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LLNL-TR-700298

Characterizing Enzymatic Deposition for Microelectrode Neurotransmitter Detection

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August 12, 2016

Disclaimer

This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344.

Abstract:

The enzyme immobilization process, one step in creating an enzymatic biosensor, was characterized and analyzed as a function of its physical properties. The neural glutamic biosensor is a flexible device, effectively minimizing trauma to the area of implantation. The Multi-electrode Array (MEA) is composed primarily of a proprietary polymer which has been successfully implanted into human subjects in recent years. This polymer allows the device the pliability that other devices normally lack, though this poses some challenges to implantation.

The electrodes are made of Platinum (Pt), and can range in number from eight to thirty two electrodes per device. These electrodes are electroplated with a semipermeable polymer layer to improve selectivity of the electrode to the neurotransmitter of interest, in this case glutamate. A signal is created from the interaction of glutamate in the brain with the glutamate oxidase (GluOx) which is immobilized on the surface of the electrode by using crosslinking chemistry in conjunction with glutaraldehyde and Bovine Serum Albumin (BSA). The glutamate is oxidized by glutamate oxidase, producing α-ketoglutarate and hydrogen peroxide (H_2O_2) as a by-product. The production of H_2O_2 is crucial for detection of the presence of the glutamate within the enzymatic coating, as it diffuses through the enzyme layer and oxidizes at the surface of the electrode. This oxidation is detectable by measurable change in the current using amperometry. Hence, the MEA allows for *in vivo* monitoring of neurotransmitter activity in real time. The sensitivity of the sensor to these neurotransmitters is dependent on the thickness of the layer, which is investigated in these experiments in order to optimize the efficacy of the device to detecting the substrate, once implanted.

Objective:

The goal of these experiments is to first homogenously coat individual microelectrodes, of which the diameter is 50μm. Varying coat thickness ought to correlate linearly with the current increase produced by its corresponding microelectrode, i.e. a twenty-layer coating would produce less of a response than a microelectrode coated with fifty layers, until the layer is too thick to allow the H_2O_2 produced by the reaction to fully contact the surface of the microelectrode. These layers can then be quantified via wet/dry weighing of the droplet, as well as 3D imaging to determine volume and height of the enzymatic coating that produces an optimal current. This will be tested using *glucose* oxidase in place of *glutamate* oxidase, so glucose in place of glutamate as a substrate, to reduce cost and optimize repeatability.

Introduction:

A biosensor at the basic conception consists of three main components: (1) a biological recognition molecule which identifies and differentiates the target molecules in the presence of various interfering chemicals; (2) a transduction element that translates the biological signal to one that is both measurable and quantifiable, and (3) a processing signal to convert the response into a coherent reading. A recognition molecule could be an enzyme, biomarker, receptor, antibody, nucleic acid or even a microorganism. The transduction element is most often electrochemical, but could also be optic, magnetic, piezoelectric, or thermometric. Lastly, the processing signal is most often read amperometrically, as in the case of the glucose and glutamate sensors, but may also be recorded via the potential or conductance. Potentiometry and amperometry are both similar in electrochemical sense, except that potentiometry changes the voltage in the working electrode so that difference in potential between the reference and

working electrode is what is recorded, versus recording at a fixed potential in the working electrode to sense the current generated when electrons are exchanged at the surface of the microelectrode (Weltin, Yoo 4560).

Implantable amperometric biosensors pose as many applications as they do challenges. The implications of successful implantation as a neural interface are many, including monitoring baseline neurotransmitter activity, restoring retinal and cochlear hypofunction, and detecting hypoxia, hemorrhage and other trauma induced conditions that cause fatalities that are only detectable once the damage has manifested itself (Zhou 10). To date, the most prevalent application of biosensors is the glucose biosensor, most commonly used in the treatment of diabetes. In this sensor, an electrochemical method is used to transduce the chemical signal of blood glucose to either a charge or a current, depending on whether the measurement is coulometric or amperometric, respectively (Wassum). The main difference between these two methods is mainly the time scale, in which coulometric measurement would be taken over an interval of time, versus amperometric in which the current is measured at a specific time. Both charge and current are a result of the reaction of glucose with glucose oxidase (GOx) and the subsequent oxidation of hydrogen peroxide at the surface of the electrode. Enzymatic biosensors have thus existed for the past 50+ years, though their viability as implants are only currently being investigated due to the complex and dynamic nature of enzymes, as well as the body's response to implants (Kotanen et.al).

In this experiment, GOx is utilized as the enzyme to be immobilized on the surface, since it has the highest selectivity for glucose, and has greater ionic strength as well as resistance to pH and temperature fluctuations. Many sensors require the presence of FADH to produce a redox reaction, but this is not necessary here, as the coating produces the oxidation cofactor.

Materials & Methods:

Several steps were taken in the development and execution of this experiment. The first challenge was to fashion a device capable of depositing the enzymatic solution upon the surface of the electrode that could be fully manually manipulated, while still staying within the perimeter of the 50µm electrode and maintaining the integrity of the previously deposited enzymatic layers. To accomplish this, a single eyelash brush was coated in polydimethylsulfoxane (PDMS), to a tip diameter of 43.23 µm. This PDMS coating was necessary since the microdroplet on the tip of the uncoated brush would not leave the tip or would evaporate before deposition onto the microelectrode. By coating with this polymer, the enzymatic droplet is more easily transferred from the tip of the brush to the surface of the electrode.

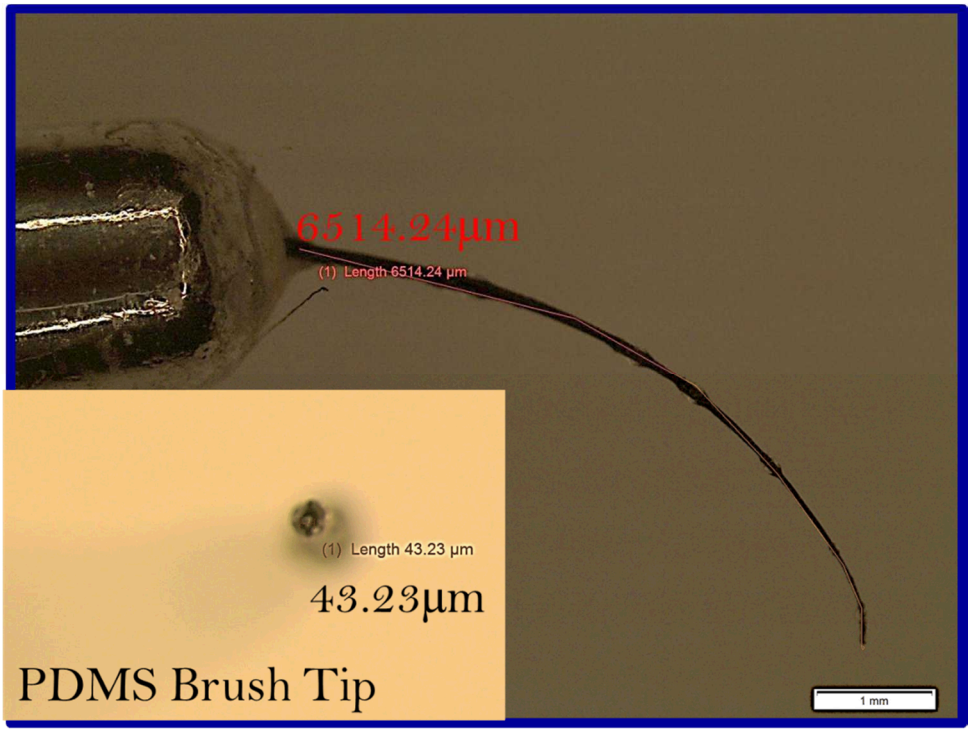

Figure 1.1: The applicator fashioned for enzymatic deposition. In the bottom left insert, the diameter of the brush tip can be observed, coated in PDMS. The upper right photo shows the 6.5 mm PDMS coated eyelash, cured overnight at 60ºC.

The 60 x 20 µm diameter microelectrode, 1333 μm^2 in surface area, was then coated using this brush in a two-step dabbing process, to assure full coverage of the surface. This was done by depositing a pool of enzymatic solution consisting of 10 mg BSA, 4 µL of Glutaraldehyde, and 0.2 µL GOx. A 0.1-2 µL micropipette was then used to dispense a volume of 0.100 µL near the array, into which the brush was dipped and lightly "painted" across the surface, as is shown below.

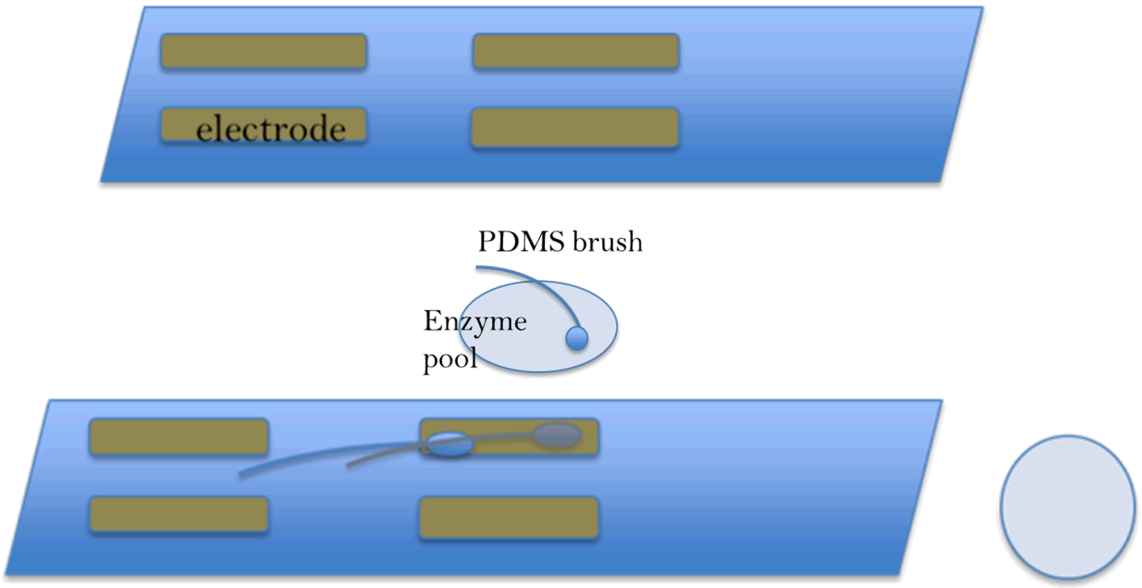

Figure1.2: Enzymatic Deposition using PDMS coated brush: A droplet of enzyme + BSA + Glutaraldehyde is placed adjacent to the probe tip of the electrode. The PDMS brush is dipped into the solution, and dabbed twice onto the surface for even distribution of the solution, constituting a single enzymatic layer.

The GOx is suspended in a solution of BSA and glutaraldehyde for many reasons: one to extend its lifetime on the surface of the electrode by encapsulating it and making it water insoluble, and two, in order to immobilize the enzyme on the device. Crosslinking occurs between the covalent bonds of glutaraldehyde with lysine residues of BSA, resulting in a fixation of the solution onto the microelectrode. In a gentle and simple coupling method, the covalently attached H_2O insoluble molecules prevent denaturation of the enzyme by the solutes present in

an *in vivo* application. It is also useful in electrochemical testing, to prevent the coating from physical disruption due to the agitation of the solution done to evenly distribute the glucose injected.

Once the devices were coated with volumes of 15, 20, 50, and 70 layers, they were set to desiccate over the span of 48 hours to assure the enzyme is fully dry before testing. After this curing period, they were individually tested to compare the correlation of different layer volumes to sensitivity to glucose; the electrochemical cell is pictured below:

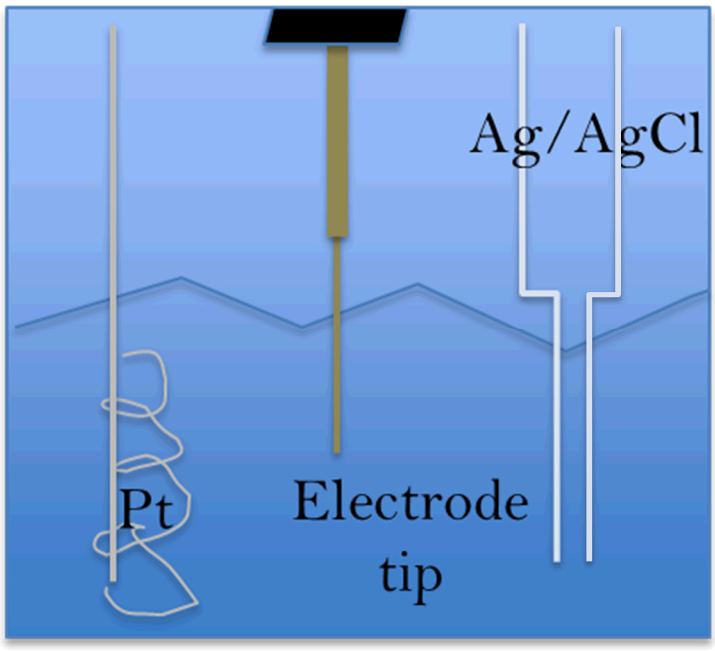

Figure 1.3: Electrochemical testing of enzymatically deposited electrodes, pictured in the middle, immersed in 10mL PBS. The Pt counter electrode is pictured on the left, and Ag/AgCl reference electrode on the right. Glucose solutions of 50, 100, & 500 mM were pipetted at ~1 minute intervals into the solution, which was mixed using a magnetic stir bar. Electrodes 5, 6, 7 and 8 on the device, as shown in figure 2, were connected to channels 1-4 of a potentiostat, and response to the glucose signal was interpreted using EC Lab and Excel.

Results:

Cumulative Molarity	Current (I) 15 layers	(I) 20 layers	Current (I) 50 layers	(I) 70 layers
0.00E+00	7.61E-05	2.61E-03	0.00847281	2.84E-03
2.50E-05	8.72E-05	5.51E-03	0.01163811	3.16E-03
5.00E-05	1.01E-04	9.47E-03	0.01580605	3.54E-03
9.98E-05	1.17E-04	1.56E-02	0.02575281	4.44E-03
1.50E-04	1.22E-04	2.29E-02	0.0433523	5.35E-03
2.49E-04	1.25E-04	3.64E-02	0.04734456	7.07E-03
3.48E-04	1.26E-04	4.97E-02	0.04948394	8.84E-03
3.97E-04	1.30E-04	5.69E-02	0.05767337	9.80E-03
4.46E-04	1.35E-04	6.32E-02	0.06457879	1.06E-02
5.45E-04	1.39E-04	7.70E-02	0.07755719	1.18E-02
6.44E-04	1.42E-04	8.74E-02	0.08157265	1.37E-02
8.40E-04	1.41E-04	1.10E-01	0.13822113	1.66E-02
1.04E-03	1.64E-04	1.34E-01	0.23778544	1.98E-02
1.28E-03	1.68E-04	1.62E-01	0.29120589	2.72E-02
1.53E-03	1.75E-04	1.77E-01	0.3287448	2.52E-02

Table 1: Current (I) (mA) as a response to increasing injection volume and molarity of glucose on 15, 20, 50 and 70 layered microelectrodes. The sensitivity of the GOx to glucose did correlate to both increasing layer volume, as well as glucose concentration, but only up to a specific layer number. As can be interpreted in the last four columns, 50 layers appears to be the ideal volume to elicit the greatest current response from the sensor.

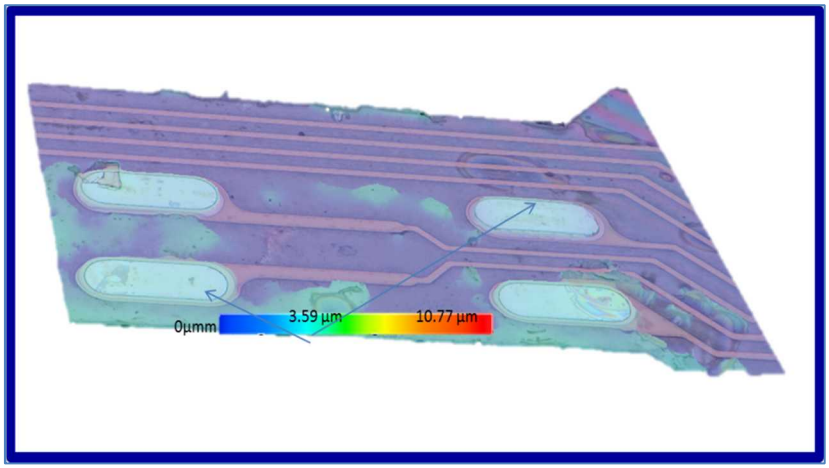

Figure 2: 3D Keyence Image (5000X) of the topography of the enzymatic deposition onto electrodes, measureing the height of 50 layers at ~3.59 µm. The purple color of the MEA surface corresponds to a null (flat) height based on the topographical mapping of the microscope, whereas the green color outside of the electrodes can be attributed to light artifact due to the reflective and translucent nature of both the array surface and enzymatic layer, respectively.

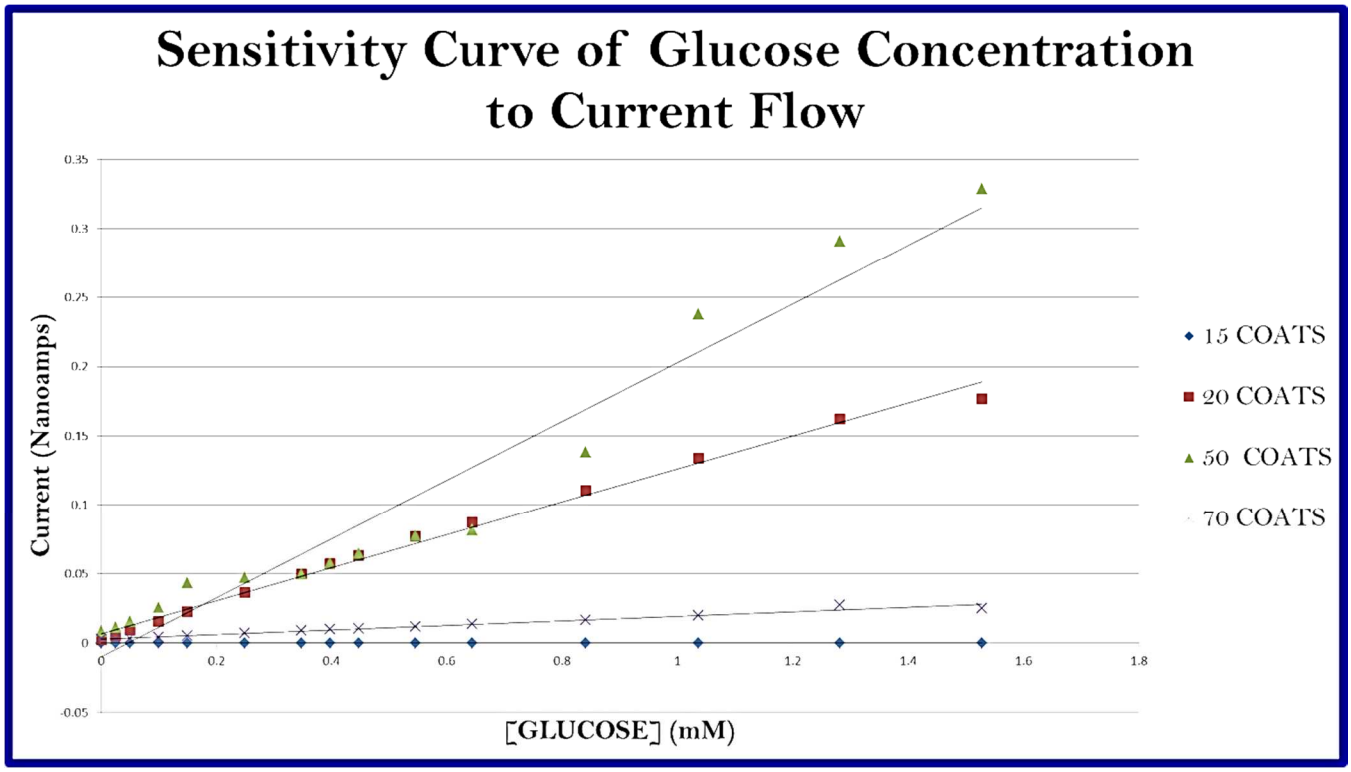

Figure 3A: The relationship of glucose concentration to current flow (nA) is linear until the enzymatic layer is too thick to allow H_2O_2 to contact the electrode surface. As shown, coats below 20 layers in depth have reduced sensitivity to glucose.

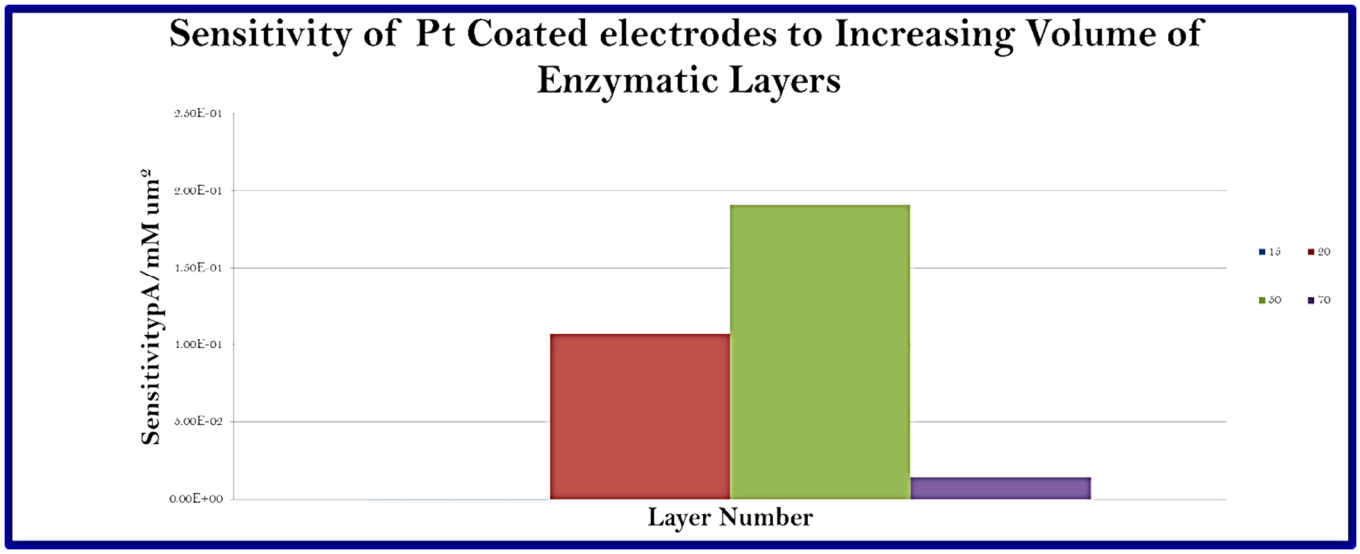

Figure 3B: Visualization of the change in sensitivity as the number of layers and corresponding concentration of glucose increase linearly.

Conclusion:

Firstly, the PDMS eyelash brush has thus far presented itself as the most viable means of manual deposition of the enzymatic solution onto the microelectrode surface. Compared to other means such as a 34 gauge metal syringe, or an even finer gauge wire, it is a safer and more effective tool for deposition as one can directly contact the Pt surface without interrupting either a preexisting layer or the metal surface of the electrode itself, which can otherwise easily be scratched or even removed by denser materials such as needles or wires. The single eyelash allows for some pliability without complete rigidity, so depressing gently onto the surface to produce a dabbing motion is optimized. This also allows for successful transfer of the enzyme-containing solution, which tends to stick and coat substrates.

Secondly, the methodology of manual deposition appears successful and consistent enough so that a discernable difference in layer volume can be seen even in slight comparisons, such as 15 layers versus 20. This data assures us that manual deposition of the enzymatic solution can be modulated according to layer volume in order to increase or decrease sensitivity to the substrate. It remains to be investigated how other layer numbers, such as 15, 20, and 70, can be measured on the 3D imaging microscope in order to measure deposition height. This could eventually be expanded to measure the volume of a single layer. This is important to streamlining the homogeneity of deposition, both manually and automatically. By accurately identifying the volume per layer, an automated deposition system could dispense the ideal volume onto single electrodes within an MEA in the time that it takes to manually coat a single microelectrode.

Thirdly, there appears to be an ideal number of layers for maximum sensitivity to glucose. As is shown in Figures 3A and 3B, 50 coats has a sensitivity of 0.1911 pA/mM μm^2, whereas 70 coats,

which one might think would produce a stronger current, only has a sensitivity of 0.0147 pA/mM μm^2. This is approximately a tenfold decrease in sensitivity compared to the 50 layer-coated microelectrodes. Even more interestingly, the 20 layer microelectrodes had an average sensitivity of 0.1072 pA/mM μm^2, but just 5 fewer layers (15), had a sensitivity of almost 0, at 0.0000485 pA/mM μm^2. Any number of coatings beyond 50 appears to hinder the reaction by diffusing the peroxide away from the surface of the electrode, rather than towards it. Too few layers, logically, does not contain enough enzyme to produce enough hydrogen peroxide to generate a discernable current signal.

A more optimized layer volume, in the vicinity of the ones identified in this paper, is currently being investigated to optimize current response. Once this "sweet spot" is identified, the experiment can then be tested using the real enzyme of interest and substrate, glutamate oxidase and glutamate.

Works Cited

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