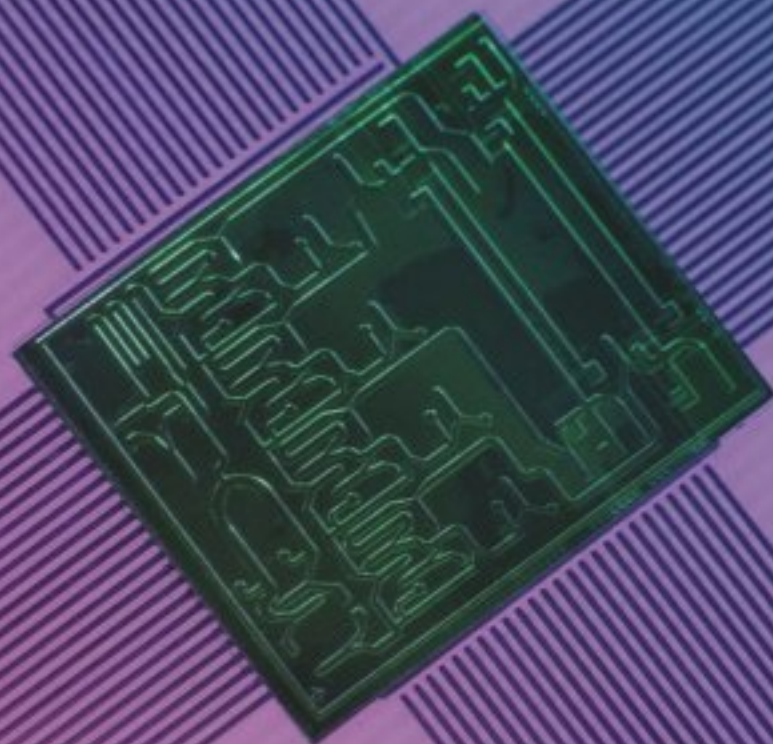


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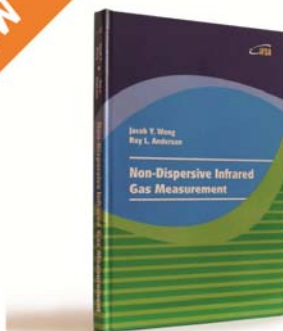
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Evaluation of Anchoring Materials for Ultra-Sensitive Biosensors Modified with Au Nanoparticles and Enzymes

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Abstract: In this report, we investigated the performance of ultra high performance sensors fabricated with identical biocomposite materials and procedures, except the anchoring conductive materials. The anchoring materials were glassy carbon, Pt, Au and Ag; the biocomposite layer consisted of polymer/Au nanoparticles/enzyme. The enzymes in the biocomposite layers are specific for the target detecting species which enable the coupling (detection) reactions to occur. The enzymes used in this study were lactate dehydrogenase (LDH), glutamate dehydrogenase (GDH), and hemoglobin. The specific target species for the detection included lactate, NH_4^+ , NO_2^- , and peroxide. The biosensors developed here were tested with solution concentrations ranged from 10^{-4} to 10^{-16} M, however, the lower concentration limit could be orders of magnitude lower. In all, the anchoring materials have drastic effect to the performance of this ultra-high performance biosensor platform; also, nature of the enzymes can alter the stability of these sensors. *Copyright © 2012 IFSA.*

Keywords: Performance, Anchoring material, Nanoparticle, Sensor, Bioenzymes.

1. Introduction

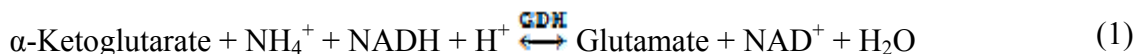
We have previously developed some of the most sensitive biosensors/electrodes modified by Au nanoparticles and enzymes that are capable of detecting concentration levels below ppb [1]. These

sensors were fabricated with composite layers of nanomaterials and enzymes anchored on conductive but non-reactive materials, such as glassy carbon and Pt. Performance of these sensors varies depending on the anchoring materials and as well as the composition of the biocomposite materials.

The important factors considered in modern era of chemical species detection are response time and concentration limit. Due to the advances of computer and nanotechnology, the fourth generation of sensor should have almost instantaneous response time and ultra-high detection sensitivity.

In this research, we examined the detection limits and performance consistency of an ultra-high sensitive biosensor platform that was anchored by various conducting materials: Au, Ag, Pt and glassy carbon. These electrode sensors coated with various biocomposite materials were then challenged by various biological and environmental samples for their performance, the target species included lactate, ammonia, NO_2^- , peroxide. Testing of performance for the anchoring materials varies from one (freshly prepared) to 28 days.

An illustration of detection principle by means of an enzymatic reaction is described in the following: glutamate and NAD^+ can be hydrolyzed to form α -ketoglutarate, NADH, and ammonium ion with the enzyme, glutamate dehydrogenase (GDH). The equilibrium constant is in favor of the formation of glutamate and thus the reverse reaction is faster kinetically:

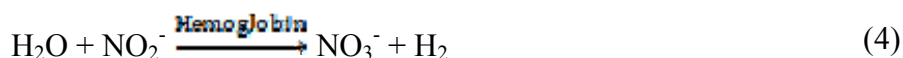


Other enzymatic reactions with similar detecting principle included in this study were lactate dehydrogenase (LDH) for the detection of lactate, and hemoglobin for the detection of H_2O_2 and nitrite. The following are the governing redox reactions catalyzed by the respective enzymes.

LDH:



Hemoglobin:



A layer of biocomposite material comprised of either one the above enzymes was deposited on the surface of the 4 anchoring electrode materials. A specified enzyme was used depending on the chemical species to be detected and the reactions were described in equations (1) to (4). For Equation (3) and (4), the end products are likely to be different within a biological system than the testing solutions as shown in the equations, the reactions would be reversible and the electron(s) generated with the peroxide and nitrite would have reacted with other electron receptors to form metabolite(s). Equations (1) to (4) are important to biomedical systems, thus the measurements of any of the chemical species involved; in addition, NH_4^+ , H_2O_2 , NO_2^- are important species for the monitoring of environmental health. Hence, the biosensors that are being developed here can be valuable in many applications in both biomedical and environmental science.

2. Materials and Method

2.1. Electrodes

Au, Ag, Pt, and glassy carbon (GCE) electrode all had diameter of 0.2 cm. The platinum counter electrode had diameter of 0.1 cm and length of 0.5 cm. They all were purchased from Tianjin Aida Heng Sheng Co, Tianjin, China. These electrodes were coated with either GDH, LDH, or hemoglobin depending on the target chemicals to be detected [1].

2.2. Materials

All enzymes and biochemicals were purchased from Sigma-Aldrich Chemical Co, St. Louis, MO, USA. Cysteamine and α -ketoglutarate, $\text{AuCl}_3\text{HCl}\cdot 4\text{H}_2\text{O}$ (Au %> 48 %) and $\text{Na}_3\text{citrate}$ were purchased from Sigma. All the other chemicals were of analytical grade or highest grade available. All the experiments were carried out under deoxygenated condition in 0.1 M phosphate buffer solution at pH 7.0 or otherwise specified.

2.3. Nanoparticles and Electrode Preparations

Nanoparticles and electrodes were prepared according to methods reported previously [1, 2].

2.4. Detections

Cyclic voltammetry was conducted by using a Gamry 600 Potentiostat. Voltammetric potential was measured against a saturated chloride electrode (SCE).

3. Results and Discussions

3.1. GDH Coating on GCE, Au, and Pt Electrode for NH_4^+ Detection

Figs. 1 to 3 show the voltammetric responses of the modified GCE, Au, and Pt electrode with GDH biocomposite. As it is shown, the characteristic peak of α -ketoglutarate and NH_4^+ conversion to glutamate was detected at about 750 to 800 mV at pH 7.0. In comparison, the responses of Pt electrode (Fig. 3) were much preferred since they were “smoother” responses stepwise and larger in magnitude.

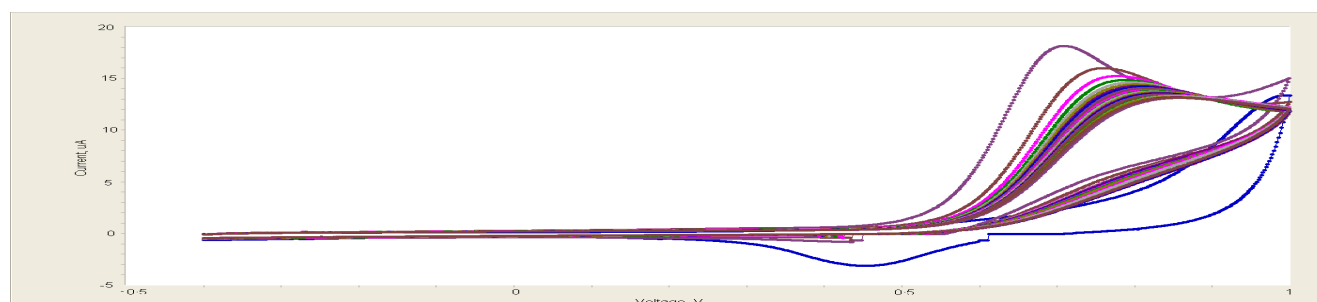


Fig. 1. Voltammetric responses of a GCE coated with GDH at pH 7.0 on Day 1. Responses were stepwise additions of α -ketoglutarate with NH_4^+ from 1.0×10^{-16} mol/L to 1.0×10^{-4} mol/L at 750 mV. The concentration of NADH was 3×10^{-3} mol/L.

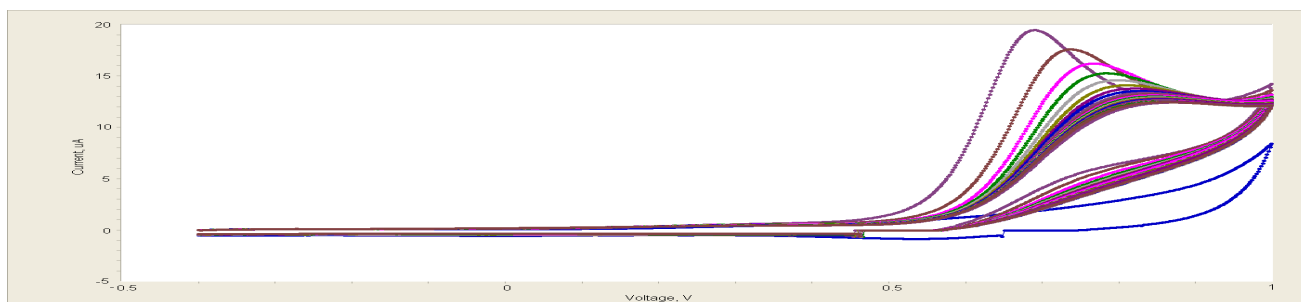


Fig. 2. Voltammetric responses of an Au electrode coated with GDH at pH 7.0 on Day 1. Responses were stepwise additions of α -ketoglutarate with NH_4^+ from 1.0×10^{-16} mol/L to 1.0×10^{-4} mol/L at 800 mV. The concentration of NADH was 3×10^{-3} mol/L.

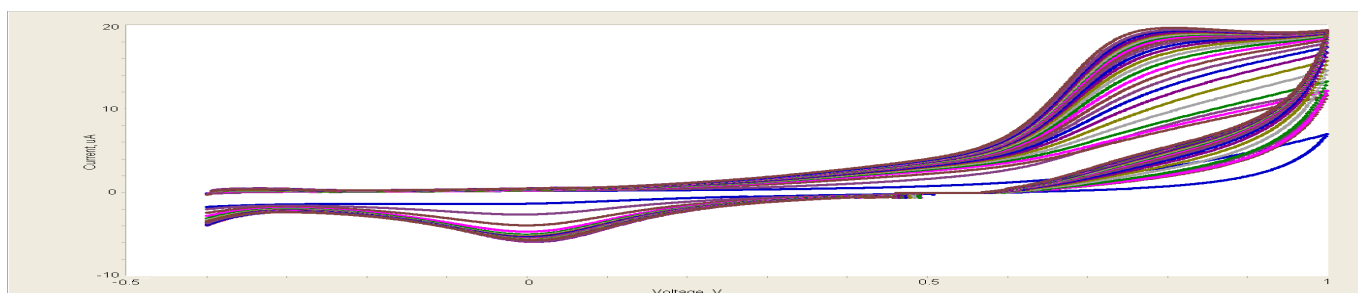


Fig. 3. Voltammetric responses of a Pt electrode coated with GDH at pH 7.0 on Day 1. Responses were stepwise additions of α -ketoglutarate with NH_4^+ from 1.0×10^{-17} mol/L to 1.0×10^{-4} mol/L at 800 mV. The concentration of NADH was 3×10^{-3} mol/L.

Fig. 4 shows the accumulative current responses with the Au, Pt and GCE. As seen, current increased with concentration for the Pt electrode which indicated oxidation while current decreased with concentration for the Au and GCE which indicated reduction. Nevertheless, all three electrodes can be used for sensing purpose as long as their responses are consistent; however, Pt appeared to be the best anchoring material in this application of NH_4^+ detection. The concentration range used in this test was between 1×10^{-16} to 1×10^{-4} M.

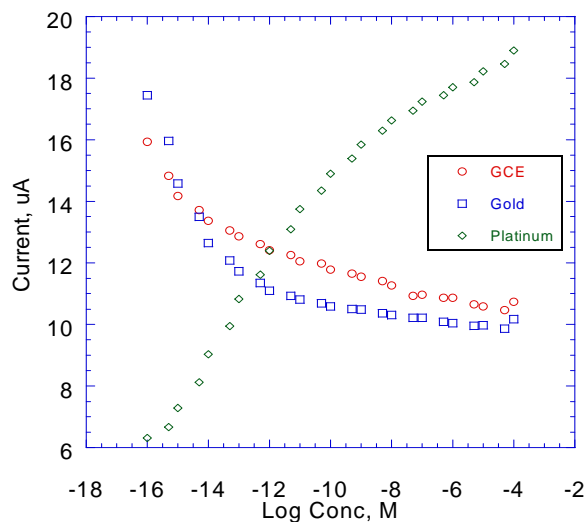


Fig. 4. Performance comparison by current measurements at peaks for NH_4^+ detection on Day 1 with Au, Pt and GCE at pH 7.0.

3.2. LDH Coating on Pt, Au, and GCE for Lactate Detection

Fig. 5 and Fig. 6 show the voltammetric responses of the modified Au and Pt electrode with LDH biocomposite. As it shown, the characteristic peak of lactate conversion to pyruvate was detected at 280 mV at pH 7.0. The characteristic peak was somewhat upstream from the peak at above 260 mV reported in some literature reports, but was similar to other reports that found the peak at about 280 to 300 mV [3, 4].

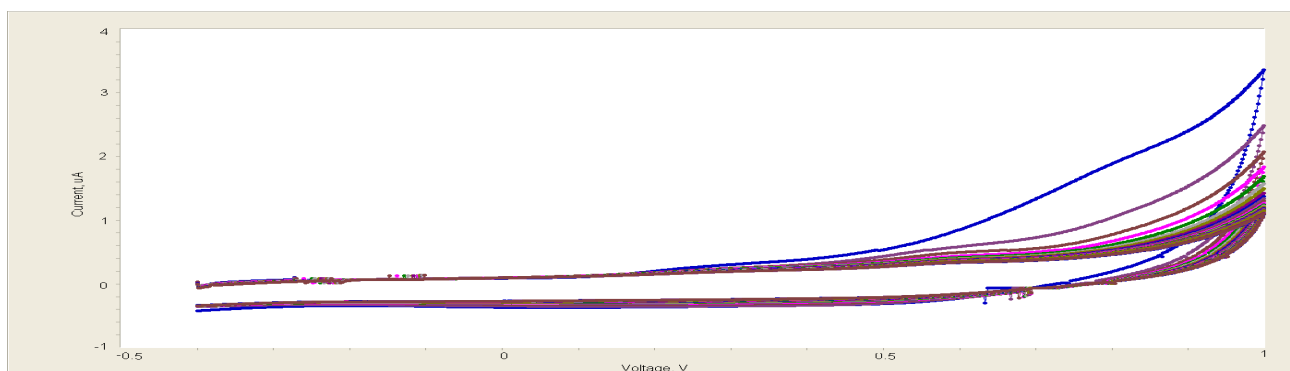


Fig. 5. Voltammetric responses of an Au electrode coated with LDH at pH 7.0 on Day 1. Responses were stepwise additions of lactate from 1.0×10^{-16} mol/L to 1.0×10^{-4} mol/L at 310 mV. The concentration of NAD^+ was 3×10^{-3} mol/L.

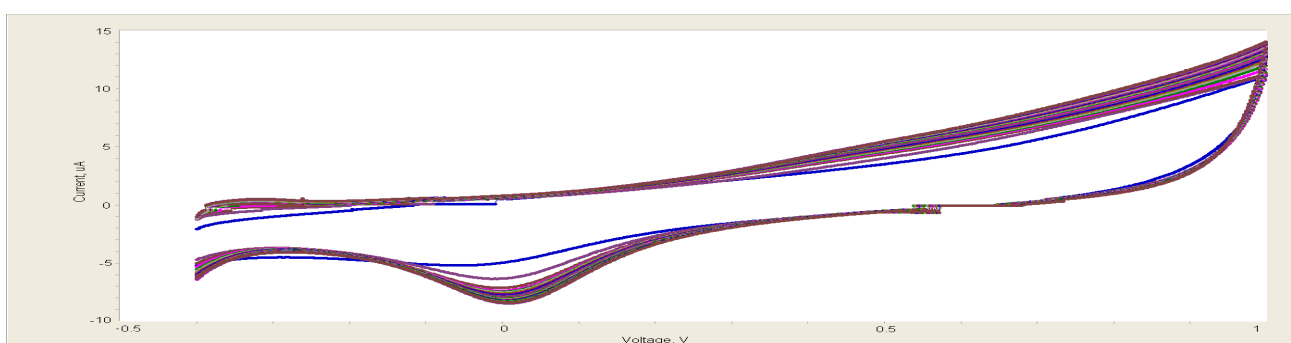


Fig. 6. Voltammetric responses of a Pt electrode coated with LDH at pH 7.0 on Day 1. Responses were stepwise additions of lactate from 1.0×10^{-16} mol/L to 1.0×10^{-4} mol/L at 310 mV. The concentration of NAD^+ was 3×10^{-3} mol/L.

Fig. 7 shows the comparison of performance for the GCE, Au, and Pt electrode. As seen in Equation (2), the reaction is a reversible catalytic reaction with which either oxidation or reduction can occur depending on the reacting condition. Our results shown here indicate a relative weak reductive reaction for GCE and Au electrode, and a strong oxidative reaction for the Pt electrode in the tested concentration range. Equation (1), hence detection of lactate, proved to be difficult to control. Fig. 7. shows a weak reductive curve for the Au electrode; but we have observed that an oxidative curve can also be generated under the same testing procedures and condition, and the current signal detected with our biosensors is just as sensitive or better than reported in literature [3-5]. The reason that causes the oscillation of redox direction of Equation (2) with the Au electrode is still unclear at this point. Our results indicated that Pt electrode is the anchoring material of choice in lactate detection especially for very low detection concentrations that is below 10^{-7} M; the sensitivity of this ultra-high performance (Pt) for LDH detection is not found thus far in the literature. A slight drawback of the Pt electrode was

that it tended to have higher signal perturbations at concentrations above 10^{-7} that were not seen for the other 2 electrodes.

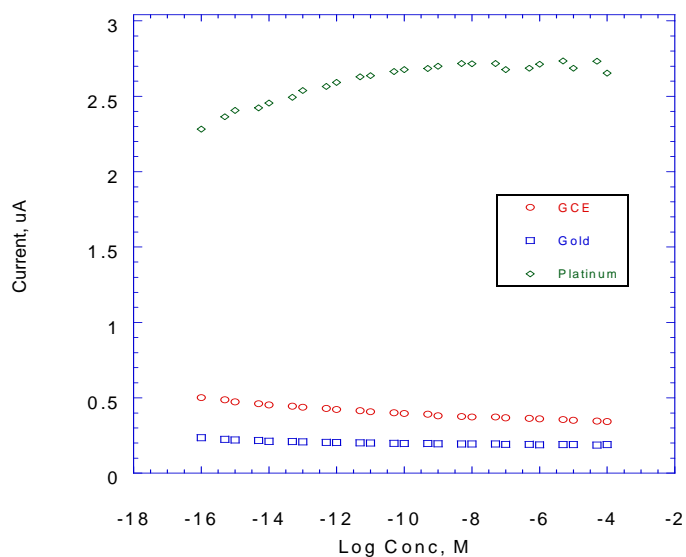


Fig. 7. Comparison of performance for the GCE, Au, and Pt electrode for lactate detection on Day 1 at 280 mV in concentrations from 1.0×10^{-16} mol/L to 1.0×10^{-4} mol/L.

3.3. Hemoglobin Coating on GCE, Pt, Au Electrode for H_2O_2 and Nitrite Detection

Hemoglobin is a biocatalyst that aids many oxidation processes in biological systems. Similar redox reactions can be observed when oxidation and reduction agents are mixed in the presence of hemoglobin molecules. As shown in Fig. 8, a distinguished reductive peak (Equation (3)) was observed at about 1.1 V for a freshly prepared GCE at pH 7. A similar peak could also be detected by using a Pt electrode but the peak is an oxidative peak, and the current signals were diminishing with time for the two electrodes in the concentration range investigated in this study from 10^{-4} to 10^{-16} M. However, the Au electrode revealed a very different behavior with time for the same biocomposite coating: its redox reaction shifted directions with time and eventually exhibited a reductive peak after 28 days (more discussion with the redox reaction oscillation of the Au electrode in later section).

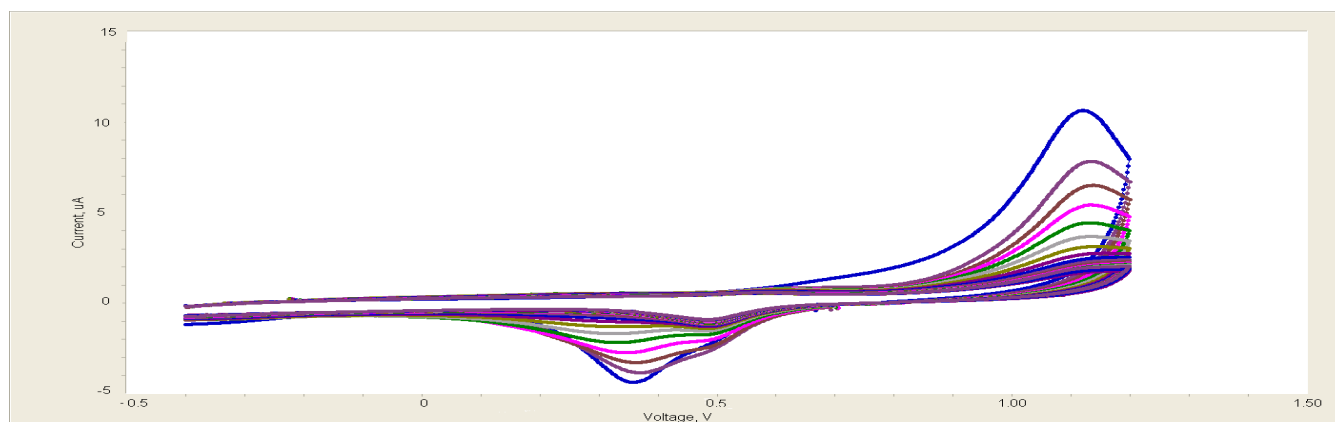


Fig. 8. Cyclic voltammogram of oxidation of H_2O_2 with a GCE coated with a layer of hemoglobin biocomposite at pH 7.0 with concentrations ranged from 10^{-4} to 10^{-16} M on Day 1. The characteristic redox peak of H_2O_2 was detected at approximately 1.1 V.

Fig. 9. shows the cyclic voltammogram of a highly modified Pt electrode with hemoglobin biocomposite for the detection of NO_2^- on Day 28, which exhibited a characteristic oxidative peak at 800 mV. The characteristic peak may shift downstream/upstream with time which is also an indicator that there are intrinsic changes within the biocomposite which affect the activation potential of the electronic transfer for the oxidation of NO_2^- ; for the Pt electrode, the peak shifted from 850 mV on Day 1 to 800 mV on Day 28. The lowering of activation potential implies lowering of potential requirement for the electron transfer of the oxidation of nitrite to nitrate, or a more efficient catalytic arrangement by the hemoglobin biocomposite.

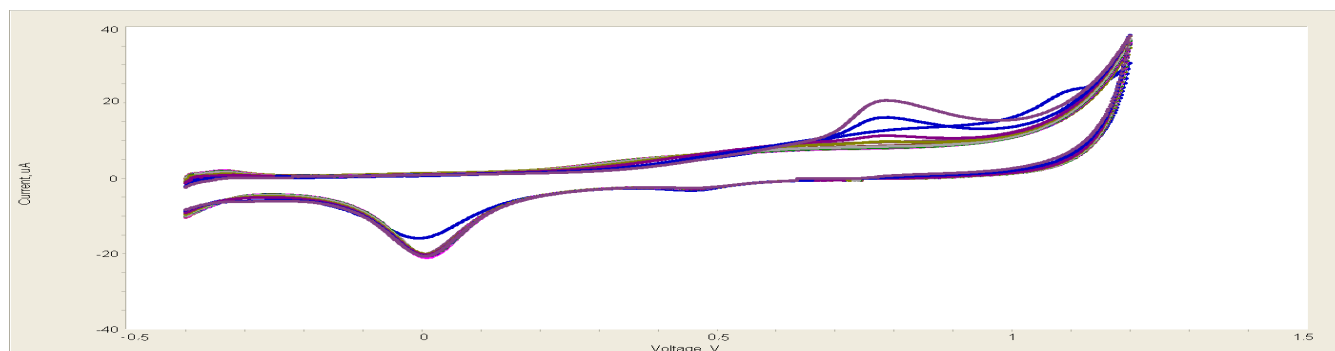


Fig. 9. Cyclic voltammogram of oxidation of nitrite with a Pt electrode with a layer of hemoglobin composite at pH 7.0 with concentrations ranged from 10^{-4} to 10^{-16} M on Day 28. The characteristic redox peak of nitrite was detected at approximately 0.8 V.

Fig. 10 shows the performance comparison of GCE, Au, and Pt electrode for nitrite detection from concentrations of 10^{-4} to 10^{-16} M. As shown, Pt electrode provided the largest current signal for the concentration range thus is the best anchoring material for nitrite detection within these concentrations. In fact, other than at the extremely low concentrations, Pt electrode was the only electrode with moderate current increase with concentration until a “cutoff” concentration at about 10^{-6} M, then all three electrodes provides reasonable current responses. In general, the three materials worked well as anchoring materials for the nitrite detecting sensor when concentrations were higher than 10^{-6} M, the ranking of materials was Pt > Au > GCE on Day 1; as the enzymatic biocomposite material aged, the ranking of materials herein remains the same, but the magnitude of current increased with time for Pt and Au, whilst the current decreased with time for GCE. Overall, Pt suited the best as anchoring material for the detection of nitrite with this biocomposite material.

3.4. Specificity of the Ultra-High Performing Electrode Sensor

Fig. 11 shows the cyclic voltammetry responses of the specificity test on GDH coated on a Pt electrode. In the specificity test, NO_2^- , and H_2O_2 were used to replace NH_4^+ in Equation (1) and the concentration range used was between 10^{-16} and 10^{-6} M. Comparing with Fig. 3, Fig. 11 shows that almost no separation (or difference in current signal) was observed at 800 mV due to concentration variations at which NO_2^- , or peroxide was added in the testing solutions, that implied the GDH coating has selectivity.

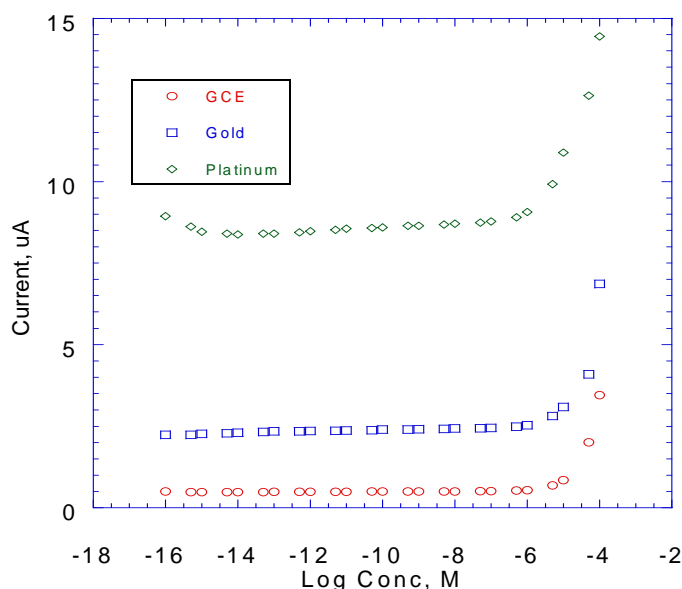


Fig. 10. Current outputs of oxidation of nitrite with the GCE, Au, and Pt electrode from 10^{-4} to 10^{-16} M on Day 1. Pt electrode provided the largest current output (signal) thus is the most ideal for nitrite detection.

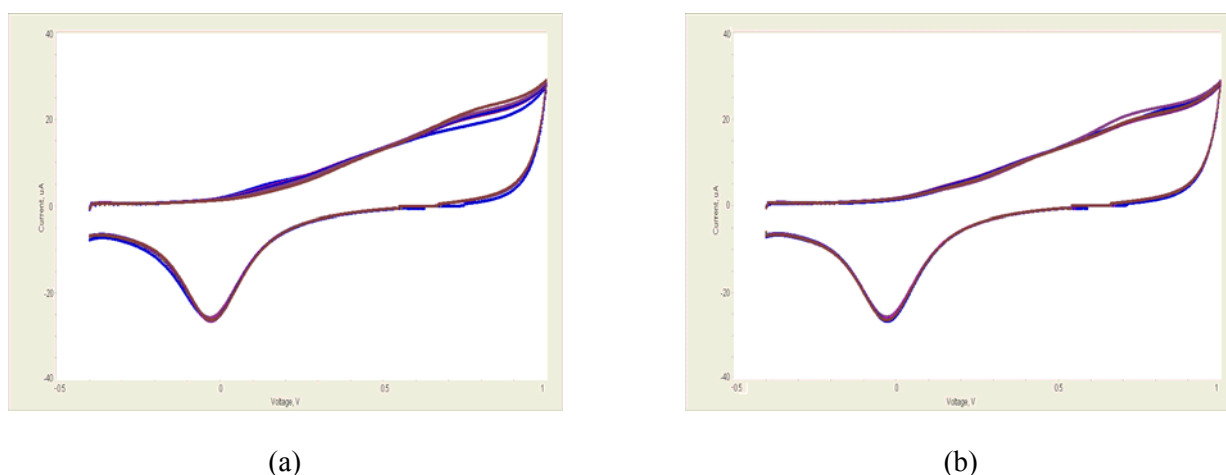


Fig. 11. Voltammetric responses of a Pt electrode coated with GDH biocomposite coating. The coating was designed for NH_4^+ detection as described in Equation (1), but the NH_4^+ was replaced by (a) NO_2^- , and (b) H_2O_2 as tested.

3.5. Stability of Biosensor Electrodes with Time

In general, the sensitivity of this high performance biosensor decreased with time after the sensor was prepared. The performance stability of this sensor was tested after 1, 5, and 28 days with the anchoring materials made of Pt, glassy carbon, and Au. As it is shown in Fig. 12, the performance of the sensor steadily declined with time which is common as expected for sensor comprised of enzymes as part of the detecting mechanism. Although the matrix of the compositional materials stabilizes and decelerates the enzymatic decay substantially, the decay is still inevitable after a prolonged period in the buffer solution (pH 7). However, the duration of the decay, herein reversely proportionally to sensitivity of the sensor, varies greatly with the anchoring material and nature of the enzyme(s) used in the fabrication process. Fig. 13 shows the performance of a Pt electrode coated with hemoglobin for nitrite/nitrate detection on Day 1, Day 5, and Day 28. The sensitivity of sensor remained about the

same with time and actually increased slightly on Day 28, which implies that the hemoglobin molecular structure within the biocomposite layer remain intact and may even open the binding sites with NO_2^- slightly more after 28 days of aging, thus generating higher current or signaling.

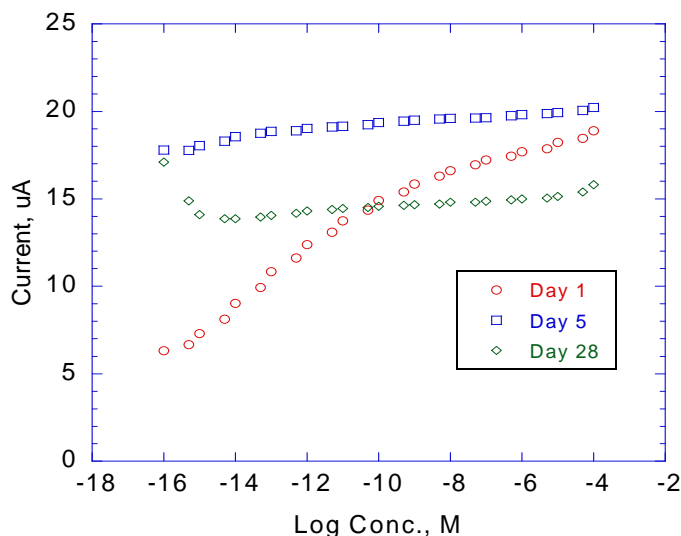


Fig. 12. Performance stability of Pt electrode coated for NH_4^+ detection on Day 1, Day 5, and Day 28 with cyclic voltammetry at 750 mV. Sensitivity of the sensor steadily declined with time.

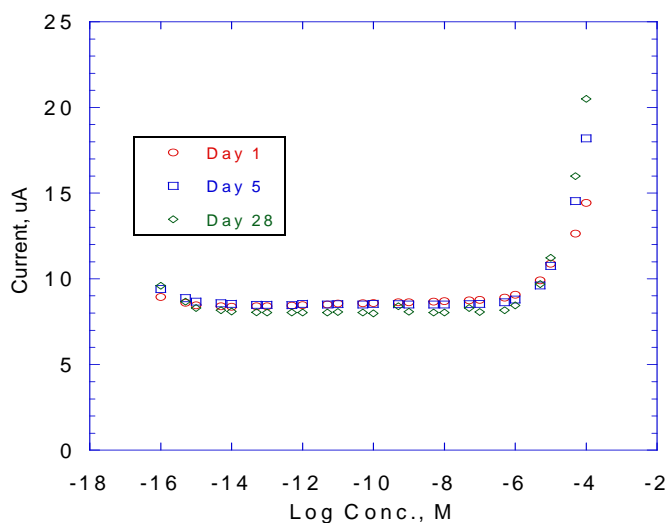


Fig. 13. Performance of a Pt electrode coated with hemoglobin for nitrite/nitrate detection on Day 1, Day 5, and Day 28. The sensitivity of sensor remained about the same with time.

Fig. 14 shows the performance of an Au electrode coated with GDH biocomposite for NH_4^+ detection. Notice that the current consumption (oxidation) of the electrode on Day 28 is larger than on Day 1, while the current signal on the Day 5 was nearly flat or increased lightly (reduction) with time which signified the reverse reaction of the enzymatic reaction that was used for the NH_4^+ detection scheme (Equation (1)). The observation is rather interesting, definitely is not impossible in utilizing the enzymatic reactions for chemical species detection: GDH is a rather complex, large enzyme which exists as a multi-unit moiety; as a consequence, it is also a stable enzyme comparatively. It is believed that the enzyme opened up more binding sites with NH_4^+ on Day 28; its transition of configuration on Day 5 became more favorable for the reverse reaction (Equation (1)). The observation was consistent throughout our same repeated experiments and it only occurred with the Au electrode as anchoring

material. Similar phenomena were also observed with the Au electrode when more modifications of the biocomposite layer were done to enhance the sensor performance [2, 6].

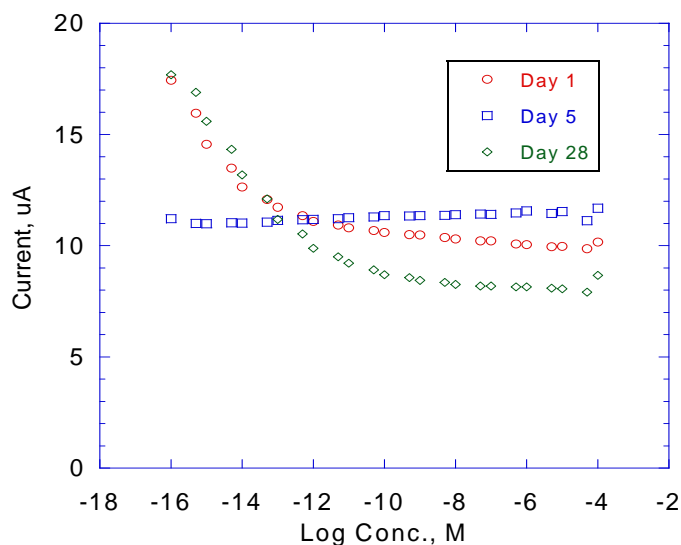


Fig. 14. Performance of an Au electrode coated with GDH for NH_4^+ detection on Day 1, Day 5, and Day 28.

Another evidence of the transition of enzymatic configuration within the biocomposite that can significantly affect the performance of the biosensor was observed with the Au anchoring electrode that is shown in Fig. 15. The signal outputs from Day 1 to Day 28 changed gradually: the sensor went through 2 cycles of redox reactions (Equation (2)) over the concentration range of 10^{-16} to 10^{-4} M for the Day 1 measurements, and slowly reduced to one cycle of redox reaction on Day 28. From the sensor detection point of view, performance of the sensor on the latter time was much preferred which provides an effective concentration range of 10^{-6} to 10^{-16} M (with only reductive reaction in the concentration range). The signals given by the Au electrode on Day 28 were similar to the signals given by a GCE on Day 1 measurements (Fig. 16). The sensor performance shown in Fig. 16 is more common than what is shown in Fig. 15.

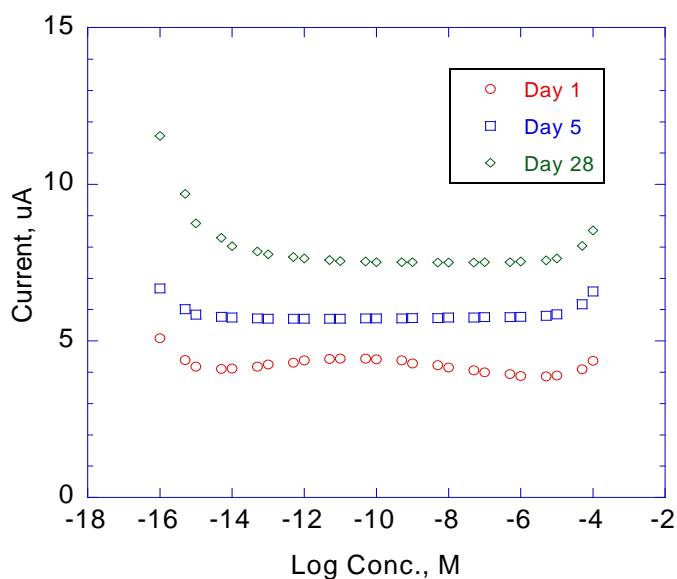


Fig. 15. Biosensor signal affected by transformation of enzymatic configuration. An Au electrode coated with hemoglobin for H_2O_2 detection.

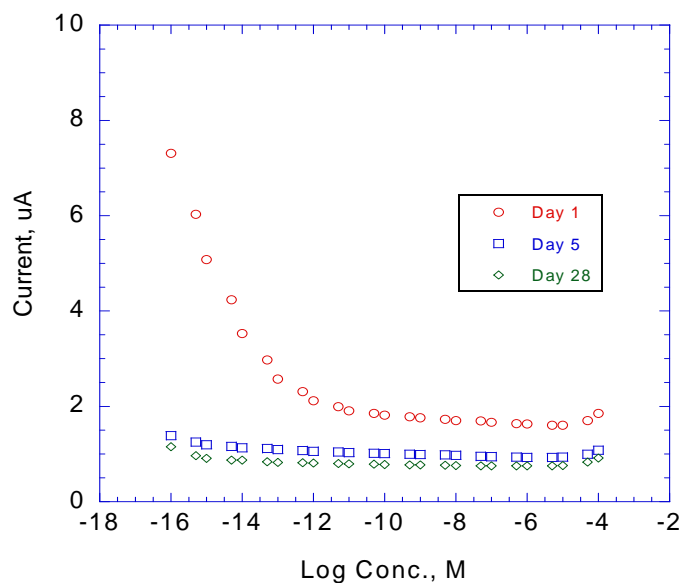


Fig. 16. GCE coated with hemoglobin for H_2O_2 detection at pH 7.0. The sensor lost essentially all its sensitivity for H_2O_2 detection on Day 5 and Day 28, but exhibited strong signals for the measurements on Day 1, in particular at very low H_2O_2 concentrations.

4. Conclusions

We successfully modified our ultra-high performance biosensor platform with Au, Ag, Pt, and glassy carbon as anchoring materials. These electrode sensors could detect target chemical concentrations routinely at 1×10^{-16} M and lower, depending on the enzyme couplings and anchoring materials.

Among the 4 metals used in the testing, Pt was thought to be the most ideal metal due to its inertness to chemical reactions and often provided the strongest signals. However, Pt appeared not to bind well with our linker polymers that was part of the biocomposite layer and thus did not generate characteristic signals as distinctively as other metals for at least the coupling enzyme, LDH; further research should be given to this direction. Despite of this observation, Pt electrode appeared to be functioning competitively with GDH and hemoglobin as part of the composite layer for the corresponding coupling reactions, thus the detections of target species. Glassy carbon worked competitively as compared to the metal electrodes, but is the most expensive among all electrodes tested. Ag electrode was easily oxidized and did not provide signals consistently in repeated testings and thus was not considered in the reported results, despite that it is the most inexpensive electrode.

It can be concluded that the anchoring materials have drastic effect to the performance of this ultra-high performance biosensor platform, their effect is mutually inclusive with the enzymes used and time (age) which affect the sensor's stability. These factors should be considered when diversified applications are to be developed.

Acknowledgement

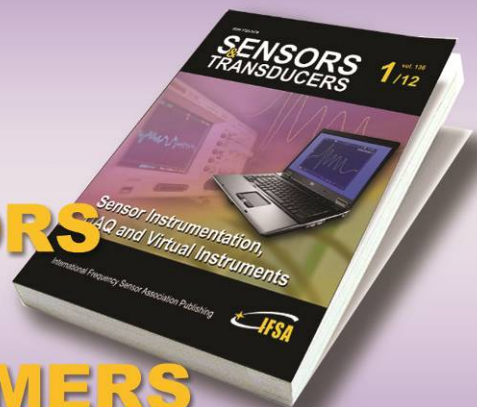
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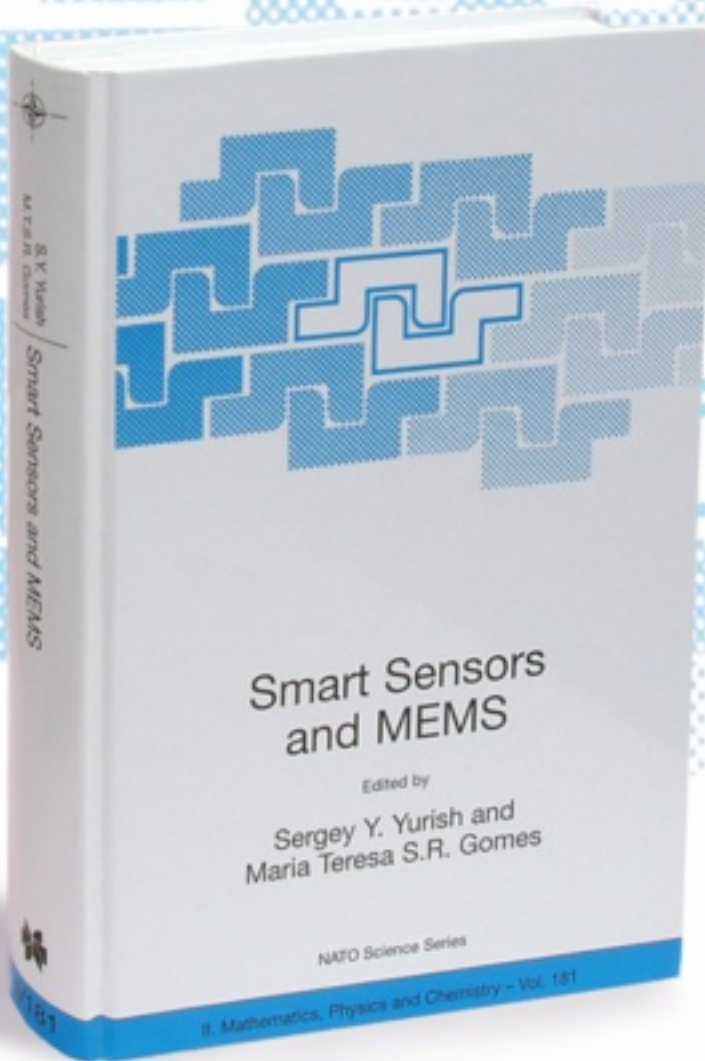
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