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A high sensitivity MEA probe for measuring real time rat brain glucose flux

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ABSTRACT

The mammalian central nervous system (CNS) relies on a constant supply of external glucose for its undisturbed operation. This article presents an implantable Multi-Electrode Array (MEA) probe for brain glucose measurement. The MEA was implemented on Silicon-On-Insulator (SOI) wafer using Micro-Electro-Mechanical-Systems (MEMS) methods. There were 16 platinum recording sites on the probe and enzyme glucose oxidase (GOx) was immobilized on them. The glucose sensitivity of the MEA probe was as high as 489 $\mu A\,mM^{-1}\,cm^{-2}$. 1,3-Phenylenediamine (mPD) was electropolymerized onto the Pt recording surfaces to prevent larger molecules such as ascorbic acid (AA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and dopamine (DA) from reaching the recording sites surface. The MEA probe was implanted in the anesthetized rat striatum and responded to glucose levels which were altered by intraperitoneal injection of glucose and insulin. After the in vivo experiment, the MEA probe still kept sensitivity to glucose, these suggested that the MEA probe was reliable for glucose monitoring in brain extracellular fluid (ECF).

1. Introduction

Neuronal activity depends on ion gradients which are maintained by energy-requiring active transport. For this the brain requires a constant supply of glucose and oxygen, which were delivered by the cerebral circulation (Lowry and Fillenz, 1997). Glucose is a main nutrient in the brain and the well-documented effects of circulating glucose on cognitive function has suggested that glucose acts on brain systems important for memory formation (Dong et al., 2003). Understanding the glucose mechanisms, which underlies the functioning of the human brain, stands at the forefront of modern science.

Recently, different studies have been reported on glucose concentration detection in extracellular fluid (ECF) (Lowry et al., 1994, 1998a, 1998b, 1998c; Zilkha et al., 1994; Zhang et al., 2004; Fillenz and Lowry, 1998; Yang et al., 1995a; Yu et al., 2013; Vasylieva et al., 2011a; Shi et al., 2000) or on the relationship between ECF glucose and neuronal activity (Sanduskya et al., 2013; Fellows et al., 1992; Silver and EreciAska, 1994; Netchiporouk et al., 1996). Among these works, microdialysis has become one of the standard and widely accepted methods for in vivo monitoring (Sanduskya et al., 2013; Obrenovitch and Zilkha, 2001; Zhang et al., 2004; Yang et al., 1995a; Yu et al., 2013; Shi et al., 2000). However, this method is generally coupled to offline analysis by high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) or enzymatic electrode (Woitzik et al., 2001; Zhou et al., 1999; Malone et al., 1995; Zhang et al., 2004), and limited by its poor temporal resolution for most collection methods limited to the range of minutes. Though higher temporal resolution is possible with recent advances in dialysate collection and analysis (Morales-Villagrán et al., 2012), the real time data acquisition made by microdialysis for different brain region at the same time on-line analysis is not currently possible (Lowry et al., 1998a, 1998b, 1998c).

In comparison with microdialysis, implanted enzymatic electrode possesses simplicity of operation. Recent advances in biocompatible nano-materials and biotechnology make it possible to develop a biosensor with high sensitivity to detect the neurotransmitter and metabolites (Fillenz and Lowry, 1998; Heller and Feldman, 2008; Dale et al., 2005), and also be able to rapidly detect changes in cerebral glucose (Boutelle et al., 1986; Shram et al., 1997; Vasylieva et al., 2011b). Furthermore the electrochemical sensors are small, so that they can record at a sub-second temporal resolution and cause less disruption in surrounding tissue compared to microdialysis guide cannula and probes (Bungay et al., 2003; Borland et al., 2005). The high specificity and temporal resolution of sensors make them ideal for measuring the relationships between neurochemistry, metabolism and neural activity (Lowry and O'Neill, 2006).

Most of the implanted assays were carried out by using a carbon fiber (Netchiporouk et al., 1996; Harada et al., 1992;

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Michael and Adrian, 1995) or metal wire (Lowry et al., 1998b; Silver and ErreciAska, 1994; Fillenz and Lowry, 1998; Pernot et al., 2008; Vasylieva et al., 2011a) microelectrode based on the enzymatic method. In contrast to the manual construction of the micro-wire based arrays, the silicon-based microelectrodes are in mass production and also in a wide variety of shapes and sizes with reproducible submicron precision and with low restrictions in their electrode contact configuration (Grand et al., 2011). These structures are biocompatible with the brain tissue (Kotzar et al., 2002). Since the 1970s, a wide variety of probes have been constructed by using different silicon fabrication methods (Kotzar et al., 2002; Anderson et al., 1989; Bai et al., 2000; Campbell et al., 1991; Ghovanloo et al., 2003; Najafi et al., 1985; Rakwal et al., 2009; Wise et al., 1970). However, few of these were used as glucose biosensor.

In the present study, a novel implantable Multi-Electrode Array (MEA) probe made by MEMS methods was designed for continuously monitoring changes in ECF glucose concentrations. Calibration results showed the MEA probe had high sensitivity and good selectivity. Measurements were made in anesthetized rat brain when plasma glucose level was normal and altered by an injection of insulin or glucose.

2. Experimental

2.1. Reagents

Glucose oxidase (GOx, 233 U/mg) was purchased from Aspergillus niger in powder form, D-glucose (≥ 99.5%) and insulin from Sigma; ascorbic acid (AA, ≥ 99%), 3,4-dihydroxyphenylacetic acid (DOPAC, ≥ 98%) and serotonin (5-HT, 99%) from Alfa Aesar; dopamine (DA, ≥ 99%) from Acros Organics. Saline (0.9% NaCl) was purchased from Shuanghe company (Beijing, China), chloral hydrate from Sinopharm Chemical Reagent Co., Ltd. 1,3-Phenylenediamine (mPD, ≥ 99%) was purchased from Aldrich, bovine serum albumin (BSA, ≥ 99%) from Amresco, glutaraldehyde solution (25%) from Shanghai Chemical Reagent Company (Shanghai, China). The phosphate buffer saline (PBS, 0.1 M Na_2HPO_4–NaH_2PO_4–KCl, pH 7.4) was prepared from a PBS tablet (Sigma) with deionized water.

For in vitro calibrations, stock solutions of 1 M glucose were prepared, left for 24 h at room temperature to allow equilibration of the anomers and then stored at 4 °C. DOPAC, DA, 5-HT and AA solutions were prepared in a phosphate buffered saline (PBS) solution, 0.1 M, pH 7.4 just before use because of their gradual decomposition.

For the in vivo experiments, solutions of insulin (1 ml, 15 U/kg) were administered intraperitoneally (i.p.). Insulin solutions were prepared in normal saline and sonicated with heating for 5 min to ensure complete dissolution. 1 M glucose was prepared in normal saline.

2.2. Apparatus

All electrochemical measurements were performed on a Gamry electrochemical workstation (Gamry Reference 600, Gamry Instruments, USA). A Dell E5400 notebook PC was used to collect electrochemical data.

2.3. Fabrication of the MEA probe

Main steps of the MEA probe fabrication were referred to the paper (Wei et al., 2013). Each MEA probe contains four shafts capable of penetrating the brain and a bonding pads area which connects to external devices. The probe has 16 round recording sites (diameter of 15 μm), four on each shank. The distance between shanks is 200 μm. Every shank is 7 mm long or longer and 38 μm thick. Along the shank, the center to center distance between recording sites is 100 μm. After being assembled to a soft printed circuit board (PCB) holder the microelectrodes is applicable for recordings in rats.

2.4. Enzyme immobilization

The MEA probe was cleaned by reactive-ion etching first. The final enzyme solution used for biosensor preparation contained 0.5 U/μL glucose oxidase, 1% bovine serum albumin, and 0.125% glutaraldehyde in phosphate buffered saline solution (0.01 M, pH 7.4). Glutaraldehyde is one of the most widely used fixative reagents for enzymatic biosensor fabrication (Vasylieva et al., 2011a) and is easy to operate. The GOx layer was deposited by dipping the tip of the MEA probe in the enzyme solution. The electrodes were placed at room temperature for at least 72 h before their first use. The modified electrodes were stored at 4 °C when not in use.

2.5. Modification by 1,3-phenylenediamine

For in vivo experiments, mPD layer was deposited by electropolymerization for 15 min in 0.5 mM mPD solution in 0.1 mM PBS at pH 7.4 under a constant potential of +600 mV versus an Ag|AgCl reference electrode. At 15 min, the microelectrode tip was removed from the mPD solution, rinsed with deionised water, and stored at room temperature for 24 h prior to calibration (Michael and Borland, 2006). The process of eletropolymerization with 1,3-phenylenediamine was followed by immobilization of enzyme. When the 1,3-phenylenediamine was eletropolymerized before the enzyme immobilization, the sensitivity was too low to detect any glucose.

2.6. In vitro conditioning and characterization

For in vivo determinations using the glucose biosensor, regular calibration of the sensors with standard glucose solutions is crucial. Calibrations were carried out in standard PBS (0.1 mM, pH 7.4) solutions. An Ag|AgCl electrode was used as the reference electrode. The electrodes were held at a constant potential of +700 mV, which is the value generally used for H_2O_2 detection (Lowry et al., 1994). All calibrations were carried out at room temperature. Glucose and interfering chemicals were added according to the experimental protocol.

2.7. Surgical procedures

A male Sprague–Dawley rat weighing 200 g was anesthetized with chloral hydrate (350 mg/kg, i.p.) and placed in a stereotaxic frame. Electrodes were then implanted following a previously described procedure (Lowry and Fillenz, 1997). The glucose MEA probe was implanted into the striatum, coordinates with the skull leveled between bregma and lambda, were A/P +2.0 from bregma, M/L +2.5 and D/V −5.0 from dura (Lowry et al., 1998b). The glucose MEA probe was used as a working electrode to detect the glucose concentration in the rat brain. A silver wire which had electroplated AgCl (Ag|AgCl) was placed in the cortex used as the reference electrode. The electrodes were held at a constant potential of +700 mV. The electrodes and probe were fixed to the skull with dental screws and dental acrylate (Heraeas Kuizer Dental Ltd., Shanghai, China). An earth wire attached to one of the support screws. The sensors were tested using chronoamperometry responses. Once the background current for the MEA probe had stabilized (typically 30–45 min) experiments were begun. All signals

were recorded at 0.1 s intervals (Lowry et al., 1998b). All procedures above complied with the guidelines of State Scientific and Technological Commission for the care and use of laboratory animals.

3. Results and discussion

3.1. Amperometric response for the glucose MEA probe

To further characterize the performance of the MEA probe modified with GOx and mPD, we determined the sensitivity and linear range first. A typical current-time response of the mPD modified glucose sensor at different glucose concentrations was shown in Fig. 1A. The concentration of glucose in SD rat brain was obtained between 0.27 mM and 1.5 mM in the literatures (Boutelle et al., 1992; Fray et al., 1997; Yang et al., 1995b; Kealy et al., 2013; McNay and Gold, 1999; Rex et al., 2009; De Bundel et al., 2009). We constructed a calibration plot for glucose concentrations in the range of 0.1–1.8 mM, which covered a concentration range sufficient for measuring brain glucose. The calibration data indicated that the biosensor showed good linear relationship with this glucose concentration beyond the range of values encountered in this work. Fig. 1B was the resulting linear plot of the glucose MEA probe. Since the microelectrode array fabrication procedure was highly rigorous, our research has found that the glucose responses result in a linear regression curve fitted with correlation coefficient of 0.986. The sensitivity of the MEA probe for glucose was 489 μA mM^{-1} cm^{-2}. The detection limit, based on the signal-to-noise ratio of three, was calculated to be 3 μM. The relative standard deviations (RSD) for one sensor was 3.5% ($n=3$), so the reproducibility of measurements for one sensor was 96.5%. The reproducibility of measurements between several sensors was 62% ($n=3$). The slope of the response and the amount of current on individual electrodes were different for the platinum surface and the dip-coating of GOx were not necessarily reproducible.

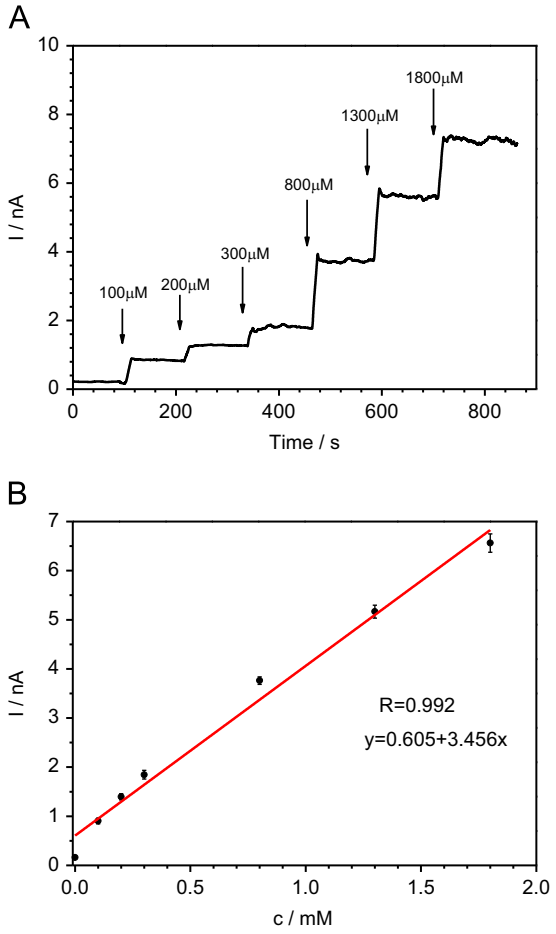

Fig. 1. (A) Current–time response of the glucose sensor at different glucose concentrations; (B) average steady state current ($n=3$) as a function of glucose concentration and the resulting linear plot of the glucose MEA probe. The $R=0.986$ for linear range of operation.

The figures of merits towards glucose detection obtained with some other relevant approaches are summarized in Table 1. As compared in Table 1, the present glucose electrode demonstrated good electroanalytical performances.

The glucose oxidation in the polymer/enzyme membrane has a relationship with the membrane thickness. The diameter of glucose oxidase is 8.6 nm and the growth of non-conducting polymers is self-limiting, which will produce thin polymer (<10 nm). In this work, the GOx was adsorbed on the Pt before electropolymerization of mPD and would be immobilized in mPD film, the polymer/enzyme membrane thickness estimations under these conditions were typically in the region of 10–30 nm (O'Neill et al., 2008; Zhou et al., 2004; Sasso et al., 1990; Malitesta et al., 1990). Since mPD is non-conductive, the oxidation of H_2O_2 occurs only after H_2O_2 diffuses to the surface of the Pt electrode. The diffusion rate of H_2O_2 in the membrane is related to the morphology of the film. If the membrane was thick, the liberated H_2O_2 diffusing to the electrode surface may be hampered. If the membrane was thin, the response time of the electrode decreases with decrease of film thickness, as the diffusion distance decreases. However, thin films limit the peroxide diffusion and cause superfluous reaction of H_2O_2, which would result in low sensor sensitivity (Day et al., 2006). As observed above, the good response behavior of the electrode implied that the membrane thickness was appropriate.

3.2. Study on the interference

As we were interested in developing a glucose sensor for neurochemical applications in the living brain, typical problems associated with analysis of such a complex biological environment include fouling by electrode poisons (e.g., proteins) and surfactants (e.g., lipids) and direct faradaic interference. For example, the increase in the current caused by adding glucose was clearly less in the presence of AA compared with its absence. There were at least three possible mechanisms that would lead to reduced sensitivity to glucose in the presence of AA: (i) fouling of the Pt surface by AA; (ii) "clogging" of the membrane by AA; (iii) a homogeneous reaction between AA and H_2O_2 (Lowry et al., 1994; Lowry and O'Neill, 1992; Matuszewski and Trojanowicz, 1990; Inamdar et al., 1991). AA is present at a

Table 1
Performance comparison of some relevant electrodes towards the electrochemical detection of glucose.

Modified electrode	Sensitivity	Detection limit (μM)	Reproducibility	References
Pt/PPD/GOx	1.56 pA/μM	n.d.	n.d.	Kealy et al. (2013)
Carbon fiber/PPD/GOx	34.5 ± 2.8 μA mM^{-1} cm^{-2}	n.d.	80%	Netchiporouk et al. (1996)
Glassy carbon/mPD-Prussian blue/GOx	2.54 pA/μM	1	97.8%	Tao et al. (2013)
MEMS MEA/mPD/GOx	489 μA mM^{-1} cm^{-2} or 3.46 pA/μM	3	96.5%	This study

concentration of 250–500 μM in the brain fluid. As demonstrated in other reports, a variety of electrochemical active species, such as DA (≤ 100 nM in ECF), 5-HT (≤ 1 μM in ECF) and DOPAC (20 μM in ECF) were coexisting in rat brain with their good electrochemical activity and thus become the potential interferences for the in vivo on-line detection of glucose (Shi et al., 2003). Some microdialysis systems used expensive chromatography to separate the interferents (Yang et al., 1995a), which used too much time, and may lose the change of signal. Herein organic molecule 1,3-phenylenediamine (mPD) was utilized to provide minimal interference from endogenous species such as AA, DA, 5-HT and DOPAC over physiologically relevant concentration ranges (McMahon and O'Neill, 2005; Lowry and Fillenz, 1997; Netchiporouk et al., 1996; Friedemann et al., 1996; Ryan et al., 1997).

Fig. 2A was the typical amperometric responses of DOPAC (20 μM), DA (1 μM), 5-HT (1 μM), AA (250 μM) on the electrode without mPD (top curve) and the mPD modified electrode (below curve) in the on-line detection system at the working potential of+700 mV. Selectivity refers to a ratio of the microelectrodes sensitivity for glucose over interferents such as AA. With a selectivity of 50:1, the microelectrode is 98% effective at blocking AA. This result showed that the respective selectivity for DA and AA was 19:1 and 286:1 and the response of DOPAC and 5-HT were close to zero for the mPD modified electrode. These convincingly indicated that the simultaneous measurement of glucose was interference-free from these electroactive species.

Fig. 2B was the typical amperometric responses of glucose on the electrode without mPD (top line) and the mPD modified electrode (below line) in the on-line detection system at the working potential of+700 mV. Final concentrations of each addition of glucose were 100 μM, 200 μM, 300 μM, 500 μM, 1300 μM and 1800 μM. After covering mPD, the sensitivity for glucose decreased 24.6 ± 2.40% (n=20). When modifying mPD, the dip coated GOx film turned into the ultrathin mPD/GOx film. Thin film would limit the peroxide diffusion and cause superfluous reaction of H_2O_2, which would result in low sensor sensitivity. This behavior has been suggested for H_2O_2 at other types of polymer electrodes (Lowry and O'Neill, 1992).

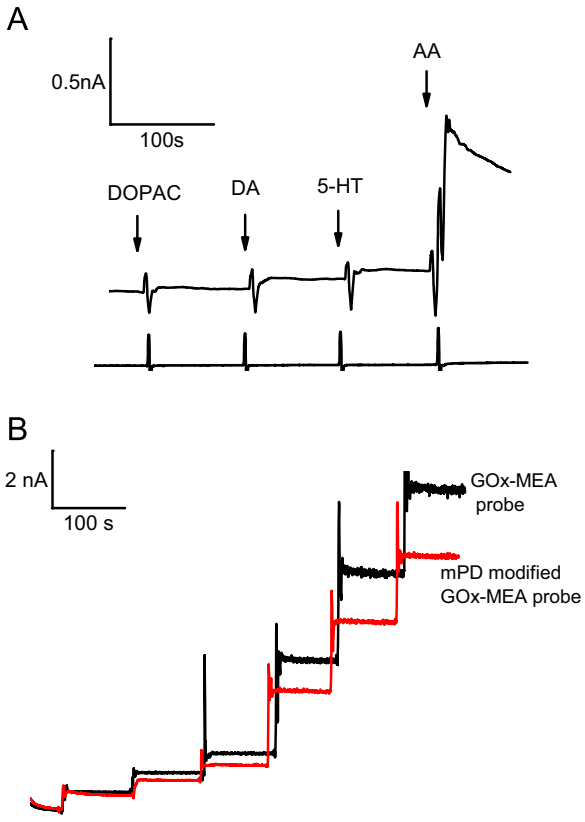

Fig. 2. (A) Amperometric responses of DOPAC (20 μM), DA (1 μM), 5-HT (1 μM), AA (250 μM) on the mPD modified electrode (top curve) and the electrode without mPD (below curve) in the on-line detection system. Applied potential: +700 mV; (B) amperometric responses of glucose on the electrode without mPD (top line) and the mPD modified electrode (below line) in the on-line detection system at the working potential of+700 mV. Final concentrations of each addition of glucose were 100 μM, 200 μM, 300 μM, 500 μM, 1300 μM and 1800 μM.

3.3. Stability of the glucose sensor

Finally, we assessed the stability of our biosensor in both operating (i.e. exposed to glucose) and storage conditions. Glucose biosensor could be used repeatedly for in vitro and in vivo experiments lasting 10 h. This glucose sensor was used intermittently and stored at 4 °C for 65 days, and it maintained 92% of its original activity and still displayed an excellent response to glucose.

3.4. Effect of glucose and insulin on biosensor response in vivo

We demonstrate the glucose MEA probe respond to changes in glucose in brain ECF by examining the effect of glucose and insulin on the in vivo response. Fig. 3 was an example of the MEA probe recording in vivo. The basal glucose level was monitored during at least 2 h before injection of insulin or glucose. After an injection of glucose (1.8 g/kg), the mean baseline glucose current increased from 285.1 pA to 295.4 pA. In the work of Lowry et al. (1998a, 1998b, 1998c), injecting glucose (0.36 g in 2 mL i.p.) on the in vivo response of the implanted biosensor in awake normoglycemic animals did not make significant difference (p > 0.46, n=3) in the signal before (12.59 ± 1.1 nA, n=3) and after (12.89 ± 0.7 nA, n=3) injection. This and our similar observation, agree with results that i.p. injections failed to produce an increase in the extracellular concentration (DiMattio and Streitman, 1988). In addition, chloral hydrate was used as anesthetic in present work and it might cause a decrease in glucose current (Lowry and Fillenz, 2001).

Insulin, which lowers plasma glucose levels and has also been reported to lower brain ECF levels (Vasylieva et al., 2011b; Lowry et al.,1998b), was injected i.p. (15 U/kg) into anaesthetized rats. The mean glucose current decreased from 295.4 pA to 130.8 pA 1.5 h after insulin injection (1 mL, 15 U/kg), representing a decrease in response of 55.7% (Fig. 3). There was then a gradual return to baseline values. When another injection of insulin (1 mL, 15 U/kg) was given, the baseline glucose current decreased from 233.4 pA to

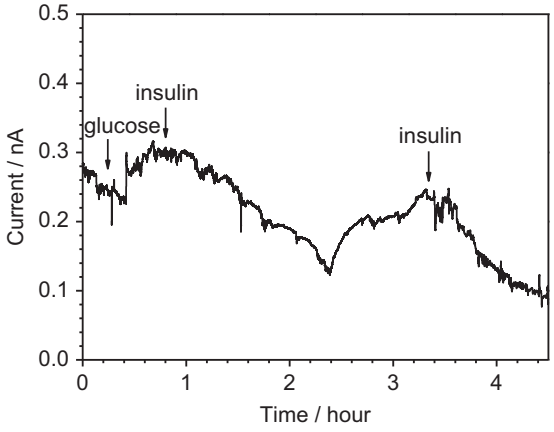

Fig. 3. Glucose monitoring in vivo using a glucose MEA probe implanted in the striatum of an anesthetized rat. Brain glucose levels increased following a glucose injection (1.8 g/kg) and decreased following two intraperitoneal injection of insulin (15 U/kg).

97.4 pA, decrease in response of 58.3%. These results suggest that glucose MEA probe respond to changing glucose levels in the ECF. A fall in brain glucose in animals which were given insulin has previously been reported by several groups using GOx-modified microelectrode sensors. Boutelle et al. (1986) observed a decrease of ≈10% in awake freely moving animals and Lowry et al. (1998b) observed a decrease of ≈14% with the same dose of insulin. Silver and ErreciAska (1994) and Netchiporouk et al. (1996), however both observed decreases of ≈80–90% of control. This difference may be due to a combination of factors including the use of higher doses of insulin (25 and 40 U/kg, respectively) and the fact that both these reports involved acute experiments on anaesthetized animals where a decrease in the extracellular concentration of glucose would be expected (Fillenz and Lowry, 1998). In our experiment the rat was anaesthetized and the quantity of insulin was 15 U/kg, the current decrease between 10% and 90% was reasonable.

After in vivo implantation, calibration experiments performed in the MEA probe removed from the rat brain and revealed that the sensitivity was 74% of the pre-implantation probe. The direct contact of biosensors with biological samples can lead to a decrease in sensitivity due to surface fouling by proteins and other biomolecules. This decrease in sensitivity generally varies between 20% and 50% and occurs over several hours exposure to brain tissue. For Pt/PPD/GOx electrode, the GOx component was lost during the implant procedure and the post-implantation calibration data was not available (Lowry et al., 1998c). In this work, the decrease of probe sensitivity may decrease a little bit of the current, but it was not the main reason for the change of the currents, because: (i) the decrease slope of probe sensitivity could not be so large; (ii) pre-implantation calibration data in vitro cannot be bused directly to estimate in vivo concentrations, for the differences between a free solution and a tissue matrix. The post-implantation calibration data was proximate to the in vivo electrode sensitivity (Kealy et al., 2013). The fact that we could detect glucose changes with the glucose MEA probe therefore indicates that our method was well suited for in vivo brain glucose monitoring.

4. Conclusions

In the present study, a MEA probe which was made by MEMS methods has been used as a glucose biosensor for rat brain research. The in vitro experiments emphasize that the sensor exhibited good linearity and high sensitivity for direct and specific glucose monitoring. In the end, continuous in vivo detection of glucose was realized in rat brain with an excellent anti-interference ability. This provided a reliable, simple and sensitive biosensor for use in the study of brain metabolism. The next step would be detecting some neurotransmitters like glutamate in the rat brain, and the 16Pt recording sites modified with different enzyme at the same time. In this way, different neurotransmitters would be detected at the same time, which was important for the neurobiology study.

Acknowledgments

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