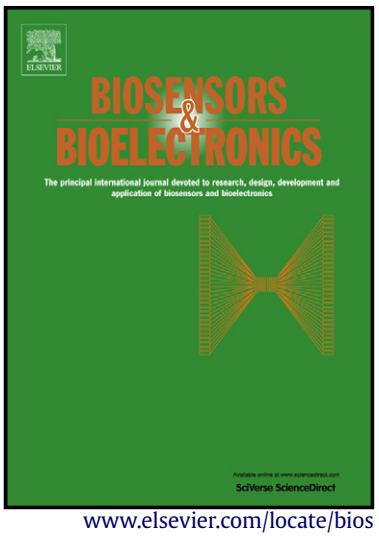

www.elsevier.com/locate/bios

Polymer-based, flexible microsensors for glutamate and lactate for *in vivo* application

Andreas Weltin, Jochen Kieninger, Barbara Enderle, Anne-Kathrin Gellner, Brita Fritsch, Gerald A. Urban

PII: S0956-5663(14)00347-9
DOI: http://dx.doi.org/10.1016/j.bios.2014.05.014
Reference: BIOS6776

To appear in: *Biosensors and Bioelectronics*

Received date: 2 April 2014
Revised date: 2 May 2014
Accepted date: 6 May 2014

Cite this article as: Andreas Weltin, Jochen Kieninger, Barbara Enderle, Anne-Kathrin Gellner, Brita Fritsch, Gerald A. Urban, Polymer-based, flexible microsensors for glutamate and lactate for *in vivo* application, *Biosensors and Bioelectronics,* http://dx.doi.org/10.1016/j.bios.2014.05.014

Polymer-based, flexible microsensors for glutamate and lactate for *in vivo* application

Andreas Weltin[a*], Jochen Kieninger[a], Barbara Enderle[a], Anne-Kathrin Gellner[b], Brita Fritsch[b], Gerald A. Urban[a]

a) Laboratory for Sensors, Department of Microsystems Engineering – IMTEK, University of Freiburg, Germany

b) Department of Neurology, University of Freiburg, Germany

**Corresponding author: Andreas Weltin, Laboratory for Sensors, Georges-Köhler-Allee 103, 79110 Freiburg, Germany, +49 761 203-7263, weltin@imtek.de*

Abstract

We present a flexible microsensor, based on a polymer substrate, for multiparametric, electrochemical *in vivo* monitoring. The sensor strip with a microelectrode array at the tip was designed for insertion into tissue for fast and localized online monitoring of physiological parameters. The microsystem fabrication on a wafer-level is based on a polyimide substrate, includes the patterning of platinum microelectrodes as well as epoxy and dry-film-resist insulation in a cost-effective thin-film and laminate process. A stable, electrodeposited silver/silver chloride reference electrode on-chip and a perm-selective membrane as an efficient interference rejection scheme are integrated on a wafer-level. Amperometric, electrochemical, enzyme-based biosensors for the neurotransmitter L-glutamate and the energy metabolite L-lactate and have been developed. Hydrogel membranes or direct cross-linking as stable concepts for the enzyme immobilization are shown. Sensor performance including high selectivity, tailoring of sensitivity and long-term stability is discussed. For glutamate, a high sensitivity of 2.16 nA mm^{-2} μM^{-2} was found. For lactate, a variation in sensitivity between 2.6 and 32 nA mm^{-2} mM^{-1} was achieved by different membrane compositions. The *in vivo* application in an animal model is demonstrated by glutamate measurements in the brain of rats. Local glutamate alterations in the micromolar range and in nanolitre-range volumes can be detected and quantified with high reproducibility and temporal resolution. A novel, versatile platform for the integration of various electrochemical sensors on a small, flexible sensor strip for a variety of *in vivo* applications is presented.

Keywords: Microsensor; biosensor; glutamate; lactate; *in vivo*; monitoring; brain

1. Introduction

The monitoring of metabolic parameters in living organisms with sensors is found throughout life sciences and plays a central role in personalized medicine, biomedical research and biotechnological applications. Electrochemical sensors allow the continuous, precise and fast *in situ* measurement of metabolic parameters directly inside the tissue. Multiple parameters can be measured in parallel and long-term. L-glutamate is the major excitatory neurotransmitter in the central nervous system and is involved in almost every aspect of normal brain function, its diseases and the signalling with its periphery (Danbolt, 2001; Nedergaard et al., 2002). In the energy metabolism of cells, oxygen is consumed for aerobic metabolism of glucose, and L-lactate is produced during anaerobic metabolism. Energy metabolism is the prerequisite for any cellular function and its alterations play a pathophysiological role, for example during ischemia or the development of cancer (Hanahan and Weinberg, 2011; Kroemer and Pouyssegur, 2008).

By the insertion of microsensors, bio- and chemo-sensors can be placed directly in the area of interest for continuous, online monitoring in extracellular space, *in situ*. Other methods, such as microdialysis and nuclear magnetic resonance (NMR) techniques, suffer from limited temporal or spatial resolution. In microdialysis the analyte has to be pumped outside the tissue to be analyzed ex situ, leading to a time delay usually in the range of typically at least several minutes (Timmerman and Westerink, 1997; Vespa et al., 1998; van der Zeyden et al., 2008). Additionally, the analyte may be diluted in the process, hardly representing the actual levels inside the tissue. NMR methods are not only limited in spatial resolution by voxel size of typically several millimeters, but data acquisition usually takes at least several minutes for a single measurement point (Cai et al., 2012; Shen et al., 1999). Furthermore, only the integral value including intra-cellular, extra-cellular and blood metabolite levels can be measured.

Electrochemical sensors have response times within a few seconds and the analyte is directly converted at the electrodes. Near real-time monitoring of metabolite levels, comparable to the recording of electric potentials, is desired. Microelectrodes allow a high spatial resolution; a small sensor cross-section and arrays of multiple electrodes with precisely defined geometries can be implemented. Furthermore, wafer-level processing allows parallelization of the fabrication. We have demonstrated precise, online microsensor measurement of metabolic parameters in cell cultures (Kieninger et al., 2014; Weltin et al., 2014).

A number of insertion-type electrochemical *in vivo* sensors have been presented, mainly for application inside the brain for the measurement of neurotransmitters, such as glutamate or choline. Modified or capped platinum wires (Hu et al., 1994; Lowry and O'Neill, 1994; Mikeladze et al., 2001) and carbon fibers (Kulagina et al., 1999; Oldenziel et al., 2006; Shram et al., 1998) coated with glass or epoxy for isolation have been used to form microelectrodes. Microfabricated sensors with thin-film electrodes have been fabricated on silicon or ceramic substrates. Ceramic-based multi-site sensors have been used extensively to monitor, among other parameters, glutamate (Burmeister et al., 2000, 2002) and lactate in the brain (Burmeister et al., 2005). Micromachined silicon-based sensors with platinum electrodes and silicon oxide insulation have been introduced (Frey et al., 2010; Tolosa et al., 2013; Wassum et al., 2012). Often, sophisticated dry etching techniques are necessary to form thin, needle-type sensors in silicon. However, rigid sensors run the risk of breaking and cannot bend with

the tissue. Polymer-based technology has been used to fabricate shaft electrodes for electrical recording *in vivo* from polyimide (Metz et al., 2004; Myllymaa et al., 2009; Stieglitz and Gross, 2002), sometimes combined with epoxy layers (Tijero et al., 2009) or epoxy alone (Altuna et al., 2012). Few flexible biosensors for *in vivo* application have been introduced for glucose (Urban et al., 1992) and lactate (Jobst et al., 1996), both based on polyimide. Only a few approaches allow an electrode recess to contain the membrane within the electrode area (Frey et al., 2010).

The utilized biosensors are usually based on the immobilization of an enzyme that converts the analyte and produces hydrogen peroxide, which is then oxidized directly at the electrode (Hu et al., 1994) or converted by another enzyme in a bi-enzyme approach (Heller, 1992). There is variety of methods for enzyme immobilization on electrodes. Commonly, the enzyme is mixed with a crosslinker, e.g. glutaraldehyde, which links it to a supporting matrix, e.g. bovine serum albumin (Hu et al., 1994). Traditional dip coating techniques (Mikeladze et al., 2001; Shram et al., 1998) are less suitable for multi-site microelectrodes due to the limited spatial control of the immobilization and the difficulty to create different sensor types on one platform. Therefore, widely used for microelectrodes are suspending and depositing drops from a needle (Burmeister et al., 2002), polymerization into polymer matrices (Lowry and O'Neill, 1994), encapsulating in hydrogels (Jobst et al., 1996) or electrostatic deposition methods (Strike et al., 1995).

In contrast to silicon or ceramic probes, we present a new flexible microsensor based on a polymer substrate for multiparametric *in vivo* monitoring. The flexibility allows placement in flexible tissue, without the risk of breaking, while being stiff enough for insertion into the brain. Sensor strips were fabricated up to a length of 50 mm, significantly longer than available rigid probes. A new wafer-level fabrication process including a polyimide substrate, platinum micro electrodes, epoxy and dry-film-resist insulation was introduced, resulting in recessed microelectrode arrays. Multiple geometries can be realized and a versatile platform for the integration of different sensors is presented. The wafer-scale processing includes the reference electrode integration and the deposition of a perm-selective membrane for selectivity and cutting of the individual sensors, resulting in a highly parallelized and cost-efficient hybrid fabrication process. Biosensors for glutamate and lactate have been developed and the performance is discussed. Different approaches for precise enzyme immobilization are presented. Multiple layers of thin, geometrically defined membranes and tailoring of the diffusion properties allow the adjustment of sensitivity and linear range over a wide range, according to the application. The *in vivo* application of the sensor in an animal model is shown.

2. Materials and methods

2. 1 Sensor Design

The presented sensor, designed for insertion into tissue without guiding instruments, consists of a polymer strip with two working electrodes at the tip. The core of the sensor was a 50 µm thick polyimide film. A 5 µm thin epoxy insulation and a 38 µm dry-film-resist layer were added on both sides, adding up to a final thickness of approx. 100 µm. The width of the sensor was 500 µm. Alternatively, a 75 µm and 100 µm thick core was also used, increasing the sensor thickness and stiffness. For different areas of application, the length of the sensor was varied by design. Sensors between 5.5 mm and 50 mm length were fabricated. The shorter versions were designed for insertion

into the rat brain, whereas the longer variants were for application in subcutaneous or intramuscular tissue. The short versions are stiff enough for precise placement inside the brain [Fig. 1c] with a stereotaxic setup. For the longer versions, flexibility is a key feature, for placement in flexible and moving tissue. An early version has been introduced previously (Weltin et al., 2013).

The polyimide substrate acts as a mechanically durable yet flexible substrate. It allows the integration of a platinum metallization by vapor deposition and electrochemical modification of the platinum resulting in a micro-rough surface. Platinum was chosen as the electrode material for its well-known electrochemical properties in electrode modification and amperometric measurement. The electrochemical silver deposition for the reference electrode and the electrochemical deposition of a perm-selective membrane to prevent interference are possible.

In the utilized biosensor principle, hydrogen peroxide is produced as an intermediary product by the enzyme and is then oxidized at the working electrode according to Eq. 1 at an appropriate potential. For the glutamate sensors the enzyme glutamate oxidase and for the lactate sensor the enzyme lactate oxidase is immobilized. Both consume the respective analyte together with oxygen and produce hydrogen peroxide according to Eq. 2 and 3.

$$H_2O_2 \rightarrow O_2 + 2\,H^+ + 2\,e^- \quad (1)$$

$$\text{Lactate} + O_2 \rightarrow \text{Pyruvate} + H_2O_2 \quad (2)$$

$$\text{Glutamate} + O_2 + H_2O \rightarrow \text{Oxo-glutarate} + NH_3 + H_2O_2 \quad (3)$$

To reject interferents and thus ensure selectivity, the species of molecules reaching the electrode have to be limited. Hydrogen peroxide should reach the electrode, whereas larger molecules should be rejected. To ensure this, a perm-selective membrane is deposited onto the working electrodes on wafer-level. In comparison to chip-level deposition, efficiency during fabrication is increased, as up to 76 sensors, with two electrodes each, can be modified in parallel, leading to a uniform membrane layer on each electrode due to the self-limiting nature of the process.

The enzyme is immobilized on the electrode in a membrane. Hydrogels on the electrodes can act as a diffusion barrier. A schematic electrode cross-section including the biosensor principle is shown in Fig. 2b.

The two platinum working electrodes are located at the tip of the sensor strip [Fig. 1a, b]. The area is 0.095 mm^2 for each electrode with a distance of 180 µm in between. That allows a localized measurement with high spatial resolution. The reference electrode is placed behind the working electrodes and the counter electrode opens to the side on a large portion of the shaft. On the wafer, all reference electrodes and all working electrodes are connected to allow parallel electrochemical modification. The cutting of the individual chips separates the interconnections. At the end of the shaft, the sensor is widened to give room for the contact pads, which connect the electrodes to the connector.

The first insulation layer provides electrical insulation. A permanent epoxy resist (SU-8 3000) was chosen for its excellent insulation properties over weeks. It can be spin coated as a thin layer of 5 µm on the polyimide film substrate and is thin enough to allow flexibility, while maintaining adhesion. The second layer is used to form wells around the electrodes to allow the immobilization of enzyme- or

other membranes by dispensing a liquid precursor solution, which is then cross-linked to form a stable membrane. The well around the electrode forms a defined volume above the electrode. The well can hold a sufficiently large volume as the volume of the liquid precursor solution may be much higher than the dried and cross-linked membrane.

2.2 Fabrication

Wafer-level fabrication [Fig. 2a] started with the cutting of a copper-clad polyimide sheet, Pyralux AP1535 (DuPont), into wafer shape and removing the copper by etching. The remaining polyimide sheet exhibited a rough surface, was cleaned with deionized water and served as the substrate for the flexible sensor strips.

For metal structuring, a negative lift-off photoresist, ma-N 1420 (Microresist), was spin coated and patterned by lithography with a mask-aligner. Layers of 100 nm platinum as the electrode and conductor material and 100 nm titanium as a protection layer were deposited by physical vapour deposition (PVD). The following lift-off process led to the final structuring of the metal layer.

As an insulation layer, the permanent epoxy resist SU-8 3005 (Microtec) was applied by spin coating at a thickness of 5 µm and patterned by lithography on a mask-aligner. After development, the process was repeated on the back side of the wafer to minimize bending and to achieve symmetrical mechanical properties. The epoxy resist was finally hard baked at 150 °C for 3 h yielding a flat, flexible, stress free, insulated, multi-layer polymer film.

As the second layer, to form the electrode wells for enzyme immobilization, a negative dry-film-resist, Pyralux PC1015 (DuPont), was laminated and structured. The dry-film-resist was laminated with a hot roll laminator and then patterned by photolithography on a mask-aligner. It was then developed and cured at 150 °C for 3 h. This process step was repeated for the back side of the wafer.

After structuring of the dry-film-resist, the titanium protection layer on the platinum electrodes was removed by etching with hydrofluoric acid, where the platinum acts as the etch-stop layer. Hereby, a clean platinum electrode showing the typical electrochemical characteristics was revealed.

2.3 Reference electrode modification

Wafer-level electrodeposition was used to integrate the silver/silver chloride (Ag/AgCl) reference electrode on-chip. Silver was cathodically electrodeposited from Arguna S (Umicore) solution by applying a constant current density of -16 mA cm^{-2} vs. a silver wire counter electrode. By anodic oxidation at 1.6 mA cm^{-2} in 0.1 M KCl solution, the silver was then partially converted to silver chloride, resulting the in the desired pseudo-reference electrode of approx. 10 µm thickness.

2.4 Interference rejection

As a perm-selective membrane on the biosensor electrodes, which prevents interfering molecules from being oxidized at the electrode, a layer of 1,3-diaminobenzene was deposited by electropolymerization (Geise et al., 1991). Then, deposition was preformed from a 3 mM 1,3-diaminobenzene (DAB) solution in phosphate buffer electrolyte by cyclic voltammetry in a 3-electrode setup. The potential was scanned between 0.2 V and 0.9 V vs. Ag/AgCl (3 M KCl) at 2 mV/s scan speed. Platinum was used as the counter electrode. This process led to the formation of a homogenous DAB polymer layer due to the self-limiting nature of the process on all working

electrodes on the entire wafer in a single process step. The electrodes were modified, e.g. by enzyme immobilization and membrane deposition, afterwards.

2.5 Cutting

The individual sensors were cut from the wafer with a swivelling drag knife on a CNC-machine. This method allows the precise cutting through the polymer for almost any geometry. All individual sensors can be cut from one wafer in a single automated process step. In contrast to etching, dicing or laser cutting it requires no expensive equipment or additional process steps, such as the photo-patterning of structures or cleaning steps. More importantly, the process is performed at ambient conditions without any cooling fluids, large amounts of friction, heat transfer or debris production. In this way, the electrodes and electrode modifications are not exposed to any harmful conditions, such as heat or humidity. Even the enzyme immobilization can be done prior to cutting, yielding fully functional sensors after the process. The method also allows rapid prototyping, where geometries can be varied between sensors and sensors can be conveniently cut from the wafer in multiple steps.

2.6 Electrical interfacing

The platinum contact pads are designed for a regular flexible cable connector. No time consuming and costly soldering or bonding post-processing is necessary. Sensors can be removed from the electrical connection at any time. If the connection of the sensor to the connector needs to be sealed against its environment, it can be encapsulated by medical grade epoxy glue, EPOTEK 302−M (Epoxy Technology). The cable from the connector can be connected directly to a potentiostat.

2.7 Chemicals

Glutamate oxidase, bovine serum albumin (BSA), glutamate sodium salt, lactate monosodium salt and gentamicin were purchased from Sigma-Aldrich. Lactate oxidase was from Genzyme. Glutaraldehyde (50%) was from Fluka. Ascorbic acid was from Alfa Aesar, uric acid, dopamine hydrochloride and hydrogen peroxide (30%) were from Merck. 0.1 M phosphate buffered saline (PBS), pH 7.4, 0.1 M NaCl, was self-made from deionized water.

2.8 Enzyme and membrane immobilization

To immobilize the glutamate oxidase enzyme highly concentrated in a thin layer on the electrodes the enzyme was mixed with the protein bovine serum albumin (BSA) and then cross-linked by glutaraldehyde. One unit of glutamate oxidase was dissolved in 10 µl PBS containing 1 wt.% BSA using magnetic stirring. The solution was then supplied with 0.15% v./v. glutaraldehyde immediately before dispensing onto the electrodes. A higher concentration of glutamate oxidase was found to increase viscosity too much. The mixture was dispensed onto the electrode into the volume defined by the dry film resist well with a CNC-based dispenser. The dispensed volume per step was around 5 nl. The dispensing step was repeated until the desired amount of enzyme was immobilized. By the defined volume and the number of repetitions the amount of enzyme immobilized on each electrode can be controlled tightly. The sensitivity increases with the number of repetitions. However, the response time decreases as the membrane becomes thicker. Three to five dispensing steps per electrode were usually applied. For the blank electrode, the procedure was the same, only without the

enzyme. The final membranes were left to dry for at least 2 h at room temperature to complete crosslinking by the glutaraldehyde.

For the lactate sensors the enzyme was immobilized in a poly(hydroxyethyl methacrylate)-based (pHEMA) hydrogel. It stabilizes the enzyme for long-term measurements and acts as a diffusion barrier. The enzyme was mixed into a precursor solution with monomers, plasticizer and UV-cross-linker as described previously (Moser et al., 2002) . The precursor solution was then dispensed as a liquid with a CNC-based dispenser. Subsequent crosslinking by UV-light formed a stable membrane. A pHEMA hydrogel without the enzyme was dispensed onto the blank electrode. If desired, the blank membrane can also be dispensed on top of the existing enzyme membrane to act as an additional diffusion limiting layer. The pHEMA hydrogel was also dispensed on the reference electrodes. The immobilization processes are fast and precise and can also be performed manually with low-cost equipment.

2.9 *In vitro* characterization setup

The electrode characterization and biosensor calibration was performed by adding the respective dissolved analytes to PBS. All experiments were performed at 37 °C in a stirred 50 ml container. For long-term measurements 1 mg/ml of the antibiotic gentamicin was added, which showed no effect on the sensor performance. Potentiostats such as the CompactStat (Ivium Technologies) or a 4-channel potentiostat (Jobst Technologies) were used for measurement. All amperometric measurements were performed at a potential of 450 mV vs. the on-chip reference electrode in a 3-electrode setup. The signal of the blank electrode can be subtracted in order to eliminate the unspecific background. For oxygen dependency measurements, the dissolved oxygen level was adjusted with a gas-mixing station.

2.10 *In vivo* measurement setup

The *in vivo* application and performance of the sensor system was tested by intracerebal measurements *in vivo* in rats. Experiments involving animals were approved by the regional council and were performed in accordance with the respective state, federal and institutional regulations, including the guidelines of the most recent European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

Male Sprague Dawley rats, aged 9−11 weeks, were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg), until loss of the toe pinch reflex, which was controlled frequently. Anesthesia was reapplied throughout the experiment if needed. Body temperature was held constant at 37 °C during the entire procedure by a closed-loop temperature controller.

A 0.2 mm diameter stainless steel cannula (Plastics One) was permanently fixed to the shaft of the sensor with the opening placed exactly between the two electrodes at a distance of about 150 µm to the side to allow injection of glutamate. The setup was calibrated *in vitro* directly prior to the *in vivo* measurement, stored dry and then directly applied *in vivo*. All measurements were performed in a 3-electrode setup using the on-chip reference and counter electrodes.

After preparation of the skull, the sensor/cannula setup was stereotaxically placed in the neocortex (from bregma: −0.5 mm AP and −2.0 mm ML; −2.3 mm DV; at an angle of 59°) reaching a motorcortical depth of 1.7 mm.

3 Experimental results and discussion

3.1 Basic electrode characterization

The platinum electrodes on the micro-rough polyimide substrate were characterized electrochemically. Cyclic voltammetry was performed and the working point for the hydrogen peroxide oxidation was determined, so that the electrode reaction is only diffusion limited. For potentials higher than 450 mV vs. Ag/AgCl (0.1 M Cl^-) no increase in sensitivity was found. In the biosensor principle, hydrogen peroxide is the intermediary product produced by the enzymes and is then oxidized at the working electrode. Therefore, the amperometric biosensor measurements were performed at a potential, lower than typically found in literature (Burmeister et al., 2005; Frey et al., 2010), resulting in a low background current and enhanced selectivity.

The sensitivity for hydrogen peroxide at the bare platinum electrode was 8.92 nA mm^{-2} μM^{-1} ± 0.03 nA mm^{-2} μM^{-1} (n = 8) at 100 µM. Linearity of the hydrogen peroxide oxidation was found up to a concentration of 1 mM. With the electropolymer as the interference rejection layer, the sensitivity was reduced to 3.80 nA mm^{-2} μM^{-1} ± 0.09 µA cm^{-2} mM^{-1} (n = 12). The sensitivity was higher than found in all biosensors, indicating that the sensitivity of the biosensors was not limited by hydrogen peroxide conversion. The applied fabrication process yielded very reproducible electrode properties.

The reference electrode stability was characterized by long-term exposure to 0.1 M PBS at 37 °C. Electrodes were stored continuously in the solution for two weeks and no potential difference or significant drift vs. a chloridated silver wire was measured. The used electrodeposited reference electrode in combination with the pHEMA hydrogel coating formed a long-term stable pseudo-reference electrode, also under physiological conditions.

3.2 Interference rejection

The wafer-level coating of the working electrodes with a perm-selective membrane is the key to reject interfering substances at the electrodes. The selectivity can be defined as the ratio of the sensitivity of the analyte to the sensitivity found for the direct oxidation of the interferent at the electrode. A high ratio is desired, where most of the measured signal comes from the analyte. These ratios were determined by adding known biogenic interferents, such as ascorbic acid, dopamine or uric acid and measuring the resulting sensitivity at the biosensor electrode.

For the glutamate sensor, the demand is certainly the highest, as the enzyme membrane is thinnest. Thus, the diffusion of the interferents through the membrane is highest and they could potentially create the highest current. For ascorbic acid a ratio of 143 (n = 12), 52 for uric acid (n = 8) and 20 (n = 12) for dopamine was found for the highest reproducible glutamate sensitivity. For the lactate sensors the ratios are always higher, since the diffusion of the interferents through the hydrogel is slower than the diffusion of lactate. The use of a blank electrode and the subtraction of its signal additionally enhance selectivity. Since the diffusion and electrochemical properties for the blank electrode are the same as for the working electrode [Figs. 3a, 4a], cross-sensitivity is practically eliminated.

The membrane deposition process and initial exposure to hydrogen peroxide may deteriorate the semi-permeable membrane slightly. Therefore, the above mentioned ratios were always determined

measuring the analyte (e.g. glutamate) first and then the interferent. For unmodified electrodes with only the semi-permeable membrane used directly after fabrication, even higher rejection ratios in the range of 1000 were determined. During long-term biosensor measurements over several days, no significant change of the interference rejection properties could be found. Sometimes, even a slight improvement could be observed.

It was demonstrated that the implemented method of interference rejection yields excellent results for typical biogenic interferents. High and stable selectivity ratios mean a drastic reduction in cross-sensitivity of the biosensors, well suitable for application in biological environments. Furthermore, the wafer-level electrode modification before the sensor integration is a highly efficient, parallelized fabrication method, in contrast to chip-level modification.

3.3 Glutamate measurements

For the glutamate sensor, the highest stable and reproducible sensitivity was found at 2.16 nA mm^{-2} μM^{-1} ± 0.08 nA mm^{-2} μM^{-1} (n = 10). Linearity was found up to 150 µM (R^2 = 0.999). A detection limit (3 σ) of 220 nM was achieved. The rise time (0−95%) was 4.9 ± 1.9 s (n = 9) for a step of 10 µM glutamate.

The raw signal of a transient glutamate measurement for three sensors and a blank electrode is shown in Fig. 3a. Glutamate was added successively, and a fast, selective and linear response was demonstrated [Fig. 3b]. Afterwards, the interferent ascorbic acid (AA) was added. It was shown that the sensitivity for the glutamate is magnitudes higher (here approx. 95 times) than that for the interferent. It can be observed that the blank electrode has the same signal for AA. Thus, by subtraction of the blank signal, selectivity is enhanced even further.

The sensor stability was evaluated in continuous long-term measurements. The long-term exposure of the enzyme to the analyte and thus exposure the hydrogen peroxide presents a significantly different environment for the enzyme than storing the sensor in PBS and performing calibrations on different days. A mild loss in sensitivity during continuous operation is expected for this immobilization method. Over four days continuous operation at 37 °C, we found a drift in sensitivity of around −5 % per day (n = 9), for a relatively high concentration of 50 µM glutamate [Fig. 5a].

The sensors were introduced to measurement as short as 2 hours after fabrication. A longer time for crosslinking and drying was found to have no effect on sensitivity or long-term stability. Before the first measurement the sensors were stored dry at 4 °C together with a desiccant. No performance difference for dry storage up to 4 weeks could be observed. After the first measurement it is recommended to store the sensors in PBS, as repeated drying/hydrating cycles of the membrane increases the risk of crack formation.

The sensors were stored in PBS up to 4 weeks. For up to 10 days in PBS, no measurable loss in sensitivity was found. After 28 days more than 75% of the sensitivity remains. It was hereby demonstrated that the presented method results in a stable immobilization of the enzyme with a storage capability of the sensor that meets the demands of the desired application.

Oxygen dependency was investigated, since the oxygen levels in tissue are usually lower than air saturation. At dissolved oxygen levels reduced to 25% (5.23 vol.%) of the atmospheric level (20.95 vol.%), the signal remains at over 90% compared to air saturation, for a relatively high

concentration of 50 µM glutamate. For an even higher glutamate level of 250 µM, still 75% of the signal remains.

We have developed a highly sensitive, fast and reproducible glutamate sensor. It is well suitable for measuring physiological glutamate levels in the micromolar range, found e.g. in the extracellular space in the brain.

3.4 Lactate measurements

Lactate sensors consisted of an enzyme membrane covered with an optional diffusion limiting membrane of variable thickness. For a single membrane containing lactate oxidase only, sensitivity up to 32 nA mm^{-2} mM^{-1} and a detection limit (3 σ) of 2 µM could be achieved [Fig. 4b]. The minimal thickness of the membrane was only limited by its viscosity during immobilization as a liquid, which prevented the coating of the entire electrode if too little volume was dispensed.

The linear range is limited, due to the limitation in oxygen supply through the membrane. Adding a diffusion limiting membrane decreases the sensitivity but increases the linear range, since the oxygen diffusion in the membrane is significantly higher than the lactate diffusion. Thus, by adding diffusion limiting membranes of various thicknesses, or by stacking two of these membranes, the linear range of the lactate sensor can be adjusted over a wide range according to the application.

A transient measurement for successive additions of lactate in PBS is shown in Fig. 4a for two sensors with different membrane thickness. A total concentration of 1.5 mM ascorbic acid was added afterwards, showing no influence on the signal. A Calibration for three sets of sensors with different sensitivity is shown in Fig. 4b, demonstrating the variation of sensitivity. A linear range up to 10 mM was achieved. The rise time (0−95%) was always less than 15 s.

For long-term measurements the analyte was continuously measured at concentrations up to 2 mM and at 37 °C to account for the actual monitoring application. A signal drift of less than −1% per day (n = 7) was observed over up to 10 days continuous measurement [Fig. 5b]. The lactate sensors can be repeatedly (> 10) applied in measurement and stored dry in between without measurable change in performance.

Oxygen dependency measurements showed that at oxygen levels reduced to 10% (2.1 vol.%) of the atmospheric level (20.95 vol.%), more than 90% of the signal remained, for a lactate concentration of 1 mM.

We developed a highly selective, long-term stable lactate sensor, which can measure physiological lactate concentrations over days. The sensors can be applied also in hypoxic environment with low local oxygen concentrations. The sensitivity can be adjusted over a wide range according to the desired application by different diffusion limiting membrane layers.

3.5 *In vivo* measurements

After sensor placement in the rat cortex, a stable baseline was established before different volumes (50 to 200 nl) of 200 mM glutamate in 0.1 M PBS were injected using a precision syringe pump (Harvard) with a 10 µl syringe (Hamilton) at a pump rate of 500 nl per minute. The interval between the injections was 90 s. The resulting transient signal is shown in Fig. 6, where the blank signal has been subtracted. As expected for physiological values, the background concentration of extracellular glutamate was in the lower micromolar range and stable. The glutamate injections were clearly visible,

and the recorded signal responds quickly. From *in vitro* calibration it can be assumed that the peak height was not limited by sensor saturation. Peak height and area were very reproducible and correlated with the applied volume. The local glutamate concentration increased temporarily due to the injection and also decreased rapidly. In the *in vivo* situation this rapid decrease is not only explained by diffusion of glutamate, but also by additional extremely efficient active uptake through glutamate transporters on neuronal and glial cells (Danbolt, 2001; Nedergaard et al., 2002). This is reflected by a lack of accumulation of glutamate across the experiment, since the signal returned to a low baseline between the injections. The range of the measured concentrations in relation to the injected concentration is in good agreement with earlier reports (Burmeister et al., 2000; Frey et al., 2010).

The sensor was successfully applied *in vivo*. It was demonstrated that alterations of the local glutamate level in the micromolar range and in nanolitre-range volumes can be detected and quantified with high reproducibility and temporal resolution. The developed sensor platform is suitable for recording and online monitoring of extracellular analyte levels, such as in the mammalian cortex in an animal model.

4. Conclusion

We have developed a versatile microsensor platform for monitoring neurotransmitters and energy metabolism in the *in vivo* application. In contrast to systems made from silicon or ceramic, the fully flexible sensor strip is based on a polymer substrate and can be fabricated in multiple geometries. The possibilities of placement, positioning and field of application of a microsensor array in a variety of tissues are hereby enhanced. The designed microsystem was fabricated in an efficient wafer-scale hybrid process of thin-film and laminate technology, including the integration of a reference electrode and an interference rejection scheme. Electrochemical microsensors have been developed and integrated on the flexible platform. Enzymes have been immobilized to create biosensors for glutamate and lactate. Precise and reproducible immobilization strategies and a method to reject biogenic interferents have been demonstrated, as well as the tailoring of sensitivity according to the application. Highly selective, sensitive and long-term stable sensor performance from a localized microelectrode array has been demonstrated. The *in vivo* application in an animal model was demonstrated by measuring the neurotransmitter glutamate in the cortex of the rat brain. In the future, more *in vivo* applications and the integration of other sensor types are planned. We have introduced a new microsensor platform for the continuous monitoring of metabolic parameters inside the tissue for biomedical research *in vivo*.

5. Acknowledgement

Funding by the Federal Ministry of Education and Research (BMBF) within the Bernstein Focus Neurotechnology (BFNT) Freiburg*Tübingen (FKZ 01GQ0830) is gratefully acknowledged.

6. References

Altuna, A., Menendez de la Prida, L., Bellistri, E., Gabriel, G., Guimerá, A., Berganzo, J., Villa, R., Fernández, L.J., 2012. Biosens. Bioelectron. 37, 1–5.

Burmeister, J.J., Moxon, K., Gerhardt, G.A., 2000. Anal. Chem. 72, 187–192.

Burmeister, J.J., Palmer, M., Gerhardt, G.A., 2005. Biosens. Bioelectron. 20, 1772–1779.

Burmeister, J.J., Pomerleau, F., Palmer, M., Day, B.K., Huettl, P., Gerhardt, G.A., 2002. J. Neurosci. Methods 119, 163–171.

Cai, K., Haris, M., Singh, A., Kogan, F., Greenberg, J.H., Hariharan, H., Detre, J.A., Reddy, R., 2012. Nat. Med. 18, 302–306.

Danbolt, N.C., 2001. Prog. Neurobiol. 65, 1–105.

Frey, O., Holtzman, T., McNamara, R.M., Theobald, D.E.H., van der Wal, P.D., de Rooij, N.F., Dalley, J.W., Koudelka-Hep, M., 2010. Biosens. Bioelectron. 26, 477–484.

Geise, R.J., Adams, J.M., Barone, N.J., Yacynych, A.M., 1991. Biosens. Bioelectron. 6, 151–160.

Hanahan, D., Weinberg, R.A., 2011. Cell 144, 646–674.

Heller, A., 1992. J. Phys. Chem 96, 3579–3587.

Hu, Y., Mitchell, K.M., Albahadily, F.N., Michaelis, E.K., Wilson, G.S., 1994. Brain Res. 659, 117–125.

Jobst, G., Moser, I., Varahram, M., Svasek, P., Aschauer, E., Trajanoski, Z., Wach, P., Kotanko, P., Skrabal, F., Urban, G., 1996. Anal. Chem. 68, 3173–3179.

Kieninger, J., Aravindalochanan, K., Sandvik, J.A., Pettersen, E.O., Urban, G.A., 2014. Cell Prolif. 47, 180–188.

Kroemer, G., Pouyssegur, J., 2008. Cancer Cell 13, 472–482.

Kulagina, N. V, Shankar, L., Michael, A.C., 1999. Anal. Chem. 71, 5093–5100.

Lowry, J.P., O'Neill, R.D., 1994. Electroanal. 6, 369–379.

Metz, S., Bertsch, A., Bertrand, D., Renaud, P., 2004. Biosens. Bioelectron. 19, 1309–1318.

Mikeladze, E., Schulte, A., Mosbach, M., Blöchl, A., Csöregi, E., Solomonia, R., Schuhmann, W., 2001. Electroanal. 14, 393–399.

Moser, I., Jobst, G., Urban, G.A., 2002. Biosens. Bioelectron. 17, 297–302.

Myllymaa, S., Myllymaa, K., Korhonen, H., Töyräs, J., Jääskeläinen, J.E., Djupsund, K., Tanila, H., Lappalainen, R., 2009. Biosens. Bioelectron. 24, 3067–3072.

Nedergaard, M., Takano, T., Hansen, A.J., 2002. Nat. Rev. Neurosci. 3, 748–755.

Oldenziel, W.H., Dijkstra, G., Cremers, T.I.F.H., Westerink, B.H.C., 2006. Brain Res. 1118, 34–42.

Shen, J., Petersen, K.F., Behar, K.L., Brown, P., Nixon, T.W., Mason, G.F., Petroff, O.A., Shulman, G.I., Shulman, R.G., Rothman, D.L., 1999. Proc. Natl. Acad. Sci. 96, 8235–8240.

Shram, N.F., Netchiporouk, L.I., Martelet, C., Jaffrezic-Renault, N., Bonnet, C., Cespuglio, R., 1998. Anal. Chem. 70, 2618–2622.

Stieglitz, T., Gross, M., 2002. Sens. Actuat. B Chem. 83, 8–14.

Strike, D.J., de Rooij, N.F., Koudelka-Hep, M., 1995. Biosens. Bioelectron. 10, 61–66.

Tijero, M., Gabriel, G., Caro, J., Altuna, A., Hernández, R., Villa, R., Berganzo, J., Blanco, F.J., Salido, R., Fernández, L.J., 2009. Biosens. Bioelectron. 24, 2410–2416.

Timmerman, W., Westerink, B.H., 1997. Synapse 27, 242–261.

Tolosa, V.M., Wassum, K.M., Maidment, N.T., Monbouquette, H.G., 2013. Biosens. Bioelectron. 42, 256–260.

Urban, G., Jobst, G., Keplinger, F., Aschauer, E., Tilado, O., Fasching, R., Kohl, F., 1992. Biosens. Bioelectron. 7, 733–739.

Vespa, P., Prins, M., Ronne-Engstrom, E., Caron, M., Shalmon, E., Hovda, D.A., Martin, N.A., Becker, D.P., 1998. J. Neurosurg. 89, 971–982.

Wassum, K.M., Tolosa, V.M., Tseng, T.C., Balleine, B.W., Monbouquette, H.G., Maidment, N.T., 2012. J. Neurosci. 32, 2734–2746.

Weltin, A., Enderle, B., Kieninger, J., Urban, G.A., 2013. Proc IEEE Sensors.

Weltin, A., Slotwinski, K., Kieninger, J., Moser, I., Jobst, G., Wego, M., Ehret, R., Urban, G.A., 2014. Lab Chip 14, 138–146.

Van der Zeyden, M., Oldenziel, W.H., Rea, K., Cremers, T.I., Westerink, B.H., 2008. Pharmacol. Biochem. Behav. 90, 135–147.

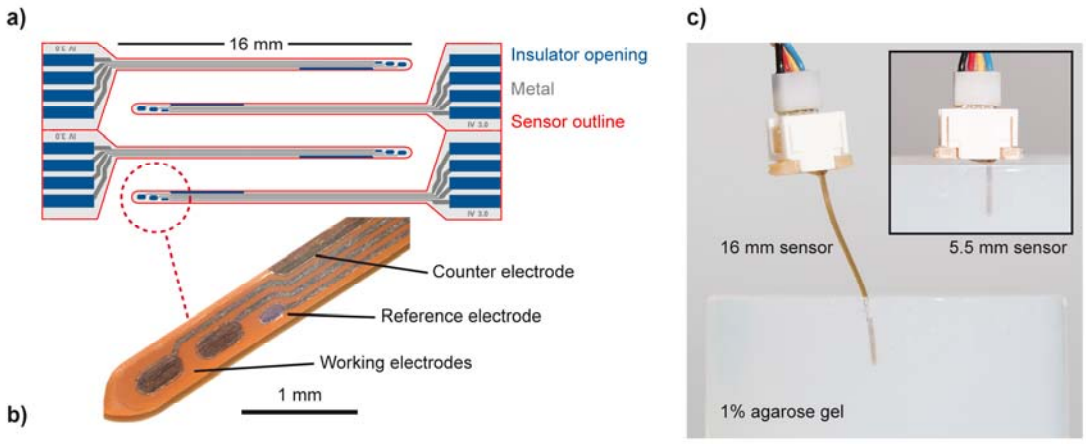

Fig. 1: a) Drawing of sensor layout as found on the wafer with sensor outline, metal layer and insulator opening.
b) Photograph of sensor tip with microelectrodes including working, reference and counter electrodes.
c) Photograph of connected sensors inserted in 1% agarose gel showing flexibility and precise placement.

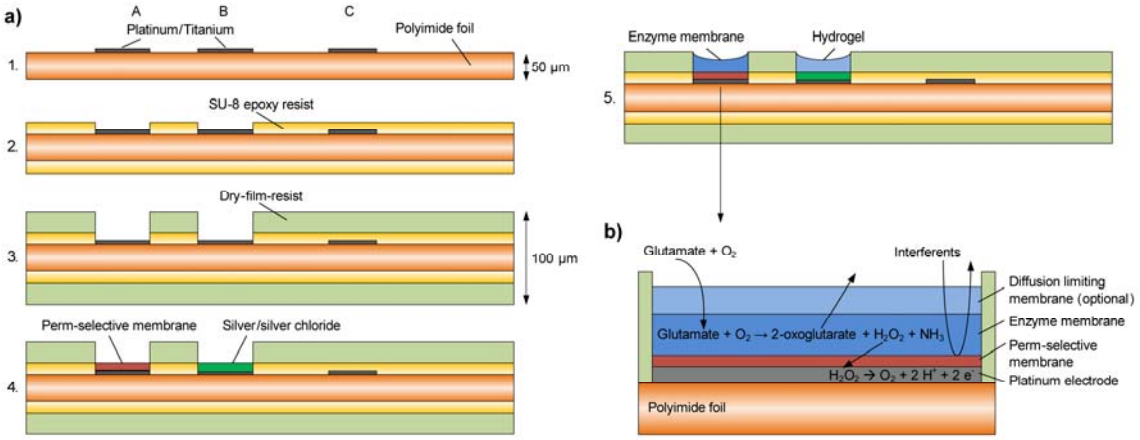

Fig. 2: a) Cross-section of the fabrication process for working electrode (A), reference electrode (B), and conductor (C): 1. Metallization. 2. Insulation with permanent epoxy resist. 3. Insulation and forming of electrode wells with dry-film-resist. 4. Electrode modification for working electrode and reference electrode. 5. Membrane integration modification for working electrode and reference electrode.
b) Cross-section of detection principle and membrane setup for the biosensors with glutamate as an example.

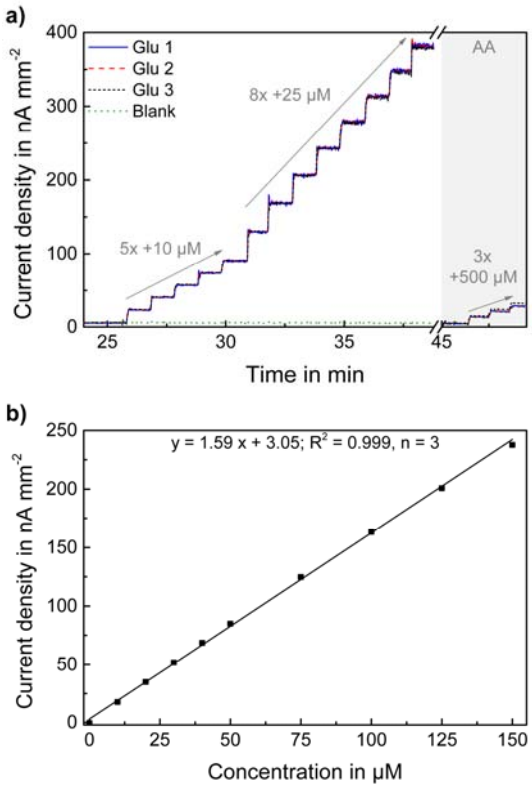

Fig. 3: a) Transient raw signal of the parallel glutamate calibration in PBS of three sensors including the blank signal. The interferent ascorbic acid (AA) was added at the end. b) Corresponding glutamate calibration with linear fit.

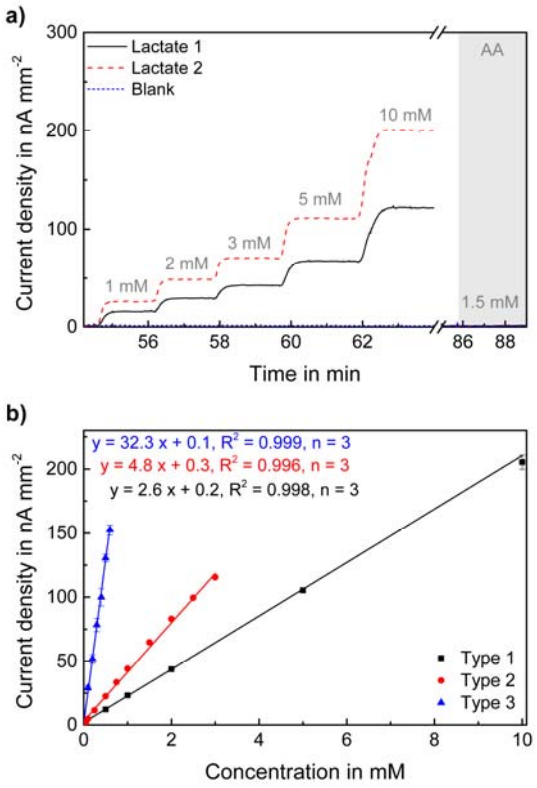

Fig. 4: a) Transient raw signal for a lactate calibration in PBS of two sensors with different diffusion limitation. The interferent ascorbic acid (AA) was added at the end. b) Calibration of three sets of lactate sensors, each with different sensitivity and linear range.

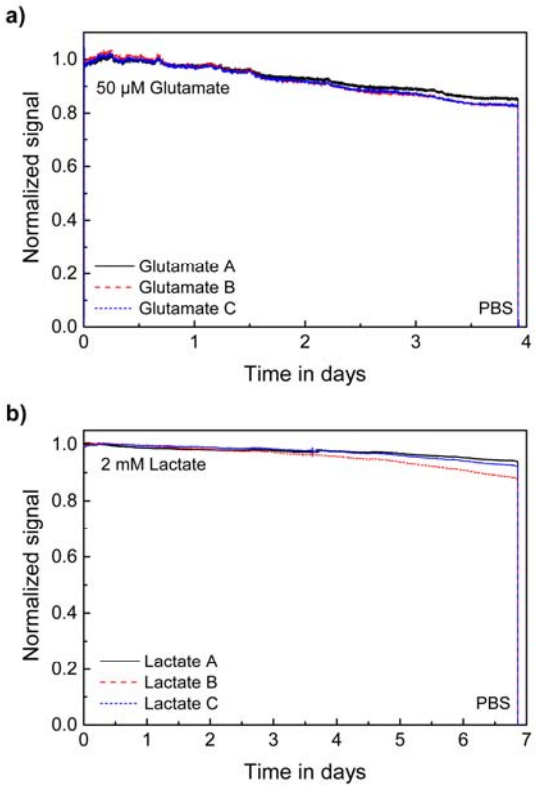

Fig. 5: a) Long-term transient raw signal (blank signal subtracted) for 50 µM glutamate in PBS during continuous measurement at 37 °C.
b) Long-term transient raw signal (blank signal subtracted) for 2 mM lactate in PBS during continuous measurement at 37 °C.

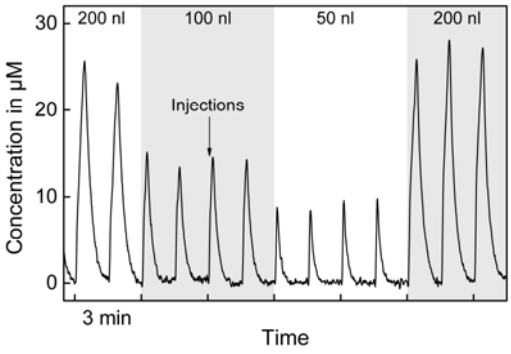

Fig. 6: ***In vivo*** **glutamate measurement of multiple glutamate (200 mM) injections of different volume (50−200 nl each) into the cortex of rats.**

- Flexible, polymer-based electrochemical sensor platform for in vivo application.
- Wafer-level fabrication in thin-film and epoxy/dry-film laminate technology.
- Long-term stable biosensors for lactate with tailorable sensitivity.
- Highly sensitive glutamate sensors with excellent selectivity and linearity.
- Precise, fast and low volume in vivo glutamate measurement in the brain of rats.