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Fabrication of Highly Sensitive Pt-black Electrochemical Sensors for GABA Detection

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***Abstract*— GABA (Gamma-aminobutyric acid) is the main inhibitory neurotransmitter in the central nervous system of mammals. It is known to be related with various neurological disorders. GABA plays a crucial role in normal neuronal activity, information processing and plasticity, and neuronal network synchronization. To date, microdialysis has been widely used to monitor the level of GABA but the temporal and spatial resolution is limited. Besides, electrochemical sensors for neurotransmitter measurement, having high temporal and spatial resolution, overcome this problem. Here, using a cost-effective method of electrodeposition of platinum black (Pt-black), a highly sensitive, GABA specific, amperometric electrochemical sensor is fabricated. Nanostructured Pt-black increases the active surface area of the electrode contributing to higher sensitivity. Along with that, a self-referencing site and an exclusion layer are integrated to increase the selectivity and the signal-to-noise ratio (SNR) of the biosensor. This provides a prototype for a highly sensitive GABA sensor that could later be used to study various neurological disorders related to GABA concentrations.**

***Clinical Relevance*— This electrochemical sensor allows real-time monitoring of major inhibitory neurotransmitter (GABA) with high sensitivity which can be used for studying various neurological disorders.**

I. Introduction

GABA (Gamma-aminobutyric acid) is the most abundant inhibitory neurotransmitter in the central nervous system and has been implicated in various neurological disorders. It has hypothesized that imbalances in the ratio of excitation to inhibition (e.g. dependent upon GABA levels) may play a crucial role in seizures, autism spectrum disorder, and normal neuronal activity [1-5]. However, GABA is non-electroactive, which requires additional enzymes to produce an oxidizable reporter molecule, making it difficult to detect in real-time *in vivo*. To date, measurement of GABA is mostly carried out through microdialysis, which requires large equipment and has limited temporal and spatial resolution [6-8]. In contrast, electrochemical sensors overcome these drawbacks with having superior temporal and spatial resolution. Also, they can be miniaturized, giving them an advantage of having arrays of detection sites for multiple measurements. With the use of a specific enzyme, namely GABase, non-electroactive GABA can be converted to L-glutamate (1) which then further gets oxidized into a secondary electroactive product (hydrogen peroxide, H_2O_2) through the reaction with L-glutamate oxidase (2). This hydrogen peroxide further generates current (3) on the electrode surface where it is detected. Thus, electrochemical sensors hold promise of providing a reliable and rapid way to measure GABA *in vivo*.

$$\text{GABA} + \alpha\text{-ketoglutarate} \rightarrow \text{SSA} + \text{L-glutamate} \quad (1)$$

$$\text{L-glutamate} + H_2O + O_2 \rightarrow \alpha\text{-ketoglutarate} + NH_3 + H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + e^- \quad (3)$$

While there has been substantial research on developing enzyme-based biosensors for L-glutamate [9-11], the major excitatory neurotransmitter which drives excitatory:inhibitory (E:I) balance together with GABA, biosensors for the detection of GABA have been limited. This owes to the fact that GABA requires more than one enzyme to convert it to a reporter molecule. Further, to detect GABA alone, alpha-ketoglutarate (α-keto) needs to be introduced separately for the reaction to fully take place. Among the few laboratories that developed GABA sensors, most of them used microelectrode arrays (MEAs) constituted of platinum (Pt) recording sites [12-14]. Pt shows superior electrochemical activity during redox reactions when compared to other metals, such as gold and palladium [15]. However, Pt probes fabricated with conventional evaporation method alone results in relatively low sensitivity. Thus, a GABA sensor with higher sensitivity is desired to obtain favorable GABA signals *in vivo*.

In this work, a facile and cost-effective method of electrodepositing Pt-black on Pt surface and its performance towards GABA sensing is introduced. For amperometric measurements, the signal detected is related to the active

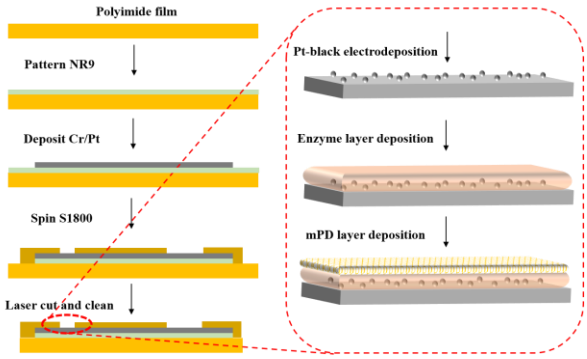

Figure 1. Fabrication process of the biosensor.

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surface area where the chemical reaction takes place, and the nanostructured Pt-black deposited through electrodeposition here enhances the effective surface area. Along with that, electrodes are further modified with phenylenediamine dihydrochloride (mPD), which acts as an exclusion layer repelling interferent molecules for better selectivity towards GABA [16]. Furthermore, a self-referencing site is integrated to a proximity with the GABA sensing site, where only L-glutamate oxidase is coated to eliminate ambient noise from molecules other than GABA. Altogether, a highly sensitive Pt-black electrochemical sensor for GABA is fabricated.

II. Materials and Methods

A. Chemicals

Phosphate buffered saline (10x PBS) and glutaraldehyde were purchased from Thermo Fisher Scientific (Waltham, MA) and L-glutamic acid monopotassium salt monohydrate, chloroplatinic acid hydrate, dopamine hydrochloride (DA), and bovine serum albumin (BSA) were purchased from Alfa Aesar (Haverhill, MA). L-ascorbic acid (AA), m-phenylenediamine dihydrochloride (mPD) and 4-aminobutyric acid (GABA) was purchased from Acros Organics (Fair Lawn, NJ) and glutamate oxidase (GOx) from US Biological Life Science (Swampscott, MA). GABase was purchased from Sigma Aldrich (St. Louis, MO).

B. GABA Sensor Fabrication

The fabrication process of GABA sensors is shown in **Fig. 1**. Using conventional microfabrication, Pt microelectrode arrays (MEA) were fabricated on a 127-nm thick polyimide film using a lift-off process by depositing a dual layer of 20 nm chromium then 200 nm platinum on a patterned NR9 (Franklin, NJ) layer followed by acetone treatment to remove NR9. After patterning another layer of S1800 for passivation, the batch of MEAs were laser-cut into single probes as, where one probe consisted of 5 pads for detection (50 μm × 100 μm for each pad). The tailored probes were connected to a printed circuit board (PCB) through a copper wire and silver epoxy paste for stable connection to the potentiostat.

After fabricating the platform for detection, the surfaces of the probes were modified platinum black (Pt-black). Pt-black was electrodeposited on the Pt electrodes to increase the active surface area. Specifically, the probes were immersed in a 0.01 M chloroplatinic acid and potential was cycled between -0.4 to +0.8 V, vs a Ag/AgCl reference electrode with a scan rate of 50 mV/s for 10 cycles. Chloroplatinic acid used here was made by dissolving chloroplatinic acid hydrate (H_2PtCl_6*xH_2O) in DI water and a commercial potentiostat (700E, CH Instruments, Austin, TX) was used. After Pt-black deposition, the probes were rinsed with DI water followed by drying in ambient environment.

For the specificity of our GABA probe, GABase were coated on top of the Pt-black deposited surface. To make the enzyme deposition mixture, 10 mg of BSA was dissolved in 985 μL of DI water followed by adding 5 μL of glutaraldehyde (25% in water) with manual agitation. The mixture was kept at room temperature for 5 minutes while the enzymes (L-glutamate oxidase and GABase) frozen at -80 C° were brought out for thawing. 0.5 μL of GOx (1 U/μL) was mixed with 4.5 μL of the BSA solution and it was applied on the pads designated to be the self-referencing with a Hamilton syringe. Next, 0.5 μL of GOx (1U/μL) and 0.5 μL of GABase (1U/μL) were mixed with 4 μL of the BSA solution and it was applied on the GABA sensing pads. The only difference between the self-referencing and GABA sensing site is the existence of GABase which makes only the sensing pads convert GABA to L-glutamate that ends up in producing H_2O_2, while the self-referencing cannot. Thus, self-referencing detects all the other background signals generated by other electroactive molecules and can be used to remove those noises by subtracting it from the GABA sensing signal. When depositing enzyme solutions, they were applied 3 times per pad giving 1 minute of curing time in between each application. Then the probes were kept at 4 C° for over 24 hours, allowing the enzyme mixture to cure and achieve full crosslinking of the protein.

Lastly, mPD, known to block interference molecules via size exclusion, was electrodeposited as an exclusion layer for the selectivity of the probes. By dissolving mPD into deoxygenated 0.05 M PBS, 5 mM of mPD solution was made. Then the probes were immersed in the solution and using a commercial potentiostat, a potential between +0.2 to +0.8 V vs a Ag/AgCl reference electrode was cycled for 15 minutes. After the mPD deposition, the probes were rinsed with DI water and kept at 4 C° for at least 24 hours before calibration.

C. Characterization and Calibration

To quantify the increase of active surface area of the electrodes after Pt-black deposition, cyclic voltammograms of the bare Pt and Pt-black coated electrodes were obtained using $K_3Fe(CN)_6$ as a redox couple. 5 mM of $K_3Fe(CN)_6$ in 0.1 M KCl was prepared and the probes were soaked in the solution. Potential between -0.2 to +0.6 V vs a Ag/AgCl reference electrode was cycled with a scan rate of 50 mV/s. Then the peak values of the cyclic voltammograms were applied to the Randles-Sevcik equation:

$$i_p = (2.69 \times 10^5) n^{\frac{3}{2}} A D^{\frac{1}{2}} v^{\frac{1}{2}} C \quad (4)$$

where i_p is the peak current, n is the number of electrons transferred in the redox reaction, which is 1 in this case, A is the electrode area (m^2), D is the diffusion coefficient (m^2/s), v is the scan rate (V/s), and C is the concentration of analytes (mol/L).

The performance of the probes was tested using amperometry. The multichannel potentiostat from Pinnacle Technology Inc. (8400-K1, Lawrence, KS) was used for simultaneous recording of both self-referencing and GABA sensing sites. Sensitivity of the probe was first measured by immersing the electrodes in 1 mM of α-keto mixed in 0.01 M PBS, under constant stirring at 37 C°. Then various concentrations of GABA (10 μM to 100 μM) were introduced into the α-keto solution while applying a constant potential of +0.7 V vs a Ag/AgCl reference electrode. The results were then analyzed to calculate the limit of detection (LOD) by dividing 3 times the standard deviation of the baseline by the least squares slope.

Same setup was used to test the selectivity of the probe where interferents such as ascorbic acid (AA) and dopamine (DA) were introduced along with GABA. Around 20~30 minutes of stabilization was performed before calibration for all the amperometric recordings.

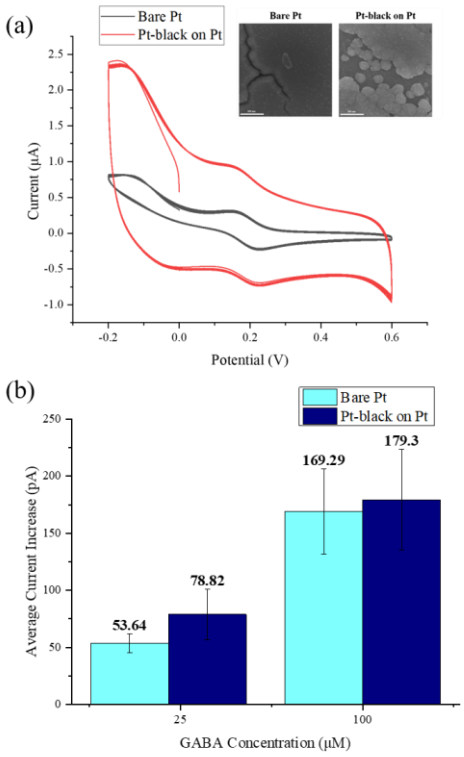

Figure 2. (a) Cyclic voltammograms of bare Pt and Pt-black coated Pt electrodes in 5 mM of $K_3Fe(CN)_6$. Inset shows scanning electron microscopic images of bare Pt and Pt-black coated Pt electrodes (bar = 500 nm). (b) Comparison of current increase from GABA between bare Pt and Pt-black coated platinum electrode (n=5 each).

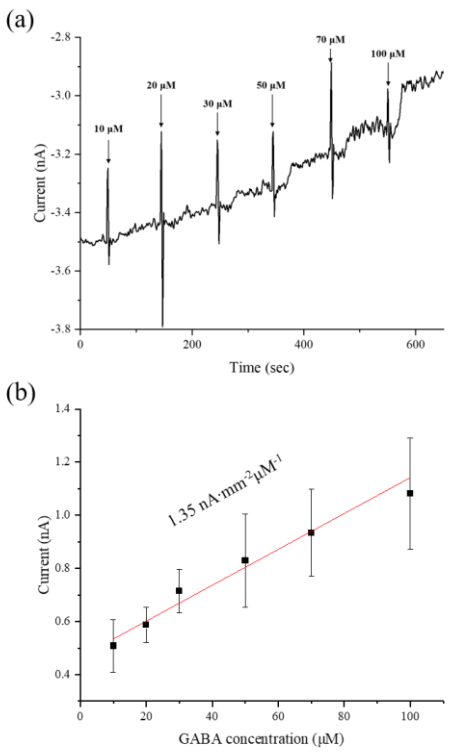

Figure 3. (a) Amperometric curve for Pt-black on platinum to sequential addition of GABA. (b) Corresponding calibration curve with sensitivity.

III. Results and Discussion

A. Confirmation of Pt-black Deposition

As described above, surfaces of the probes were modified by electrodeposition of Pt-black. Pt-black particles formed on bare Pt surfaces were expected to increase the active surface area for catalytic and enzyme immobilization for higher sensitivity. The result of this cost-effective method to electrodeposit Pt-black was first confirmed with scanning electron microscopy (SEM), as shown in the inset of **Fig. 2(a)**. When compared, granular structures were observed on top of the bare Pt surface after the Pt-black deposition. These nanostructures are thought to increase the active surface area of the electrode leading to higher sensitivity. In order to calculate the actual surface area of the bare Pt and Pt-black deposited electrodes, cyclic voltammograms of the electrodes in $K_3Fe(CN)_6$ was obtained and the peak values were put into the Randles-Sevcik equation (**Fig. 2(a)**). From the equation, the surface area of the Pt-black deposited electrode showed about 3.5 times higher active surface area compared to bare Pt, leading to higher sensitivity towards the H_2O_2 oxidation, the product of GABA reaction.

Then the actual response of the two electrodes towards GABA has been tested. The two sensors were subjected to amperometry in 1 mM alpha-ketoglutarate solution. Response for two concentrations of GABA were tested (20 and 100 μM) and the results are shown in **Fig. 2(b)**. The response at Pt-black coated Pt were higher than that of bare Pt. However, it was observed that the increase in the signal was higher at 20 μM when compared to 100 μM. This could be due to the fact that the signal reaches a plateau when the concentration gets too high, but additional experiments would be needed to validate this.

B. Calibration of Pt-black Electrode

After confirming the enhancement in active surface area on the Pt-black coated Pt electrodes, its performance was characterized. First, the response of Pt-black electrodes to GABA was tested to obtain its sensitivity. As seen in **Fig. 3**, the sensor showed linear response to various concentrations of GABA ranging from 10 to 100 μM with a sensitivity of 1.35 $nA \bullet mm^{-2} \mu M^{-1}$ (R^2=0.95) and a limit of detection of 0.04 μM.

Then the selectivity of our GABA sensor was examined. Interferents known to be present in the body along with GABA, such as AA and DA, were added along with GABA and L-glutamate. As shown in **Fig. 4**, addition GABA generated a current only at the GABA sensing and not at the self-referencing site showing the sensor's specificity towards GABA. When interferents (250 μM AA and 2 μM DA) were introduced, increase in current was not observed in both sites implying the interferent molecules are being size excluded from the mPD layer. Lastly, addition of L-glutamate generated current increase on both sites, where signal at self-referencing site can be later subtracted from the GABA signal to only extract the currents from GABA, which attributes to higher signal to noise ratio (SNR).

IV. Discussion and Conclusions

A facile, cost-effective method of Pt-black nanostructure formation on Pt electrodes has been demonstrated for the purpose of creating a sensor to detect GABA in living brain

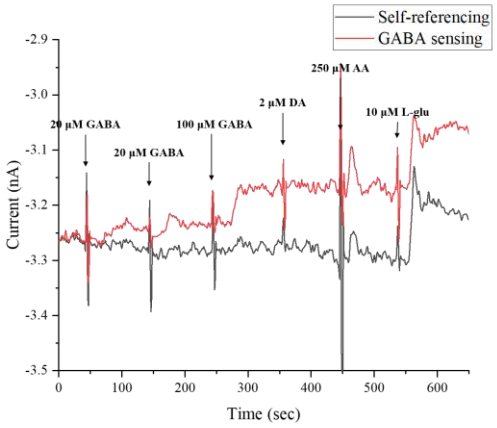

Figure 4. Selectivity test of Pt-black coated platinum electrode.

tissue. Owing to the increase in active surface area, Pt-black coated Pt electrodes showed high sensitivity towards GABA. In addition, mPD coating and self-referencing technique allowed for GABA-specific sensing. There has not yet been a report on modifying the surface of the plain electrode to obtain higher sensitivity for GABA, and the electrodeposition of Pt-black shown here demonstrates a cost-effective approach to achieve that goal.

Overall, monitoring the level of GABA *in vivo* with high temporal and spatial resolution is important when studying the role of GABA concentration in neurological disorders. While *in vivo* validations are not performed in this research, the biosensor introduced here opens a possibility of applying highly sensitive electrochemical GABA sensor to investigate the cellular and electrophysiological mechanisms underlying various neurological diseases. This platform can further be developed by integrating an additional site for L-glutamate detection. In this system, the alpha-ketoglutarate generated at the L-glutamate site through GOx reaction can take part in the GABA reaction chain so that external alpha-ketoglutarate introduction may not be needed. This will enable much-anticipated simultaneous monitoring of major excitatory and inhibitory neurotransmitters for the calculation of E:I balance *in vivo*. Finally, this platform with facile fabrication and self-referencing technique can eventually be used to explore more neurotransmitters beyond GABA.

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