutamate out of the cell. The most obvious function of the cystine-glutamate exchanger is to accumulate cystine, which is reduced to cysteine and in turn is converted to glutathione. At present it is not known if this exchanger contributes to extracellular glutamate release at normal brain functioning. Although this was initially hypothesized (Baker et al., 2002; Xi et al., 2003), recently this hypothesis was questioned (Nedergaard et al., 2002; Hertz and Zielke, 2004; Cavelier et al., 2005), as significant reversal of the transporter only seems to occur when the energy metabolism of astrocytes is compromised, for example during stroke. In addition, it is questioned whether the extracellular concentrations of endogenous cystine are high enough to trigger the exchange activity at normal conditions (Cavelier and Attwell, 2005).

The fact that astrocytes can influence synaptic activity by release of glutamate points to a complex interrelation and interaction between glutamate and astrocytes. Glutamate released by both neurons and astrocytes activates Ca\(^{2+}\) signalling in astrocytes, but astrocytes are responsible for the uptake of glutamate and thereby the control of the steady-state levels of extracellular glutamate. At present, the exact functional role of this process has not been established. However, it is possible that glutamate receptors on astrocytes sense the glutamate that is released during synaptic transmission in order to adjust its extracellular concentration, either by the modulation of uptake activity or by active glutamate release. In addition, purines (ATP and its derivative adenosine) and glutamate were found to have opposite effects in the same synaptic territory: inhibitory for purines and excitatory for glutamate (Newman et al., 2003; Zhang et al., 2003). Astrocytes could therefore exert non-stereotyped, bimodal, synaptic control through the release of these gliotransmitters. Moreover, as astrocytes also release additional gliotransmitters, which activate distinct receptors with ensuing positive or negative cooperativity, a large range of heterogenic astrocytic responses is possible.

As extracellular glutamate originates from several sources, highly specified analytical techniques are demanded in order to improve our understanding of the physiology of glutamatergic neurotransmission. The glutamate microsensor might contribute significantly to this process as discussed in chapters 6 and 7.
1.4 Outline of the thesis

Summary of the introduction

It is concluded that glutamatergic neurotransmission in the CNS is mediated by a complex interplay between neurons and astrocytes. This complexity is probably also reflected in the extracellular concentration of glutamate in the ECF. In order to improve our understanding of the physiology of glutamatergic neurotransmission, an analytical technique with a high spatial- and temporal resolution seems required. Glutamate microsensors may fulfill these requirements. Various types of glutamate microsensors have been developed and each sensor has its own typical analytical advantages and disadvantages. However, the number of reports that use a glutamate microsensor as an analytical tool in brain tissue on a routine base is very limited. Apparently, there are several technical difficulties in the construction and application of a microsensor and therefore it is concluded that the development of a reliable glutamate microsensor is still in its infancy.

A promising glutamate microsensor, referred to as a hydrogel-coated glutamate microsensor, was developed by Kulagina et al. (1999). The aim of the current research was to introduce this specific microsensor technique and to apply it as an analytical tool. However, it appeared that this introduction was a difficult task and that additional research concerning its construction and evaluation was required. Therefore, this thesis deals with the construction, evaluation and practical application of this specific glutamate microsensor.

Outline of the thesis

In the first chapters (chapters 2-4) the construction of the microsensor is investigated in detail. In chapter 2 is investigated to what extent the different aspects of the microsensor architecture affect the final performance of the microsensor. In order to improve the reproducibility in construction of the microsensor an automatic dipcoater is developed. In chapter 3 is investigated how the performance of the microsensor is affected by modification of the hydrogel constitution. The influence of the individual hydrogel constituents on the final performance of the microsensor is investigated and, in addition, an optimized coating-solution is proposed. In chapter 4 the influence of the enzyme ascorbate oxidase on the performance of the microsensor is further investigated. The influence of different batches of ascorbate oxidase is examined and an appropriate enzyme-purification step is developed. In chapter 5 various aspects of the optimized glutamate microsensor are evaluated. Several conditions that may be encountered during application in brain tissue are examined. In addition, a first series of experiments in vivo and in vitro are presented. In chapters 6 and 7 the microsensor is applied on a routine base as an analytical tool. In chapter 6 the microsensor is applied in brain slices (in vitro). The tonic release
of glutamate in acutely prepared hippocampal slices is investigated during different pharmacological conditions. In chapter 7 the microsensor is applied in vivo in the brain of anesthetised rats. In order to investigate the origin of the detected extracellular glutamate, the influence of several pharmacological compounds is examined. A general discussion and conclusion is presented in chapter 8. The application of the microsensor as an analytical tool is critically evaluated and suggestions on its future application are made.