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Glutamate sensing with enzyme-modified floating-gate field effect transistors

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ABSTRACT

Neurotransmitter release is the key factor of chemical messaging in the brain. Fast, sensitive and *in situ* detection of single cell neurotransmitter release is essential for the investigation of synaptic transmission under physiological or pathophysiological conditions. Although various techniques have been developed for detecting neurotransmitter release both *in vitro* and *in vivo*, the sensing of such events still remains challenging. First of all, the amount of neurotransmitter released during synaptic transmission is unknown because of the limited number of molecules released and the fast diffusion and reuptake of these molecules after release.

On the other hand, advances in microelectronic biosensor devices have made possible the fast detection of various analytes with high sensitivity and selectivity. Specifically, enzyme-modified field-effect (ENFET) devices are attractive for such applications due to their fast response, small dimensions and the possibility to integrate a large number of sensors on the same chip.

In this paper, we present a floating-gate FET device coated with glutamate oxidase (GLOD) layer. The surface chemistry was optimized for maximal enzyme loading and long-term stability, and characterized by quartz crystal microbalance and colorimetric assays. Enzyme loading was largest on poly-L-lysin-based surfaces combined with glutaraldehyde. The surface chemistry showed excellent stability for at least one month in Tris buffers stored at 4 °C. A glutamate detection limit of 10^{-7} M has been obtained with the GLOD-coated FET and our sensor proved to be selective to glutamate only. We show that this biosensor is a promising tool for the *in vitro* detection of glutamate and can be extended to other neurotransmitters.

1. Introduction

Changes in synaptic efficacy, including long-term potentiation and long-term depression of excitatory synaptic transmission, are considered to be the neuronal bases for learning and memory and are regulated by glutamate, amongst other neurotransmitters (Linden and Connor, 1992; Manahan-Vaughan et al., 2003). Pathological conditions related to signal transmission, such as Alzheimer's disease, require investigation at the synaptic level in order to understand the defects that occur in neuronal signaling (Walsh et al., 2002; Bell et al., 2003). Therefore, there is a large interest to investigate *in situ* glutamate levels released by neuronal cells.

The *in vivo* extracellular glutamate concentration in brain tissue measured by microdialysis is estimated to be in the range of 1–2 μM (Benveniste et al., 1984; Parrot et al., 2004; Zhang et al., 2005). However, these glutamate levels are believed to be an overestimation since the diameter of the microdialysis electrodes (200–500 μm) is 10,000-fold larger than the width of the synaptic cleft (20–50 nm) (Zuber et al., 2005). More sensitive and trustworthy techniques are therefore needed to measure glutamate release under these conditions.

Biosensor technology has proven to be very promising for the sensitive and selective detection of single analytes. Enzymatic detection of glutamate is based on its conversion to side products that can be measured by various electrochemical techniques. Among these, ion-sensitive field effect transistors (ISFETs) have many important advantages over conventional amperometric electrodes. Since their introduction by Bergveld (1970), they have been proven to be promising tools in various domains of research, including DNA genotyping, food screening and multi-analyte detection for biomedical applications. Their small dimensions, fast response and compatibility with conventional integrated circuits make them an excellent choice for biosensor applications (Alonso et al., 2003;

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Basu et al., 2006; Ingebrandt et al., 2007; Joo and Brown, 2008). Enzymatic field effect transistors (ENFETs) have an enzyme layer anchored onto the ISFET that translates the presence of the analyte into a detectable signal (Zayats et al., 2000; Chi et al., 2000; Poghossian et al., 2001; Dzyadevych et al., 2006). There has been little effort focused on the use of an ENFET for *in situ* glutamate sensing from neurons.

The principle of biochemical sensing with an ENFET is based upon the site-binding theory, first introduced by Yates et al. (1974). The gate surface of an ENFET consists of amphoteric sites which can be protonated or deprotonated. Biochemical reactions caused by the conversion of an analyte by a specific enzyme cause a change in the state of these sites, which are positioned close to the channel of the transistor. A local biochemical change on the surface will, therefore, be translated into a change in the current flowing in the transistor channel.

The FET gate surface plays an important role in the sensitivity and stability of the sensor. Each surface layer possesses certain pH sensitivity and can, therefore, detect minute changes in pH close to the electrolyte/insulator interface. Tantalum pentoxide is a promising gate oxide material for sensoric purposes, as it has a large number of surface sites that leads to a large buffer capacity (Bousse et al., 1994; Bartic et al., 2002).

In this paper, we describe the device structure and packaging of a floating-gate (FG) transistor fabricated using 0.25 μm CMOS technology. Working with advanced CMOS technology is advantageous due to its low-cost mass production. The design of the FG-FET includes a sensing area that is separated from the amplifying transistor. This not only reduces damage during the etching of the passivation layer on the sensing area, but also lends itself to more flexible geometries.

The device was coated with 100 nm Ta_2O_5, which was functionalized for the detection of low concentrations of glutamate in buffered solutions. The enzyme chosen for glutamate detection, glutamate oxidase, is ideal for ISFET sensing because it consumes only O_2 and H_2O, whereas other enzymes require co-factors or products that may interfere with the sensing. The surface chemistry was tested for its long-term stability and optimal storage conditions, and was then applied to the sensor surface to evaluate glutamate sensitivity and selectivity.

2. Experimental

2.1. pH sensitive floating-gate FETs

p-Channel floating-gate (FG) FETs with an extended gate configuration were fabricated in-house using a 0.25 μm CMOS technology. The active area contains eight individually addressable floating-gate PMOS transistors (cross-section shown in Fig. 1). To increase the gate capacitance and provide a good anchoring layer for the surface chemistry, 100 nm Ta_2O_5 was deposited by DC reactive sputtering. The floating gate was protected from the liquid by a passivation layer as well as the Ta_2O_5. The transistor gate width and length is 100 μm and 1 μm (W/L = 100), respectively. The sensor area is 10 μm in diameter. After dicing, the chips were wirebonded on customized printed circuit boards and the electrical connections were protected against liquid exposure by manual placement of a two-component epoxy (Epotek H54, Gentec, Belgium) that was cured for 3 h at 95 °C on a hotplate.

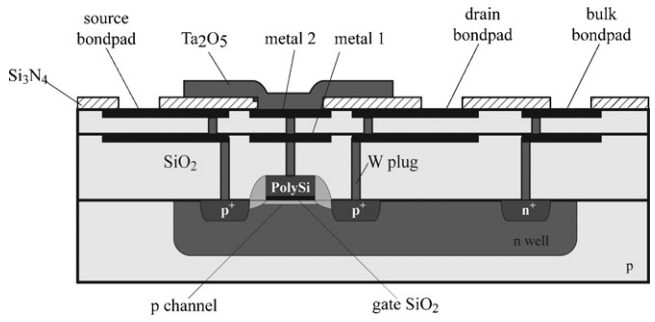


Fig. 1. Cross-section of the p-channel floating-gate FET.

2.2. Measurement set-up

The charge sensitivity of the FG FET was evaluated first in pH sensing experiments using buffers with different pH values. The device was brought into contact with the pH buffers together with an Ag/AgCl reference electrode (RE) (Metrohm, Belgium). The device together with RE form a 4 terminal srtructure that was then biased in a common source configuration. FET characteristics (i.e. $I_{DS} - V_{DS}$ for different V_{GS} and $I_{DS} - V_{GS}$ at constant V_{DS}), pH calibrations and time-lapse experiments were recorded with a Precision Semiconductor Parameter Analyzer (Agilent/HP 4156A) using a software programmable protocol (Matlab, The Mathworks). The test solutions containing glutamate or aspartate (Sigma, Belgium) in different concentrations in phosphate buffer saline (PBS, 150 mM, pH 7) were measured at room temperature.

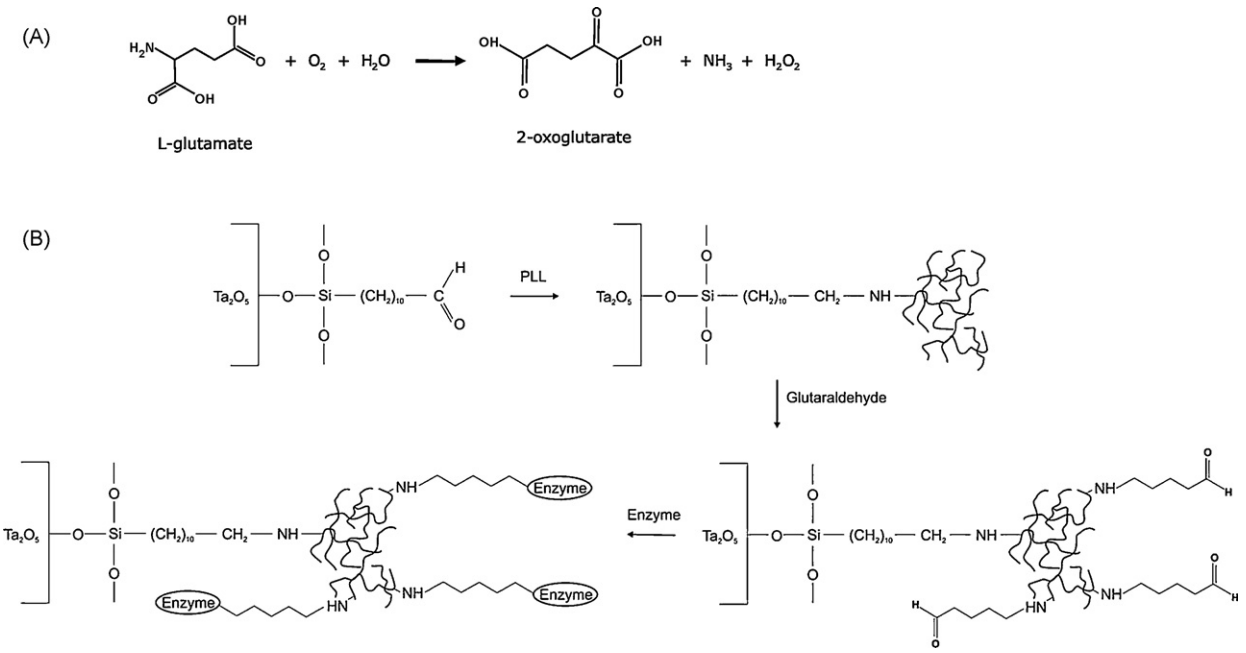
2.3. Surface chemistry

Tantalum pentoxide surfaces were cleaned using a solution of sulfuric acid and hydrogen peroxide (3:1, v/v) for 15 min followed by a UV/ozone treatment for 15 min. Samples were then immersed in an aqueous alcohol silane solution containing 2% 11-triethoxysilylundecanal (TESU) for 5 min. After rinsing with ethanol, the samples were baked on a hotplate at 110 °C for 30 minutes. Poly-L-lysine (PLL) was either adsorbed onto clean Ta_2O_5 substrates directly or was covalently bound to the TESU self-assembled monolayer (SAM) by immersion of the substrates in a 1:1 (v/v) solution of PLL (4 mg/mL in 10 mM borate buffer, pH 8) and cyanoborohydride (CNBH) for 30 min, followed by rinsing with borate buffer. Some PLL surfaces were then subjected to a glutaraldehyde (GA) treatment, which involved submersion of substrates in a 1:1 (v/v) solution of GA (5% in water) and CNBH for 30 min, followed by rinsing with water. Glutamate oxidase (GLOD) was coupled to these TESU, PLL, or GA surfaces by an overnight reaction with a 300 μg/mL GLOD solution in PBS, followed by rinsing with PBS. After GLOD coupling, some samples were treated further with CNBH for 30 min in order to create a more stable linkage of GLOD to the surface. To determine the long-term stability of the enzyme coupling, substrates were stored at 4 °C in the dark in Tris buffer (150 mM), PBS (150 mM), or air. Scheme 1B shows the reaction of the functionalization of the sensor surface.

2.4. Quartz crystal microbalance

To evaluate the amount of enzyme immobilized onto the surface, quartz crystals were covered with 50 nm Ta_2O_5. A QSense E1 system (QSense, Sweden) was used with a maximum flow rate of 2 ml/min and external temperature stabilization. The measurement volume was 80 μl and the exchange volume was 2 ml. After deposition of each new layer, the sensor crystal was rinsed with the corresponding buffer. Processing of the results was done with the QSense Software. The adsorbed mass is proportional to the frequency shift of the piezoelectric crystal and was calculated using the Sauerbrey equation, which holds for thin and rigid layers (Sauerbrey, 1959):

$$\Delta F = k\ \Delta m \quad (1)$$



Scheme 1. (A) Reaction scheme of the conversion of glutamate by glutamate oxidase (GLOD). (B) Reaction scheme of the immobilization of GLOD on the chips using silane SAMs, PLL and glutaraldehyde.

where ΔF is the change in fundamental frequency, $k = n/C$ (n is the overtone number, $n = 1, 3, 5, \ldots$, $C = 17.7$ ng/cm^2 Hz at $F = 5$ MHz) and Δm is the change in mass.

2.5. Colorimetric assay

The reaction solution contained 100 μM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), 0.25 U/mL horsradish peroxidase, 0.5 U/mL glutamic-pyruvic transaminase, 40 μM L-glutamic acid, and 200 μM L-alanine. For each assay, 150 μL of the reaction solution was pipetted onto 1 cm^2 substrates. After incubation for 30 min at 37 °C in a dark environment, 100 μL of the solution was collected from the sample and transferred to a micro-titer plate (Nunc, USA).

3. Results and discussion

3.1. Immobilization rate and efficiency of glutamate oxidase

Before implementation on the sensor surface, the glutamate-sensitive enzymatic layer was characterized using quartz crystal microbalance (QCM). This ultrasensitive mass sensor is often used to characterize formation of thin films on various surface materials in biosensor research (Marx, 2003). The Ta_2O_5 crystal surface was covered with a TESU SAM, followed by PLL serving as a coupling layer for the enzyme. The use of this polymer for the coupling of biological receptors and nucleotides in biosensors has been reported previously (Sawhney and Hubbell, 1992; Zhen et al., 2004; Braeken et al., 2008). Moreover, PLL is a cell permissive molecule extensively used for cell culturing. Fig. 2A shows the adsorption of GLOD to both the TESU and PLL layers. The results indicate that a greater binding capacity is achievable when the PLL layer is present, which is due to the more favorable physisorption on PLL compared to TESU. For covalent attachment of GLOD to the PLL layer, we included the crosslinker GA. This final GA surface leads to even greater enzyme loading because free amine groups on PLL are converted to aldehyde groups, which lead to covalent attachment of the enzyme to the surface.

In order to evaluate the enzyme immobilization efficiency, we first calibrated the activity of GLOD in solution using a colorimetric assay. This calibration curve was then used to inter-

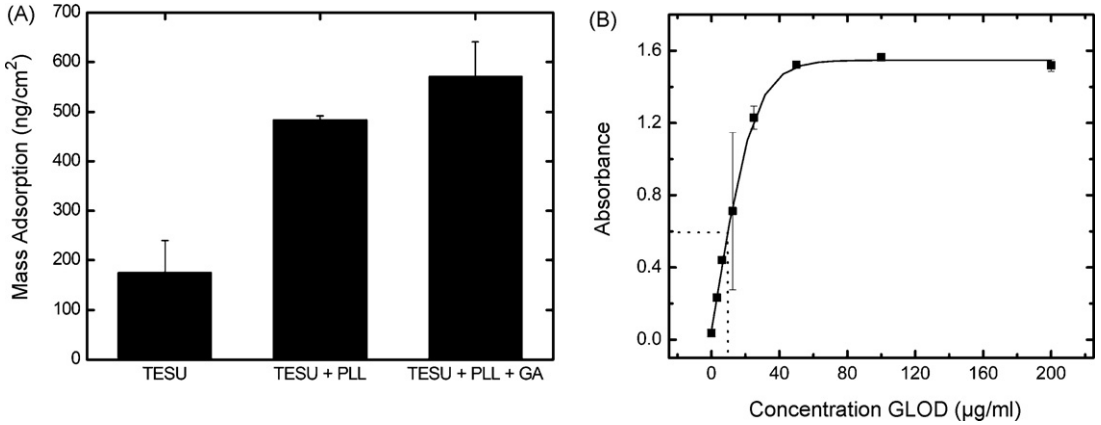


Fig. 2. (A) QCM mass adsorption (ng/cm^2) for the coupling of GLOD to TESU, PLL or glutaraldehyde (GA) surfaces. Error bars are standard deviations ($n = 2$). (B) Colorimetric calibration curve for GLOD in solution for the determination of the activity of surface-immobilized enzymes. Immobilized enzyme absorbances were compared to solution enzyme absorbances in order to extrapolate the enzyme surface concentration. Error bars are standard deviations ($n = 3$).

polate the surface concentration of GLOD immobilized using the glutaraldehyde-based surface chemistry. The absorbance value obtained corresponds to a surface concentration of 10.5 μg/ml ($A = 0.59$, see dashed line in Fig. 2B, intersection X-axis). This surface concentration can be translated into the amount of enzyme via multiplication with the total quantity of solution used for the reaction (50 μl). This yields 525 ng/cm^2 of active enzyme immobilized on the surface. Comparing this value with the total mass adsorption of GLOD on the glutaraldehyde-based surface (571.7 ± 69.5 ng/cm^2, Fig. 2A) reveals that a large fraction (91.8 ± 12%) of the immobilized enzyme remains active on the surface (assuming enzyme efficiencies on the surface and in solution are the same).

3.2. Long-term stability of the enzymatic layer

Binding of GLOD to various surface chemistries on Ta_2O_5 substrates was evaluated, as well as the long-term stability of the bound enzyme in different storage environments. Glutamate oxidase coupled to PLL or GA surfaces, either with or without TESU, was evaluated over time in different storage conditions (Fig. 3A–C). The use of a silane layer leads to the preservation of enzyme activity. This can also be seen in Fig. 3D, where initial and final absorbance values for those substrates stored only in Tris buffer are shown. While initially similar enzyme activity is seen on all surfaces, after ~50 days the enzyme activity is greater on those surfaces that use the TESU SAM. This is explained by the excellent surface organization that the silane provides, which makes sequential steps in the immobilization protocol more efficient. However, silane SAMs need ultraclean surfaces for optimal organization of the monolayer. Aggressive cleaning steps are, therefore, needed to obtain this. For packaged biosensors, this can cause practical limitations.

Dry storage of the enzyme leads to fast degradation; after 10 days, enzyme activity became minimal irrespective of the surface. However, comparing long-term stability of GLOD in Tris buffer with PBS buffer shows that Tris buffer storage is superior. The reason for this can be explained by the fact that the inert Tris buffers are optimized for enzymes and enzymatic reactions while PBS buffers may sequester divalent ions needed for enzymatic activity (Haifeng et al., 2008).

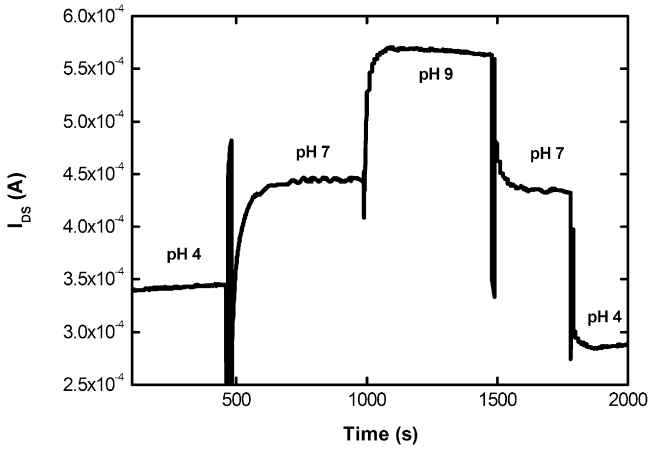
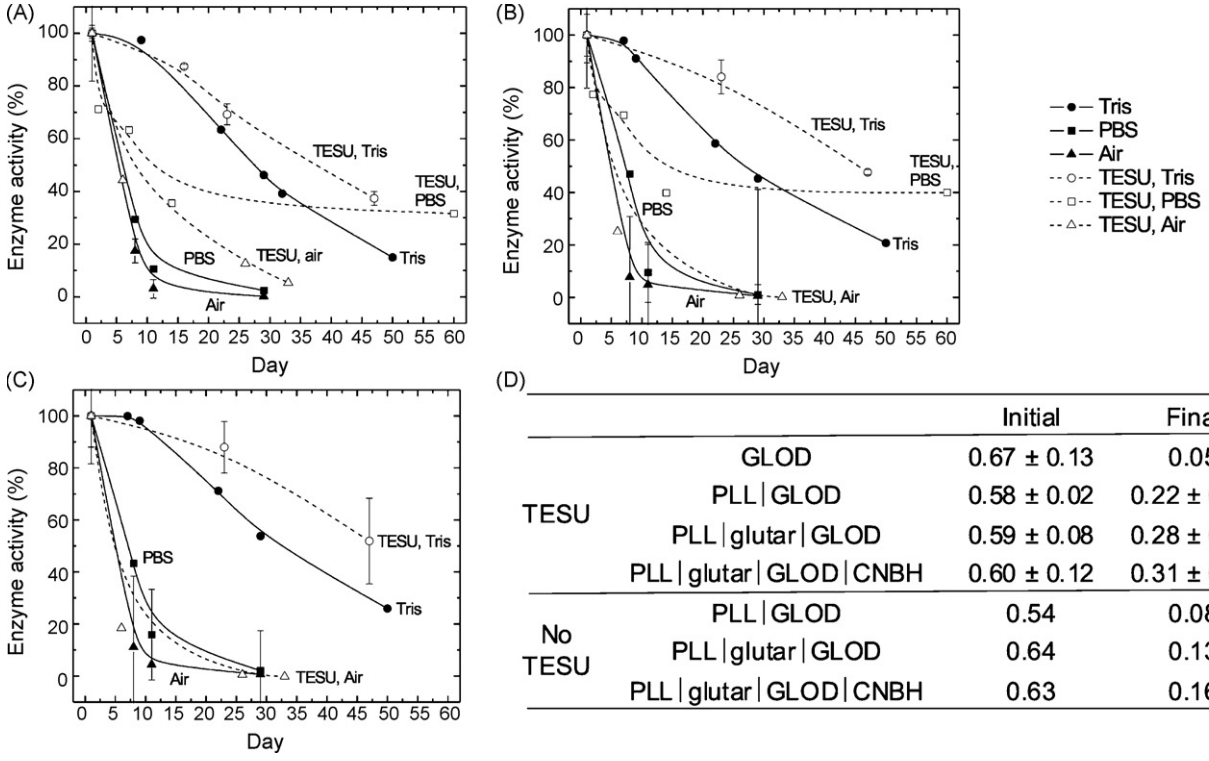


Fig. 4. pH sensitivity of the floating-gate FET with $V_{DS} = V_{GS} = -1$ V in buffers with pH 4, 7, 9.

3.3. GLOD–ENFET characterization

The detection of the neurotransmitter glutamate is based upon its catalytic conversion by glutamate oxidase (GLOD). This enzymatic conversion yields end products including H_2O_2 and NH_3 (Scheme 1A). The conversion of glutamate to NH_3 at the chip surface leads to a local increase of pH, which can be detected with a FET-based sensor.



(D)

		Initial	Final
TESU	GLOD	0.67 ± 0.13	0.05
	PLL | GLOD	0.58 ± 0.02	0.22 ± 0.03
	PLL | glutar | GLOD	0.59 ± 0.08	0.28 ± 0.01
	PLL | glutar | GLOD | CNBH	0.60 ± 0.12	0.31 ± 0.16
No TESU	PLL | GLOD	0.54	0.08
	PLL | glutar | GLOD	0.64	0.13
	PLL | glutar | GLOD | CNBH	0.63	0.16

Fig. 3. Long-term activity of GLOD coupled to (A) PLL or (B) GA surfaces with (dashed line) or without (solid line) the TESU SAM; (C) samples treated with CNBH after GLOD coupling to GA surfaces. Substrates were stored in Tris buffer (circles), PBS buffer (squares) or air (triangles). Errors bars are relative standard deviations ($n = 2$). (D) Initial and final (~50 days) absorbance values for substrates stored in Tris buffer only. Data are averages with standard deviations ($n = 2$).

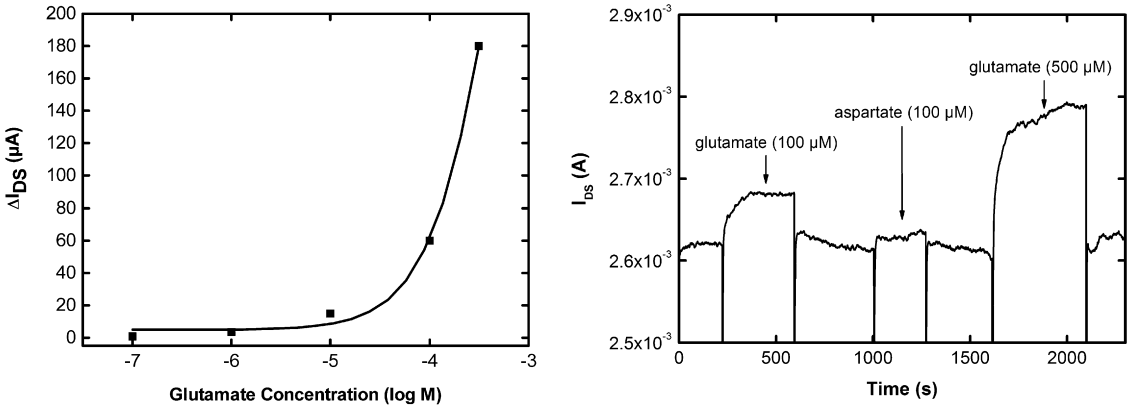


Fig. 5. (A) The change of the drain current of the transistor in function of the logarithmic glutamate concentration in PBS, pH 7. (B) ENFET response upon injection of glutamate (100 μM), aspartate (100 μM) and glutamate (500 μM). The measurement was started in PBS buffer (150 mM, pH 7), and changed to glutamate in PBS, aspartate in PBS and back to glutamate. The device was measured in constant voltage mode and biased with $V_{DS} = V_{GS} = -1$ V.

The charge sensitivity of the sensor determines, among other factors, its final sensitivity. The sensitivity of the FET-type of biosensor is reflected by the transconductance of the device, which translates a gate potential variation into a change of the current in the transistor channel. Using Ta_2O_5 as the gate material is advantageous because its numerous surface sites allow for small changes in pH to be detected. Thus, floating-gate FETs were characterized for their pH sensitivity first.

The change in I_{DS} for one pH unit ($\Delta I_{DS}/\Delta$pH) was 55.4 ± 8.14 μA ($n = 15$). An example of the response of the sensor to buffers with different pH values is shown in Fig. 4. The transconductance (g_m) calculated from the slope of the transfer characteristics in the linear transistor region, was around 1.2 mS for pH 7.

After the enzyme immobilization, the ENFET was immersed together with the reference electrode in PBS and glutamate solutions. The detection limit of the device for glutamate was tested in constant voltage mode and constant biases were applied on the reference and drain electrode with respect to the source electrode. The variation of the drain current generated by the change in glutamate concentration was monitored in time. The conversion of glutamate to NH_3 leads to the deprotonation of the hydroxyl groups at the Ta_2O_5 surface layer, and for a p-channel transistor this is translated as an increase in the drain current.

To determine the dynamic range of the sensor, it was immersed in different concentrations of glutamate and the change in I_{DS} (ΔI_{DS}) was recorded. The dose–response behavior of the ENFET is shown in Fig. 5A. The dynamic range extracted from the calibration curve is comprised between 100 nM and 500 μM.

In addition to sensitivity, selectivity to glutamate is an important parameter of the biosensor. This means that the signal detected by the ENFET should only originate from the enzymatic conversion of the analyte. We have evaluated the ability of the device to detect aspartate, a species with a similar structure and charge to glutamate. The relative activity of glutamate oxidase for glutamate compared to the amino acid aspartate is 100% and 0.6%, respectively (Kusakabe et al., 1983). While L-aspartate has a similar structure to glutamate, no significant response after its addition (100 μM) could be detected with the GLOD–ENFET (Fig. 5B). After rinsing with PBS, the sensor again shows a response to the addition of glutamate (500 μM).

Although the drift of the baseline on most sensors is significant, it is also rather slow. Because glutamate detection from cultured neurons is a fast event, monitoring only the first seconds after release is important. The drift is negligible over a short timeframe, while the glutamate detection of the sensor is rather fast. This can be seen in the rate-of-rise of the response to glutamate in the first seconds after switching the solutions. An alternative way to deal with drift is differential measurement, but because of the close proximity of the individual sensors this was not possible in our system. Future designs should include differential measurement possibilities with reference FETs on the same chip.

4. Conclusions

In this paper, we present the development of a glutamate-sensitive enzymatic FET for the electronic detection of the neurotransmitter L-glutamate. The enzyme glutamate oxidase was immobilized in a PLL matrix on Ta_2O_5 active area of a floating-gate field effect transistor. By means of QCM and colorimetric assays, we demonstrated the high enzyme loading (571.7 ± 69.5 ng/cm^2) on the sensor surface and optimal enzymatic activity of GLOD after immobilization. The long-term stability of GLOD bound to various surface chemistries was investigated in different storage conditions, the best of which was determined to be Tris buffer.

The charge sensitivity of the FG FET devices fabricated in-house was first evaluated and demonstrated in pH buffers. After implementing the optimal surface chemistry for GLOD, glutamate sensitivity was determined. The sensor was able to detect glutamate concentrations as low as 100 nM in PBS (pH 7). The sensor was selective only for glutamate, as immersion of the device in aspartate solutions did not significantly change the current in the channel of the transistor. This sensor, thus, has the potential for the dectection of the neurotransmitter glutamate and can easily be extended to other neurotransmitters by changing the enzyme system that is immobilized on the sensor surface.

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