Nano-structured Platform
for Highly Selective, Sensitive and Quantitative Electrochemical Detection of Small Redox-active Cellular messengers in aqueous buffer solution with biological interferrents

by

Tsz Lun WONG

A Thesis Submitted to
The Hong Kong University of Science and Technology
in Partial Fulfilment of the Requirements for
the Degree of Master of Philosophy
in Bioengineering

November 2012, Hong Kong
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This is to certify that I have examined the above MPhil thesis
and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by
the thesis examination committee have been made.

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Acknowledgements

First and foremost, I would like to express my deepest gratitude to my advisor, Prof. Xiao-Yuan Li, for his insightful guidance, invaluable discussion, concrete instructions, helpful suggestion, enthusiastic and timely support during the whole period of my M. Phil studies. He led me to the right way of doing research and it will benefit me for the rest of my life.

I would like to give my sincere thanks to Dr Wen-Zhan Wu, Dr Wen-Yan Tao and Miss Yan-Xin Wang for their collaboration in Chapter 2, especially on H₂O₂ detection experiments. I especially express my sincere acknowledgement to Dr Wen-Zhan Wu for her patient guidance. I would like to thank Prof. Hong-Kai Wu for providing us with the compound ascorbic acid in Chapter 2.

I am sincerely grateful to the members of my Examination Committee, Prof. Hong-Kai Wu and Prof. Zhi-Hong Guo for their advice and guidance on my thesis.

The members of Prof. Li’s group have contributed immensely to my personal and professional time at HKUST. The group has been a source of friendship as well as good advice and collaboration. I really want to thank my group mates Dr Juan Chen, Dr Jian-Bing Hu, Mr Wei-Qiang Lv, Dr Ying-Shun Li, Dr Wen-Zhan Wu, Dr Wen-Yan Tao and Dr Peng Liu for their help. Working and living with them made my stay at HKUST an enjoyable and unforgettable experience.
I would like to thank all the faculty members, researchers and supporting staff of Department of Chemistry, Division of Biomedical Engineering and MCPF for their help in my studies and research.

Last but not least, I sincerely thank my parents for their everlasting understanding, love, encouragement and firm support to me during the past years.

Tsz Lun WONG
HKUST
July 2012
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Fig. 15: The DPA curve of control of nafion- Au nano-frame electrode. For testing the fidelity. \( E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s \).

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Fig. 17: The DPA graphs of addition of different methionine concentrations from 125μM to 500μM on nafion - Au nano-frame electrode by desorption method. \( E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s \).

Fig. 18: The DPA graphs of addition of different glutathione concentrations from 500μM to 2000μM on nafion - Au nano-frame electrode by desorption method. \( E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s \).
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>cus</td>
<td>Coordinatively undersaturated</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammogram</td>
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<tr>
<td>CYS</td>
<td>Cysteine</td>
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<tr>
<td>DPA</td>
<td>Differential pulse amperometry</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>Glu</td>
<td>Glucose</td>
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<tr>
<td>GOx</td>
<td>Glucose oxidase</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
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<tr>
<td>H₂S</td>
<td>Hydrogen sulphide</td>
</tr>
<tr>
<td>MET</td>
<td>Methionine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PTFE</td>
<td>Poly-(tetrafluoroethylene)</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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Nano-structured Platform for Highly Selective, Sensitive and Quantitative Electrochemical Detection of Small Redox-active Cellular messengers in aqueous buffer solution with biological interferrents

by

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Abstract

The objective of this thesis is to develop a robust, flexible and versatile nano-structured platform for the detection of small redox-active biomessengers and demonstrate the platform by applying it to the detection of H$_2$O$_2$, CO and H$_2$S.

Endogenously generated small redox-active molecules represent a unique class of cellular messengers in biological systems. Examples include reactive-oxygen-species (ROS) such as hydrogen peroxide (H$_2$O$_2$) and superoxide O$_2^-$, reactive-nitrogen-species (RNS) such as NO, HNO and ONOO$\cdot$, as well as carbon monoxide (CO) and hydrogen sulfide (H$_2$S), among others. Their generation, targeting and regulation are associated with diverse yet critically important biological, physiological and pathological functions. For the study of their biological functions as well as the elucidation of their working mechanisms, it is highly desirable to have methodologies and techniques for their detection in a selective, sensitive and quantitative manner and in the presence of many interfering chemical species.

Chapter 1 gives a concise introduction and overview of the importance of detecting small cellular messengers, the advantages of using electrochemical method as a sensing technique and the reasons for using nanotechnology. The objective of this thesis is also included.
Chapters 2 report our effort in the development of a nano-structured electrochemical platform and its usage in the detection of H₂O₂ and glucose in aqueous buffer solution with biological interferents. This platform contained three functional layers: an analyte-dependent catalyst, a nano-structured filter and a substrate surface for immobilization of biomolecules or other desirable macromolecules. By using nano-structured platinum (Pt) as the catalyst, we were able to measure H₂O₂ by electrochemical oxidation in a broad concentration range, and in a sensitive, quantitative, and selective manner. As a natural extension to the detection of H₂O₂, we developed a glucose sensor by combining the H₂O₂-sensing platform with the immobilization of glucose oxidase (GOD) on the nano-structured surface spatially separated from that of the H₂O₂-catalysts surface. The selective detection of H₂O₂ and glucose in the presence of common interfering chemical species was also studied.

The linear range of detecting H₂O₂ we achieved was from 10μM to 5mM. The linear range of detecting glucose we achieved was from 50μM to 5mM. The detection limit was 40μM. The Km of the glucose oxidase we found on GOx - Pt nano-frame electrode was 24.02mM. The interference coefficient of ascorbic acid with respect to H₂O₂ detection by using the DPA method we developed on Au nano-frame electrode was 0.114. The measurable range of glucose without normal level (100μM) ascorbic acid significant interference (20%) was 500μM to 5mM.

Chapters 3 report a method developed recently in our group for the detection of CO in aqueous buffer solution. The method was built on the similar nano-structured electrochemical platform that we developed for the detection of H₂O₂ and glucose but with one important difference: we chose a nano-structured electro-active pseudo-catalyst (electrochemical mediator), Cu₂O, for the detection of CO. The reason was that CO is an electronically very inert molecule toward either catalytic oxidation or reduction in aqueous medium and in ambient conditions. By selecting Cu₂O as a pseudo-catalyst (electrochemical mediator), we employed a non-redox reaction, yet electrochemical active approach for the detection of CO in aqueous buffer solution selectively and quantitatively. This approach has an intrinsic advantage of immune to many interfering species. The pros and cons of our method will be discussed.
The detection limit of CO on Cu sputtering electrode was found to be 4.60μM using ΔIpc and 19.54 μM using ΔIpa. The detection limit of CO on Cu deposition electrode was found to be 4.95μM using ΔIpc and 21.27μM using ΔIpa. For low concentration range from 5 to 50μM, using ΔIpc, the sensitivity of Cu sputtering electrode was about 3 times higher than that of the Cu deposition electrode, hence the Cu sputtering fabrication method was better than the Cu deposition fabrication method.

Chapters 4 reports a very promising method for the highly selective, sensitive and quantitative detection of H2S in aqueous buffer solution and in the presence of the most common interfering species including cysteine, methionine, and glutathione. Our method was built on the nano-structured electrochemical platform we developed for the detection of H2O2 and glucose, but with the selection of nano-structured gold (Au) catalyst uniquely suited for thiol-containing compounds such as H2S. Special effort was spent on solving the interfering problems caused by cysteine, methionine and glutathione.

The linear range and detection limit of detecting H2S by desorption method we developed were from 5μM to 200μM and 2μM respectively. The linear range and detection limit of detecting H2S by adsorption method were from 10μM to 3mM and 10μM respectively. The interference coefficient of cysteine with respect to H2S detection by using the DPA desorption method on nafion - Au nano-frame electrode was 0.00915. There was no interference found from methionine and glutathione by using the DPA desorption method on nafion - Au nano-frame electrode.

This thesis reports the development of a new, robust and flexible electrochemical platform for the detection of various redox-active gaseous molecules in aqueous media. This platform has a unique advantage of integrating and supporting three nano-structured components: an analyte-dependent catalyst, a screening or filtering structure, and a substrate-surface for immobilization of biomolecules (or other desirable macromolecules). A brief summary and future perspectives are given at the end of the thesis.
Chapter 1

General Introduction
1.1 Small cellular messengers

Small cellular messengers [1-5] are a group of molecules that transmit cellular signals [6-10] within or outside a cell. Some of them are small and bear no charge, so they can diffuse very fast inside the cytosol and across the cellular membrane to achieve cellular functions [11-15], and are able to act as second cellular messengers to transmit signals both within and outside a cell.

As the small cellular messengers can travel across the membrane and their diffusion is fast, the time delay of the signal transmission from inside to outside of a cell is small. Compared to those proteins and enzymes, they can be more easily detected. Hence, they are important checkpoints of knowing what is happening inside a cell by detecting their concentration outside the cell, instead of the inside which would be more complicated. In other words, by detecting the concentration of small cellular messengers outside the cell, we can probably get some hints on which signal transduction cascade [16-20] is functioning. Conversely, by activating a known signal transduction cascade and monitoring the concentration of each molecule, we would probably know whether or not these small cellular messengers are related to the particular cascade we activated and we may also get some hints on the involvement of these cellular messengers and the signal transduction cascade under them.

H$_2$O$_2$, CO and H$_2$S are all examples of important small cellular messengers that are not charged. All of them are redox active: H$_2$O$_2$ and H$_2$S are detected by redox reactions inside cells naturally by using the amino acid side chain of different proteins [21-25], while CO is detected by the strong adsorption property toward the metal center of different proteins [26-33]. The difference between their sensing systems is due to the intrinsic properties of each molecule. As CO is inert, its oxidation or reduction requires a relatively large amount of energy (including the fabrication of a particular enzyme) and for the sole purpose of CO sensing it is not worth it. H$_2$O$_2$ and H$_2$S, on the other hand, can easily be oxidized or reduced.
H$_2$O$_2$ is a reactive oxygen species (ROS) [34-45]. It is famous for its toxicity (cell damaging role) and has been connected to stress response, apoptosis, aging and death. Recently, it has also been discovered to be an important cell growth and cell proliferation signal [46-53]. In addition, it was found to be one of the most important immune cell activating and guiding molecules [54-55]. Although it is a long discovered molecule, the significance of exploring its other functions is still there and so there is a need to measure H$_2$O$_2$ both inside and outside cells.

CO and H$_2$S are signalling gaseous molecules discovered after NO [56-65]. The discoverers of NO being a signal molecule were awarded the Nobel Prize in 1998. It opened the door to the possibility of gaseous molecules acting as signalling molecules, in other words, the possibility of discovering a new group of signalling molecules that work in different signalling transduction cascades to perform many important cellular functions. CO and H$_2$S are two signalling molecules that work similarly to NO. As it was only recently that CO and H$_2$S were found to be signalling molecules, there is an urgent demand to measure CO and H$_2$S both inside and outside cells to investigate the signalling transduction cascade involving them.

By developing a sensor of H$_2$O$_2$, CO and H$_2$S, we hope to fill the knowledge gap of those cellular messengers that we previously did not know, which may help develop new drugs with bigger effects and smaller side effects in many aspects.

1.2 Current method of measuring small molecular cellular messengers

The current methods of measuring small molecular cellular messengers such as H$_2$O$_2$, CO and H$_2$S are diversified, including top space gas chromatography [66-72], high performance chromatography [73-80], infrared spectrometry [81-85], UV-visible spectrometry [86-90], fluorescence spectrometry [91-95] and electrochemical methods. Among these techniques, top space gas chromatography and high performance chromatography cannot instantaneously detect the molecules and a quenching step is needed to stop the reaction before the measurement. Infrared spectrometry and UV-visible spectrometry are not able to be applied to
biological samples in real time or in vivo, as those techniques require samples to be transparent in a particular range of wavelengths. In addition, their measurable concentration range is small, so normally pre-treatment and dilution of the samples are required.

Fluorescence spectrometry, similar to electrochemical methods, is widely used in biological measurement both in vivo and in vitro, especially after the development of the use of green fluorescent protein (GFP) [96-100]. The major drawback of fluorescence spectrometry is the quenching effect of the fluorescent probe, which means the signals would decrease with time. Another concern is the compatibility of fluorescent probe’s applicable environment and our required experiment conditions such as pH values and ionic concentrations, which adds to the difficulty of applying it to biological measurement.

The functional change or disability of the target molecule after tagging the fluorescent probe is another concern, as after tagging the fluorescent probe, the probe would become part of the molecule. Last but not least, the selectivity of the fluorescent probe, especially for the small molecules, including H₂O₂, CO and H₂S, is the biggest problem. These molecules have very simple molecular structures, so it is nearly impossible for them to selectively bind to the probe by steric effect. A mediator can be used to react with these small molecules and we can measure the concentration of the mediator instead of that of the molecules directly. However, most mediators still have a selectivity issue.

By comparisons, electrochemical methods are better than the other current methods in terms of sensitivity, selectivity, ability to measure instantaneously and bio-compatibility [101-105]. In this thesis, electrochemical methods will be used to develop the sensory platform of small molecule cellular messengers including H₂O₂, CO and H₂S.
1.3 Nano technology

Nano technology [106-110] is the manipulation of the matter of at least one dimension on nano scale. The nano scale includes 1 to 100 nanometers. From this definition, we can have 1 dimension limited nano material, nano sheet, two dimensions limited nanomaterial, nano rod and three dimensions limited material, nano particle. Those nano confined materials were found to have some interesting chemical and physical properties, such as electrical conductivity, thermal conductivity, mechanical properties, optical properties and catalytic ability, which cannot be explained by the current theories. There is still room in nano science for us to work on. Although currently there are no existing theories to explain those phenomenon, we can still take advantage of nano materials to achieve tasks that cannot be achieved before.

There are many coordinatively undersaturated (cus) sites on the surface of nanoparticles [111-112]. Those cus sites are trusted to be the active sites undergoing the catalysis. The larger the surface area is, the more active sites there are. Since nanoparticles have a large surface-to-volume ratio compared to bulk materials, they are attractive candidates for use as catalysts. The electronic properties and arrangement of the cus sites are affected by nanoparticles’ size and shape, and changing the size and shape would exhibit different catalytic selectivity and reactivity.

By using the special catalytic activity of nanoparticles, some scientists are able to develop different electrochemical sensing devices. There are many examples of the use of gold nanoparticles as building blocks for the creation of electrochemical sensing devices. [113-118] Platinum nanoparticles have also been an intensive research subject for the design of electrodes [119-124], as platinum is known to be a good catalyst on \( \text{H}_2\text{O}_2 \) reduction and oxidation, and it can be used to cooperate with different enzymes to detect different bio-molecules. Those successful and promising results which use nanoparticles or nano surface as a component to build the electrochemical sensors show the usefulness and the adaptability of nano-technology. We hope, by using nano-technology (nano pore, nano
surface), we can build electrochemical sensors with higher sensitivity and selectivity.

1.4 Objective of this thesis

This thesis focuses on the following issues: (1) to develop a robust, flexible and versatile nano-structured platform for the detection of small redox-active biomessengers; (2) to demonstrate the application of the platform to the detection of H$_2$O$_2$, CO and H$_2$S.

1.5 References


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Chapter 2

Nano-structured Platform for Highly Selective, Sensitive and Quantitative Electrochemical Detection of Hydrogen Peroxide (H$_2$O$_2$) and Glucose in aqueous buffer solution with biological interferents
2.1 Introduction

Hydrogen Peroxide (H\textsubscript{2}O\textsubscript{2}) is one of the endogenously generated reactive-oxygen-species (ROS). It can act as either a cytotoxin or a cellular messenger, depending on the concentration and location of its endogenous generation. Increasing evidence shows that the concentration of H\textsubscript{2}O\textsubscript{2} is related to both cell growth and cell apoptosis. In order to study its functional mechanisms and regulation pathways, it is desirable to have methodologies and/or techniques for its detection in a selective, sensitive and quantitative manner and in the presence of many interfering chemical species. As a natural extension, by co-operating our platform with glucose oxidase (GOx), we can also measure glucose by measuring the local concentration of H\textsubscript{2}O\textsubscript{2}. Measuring glucose is important for the diabetics to control their blood glucose level.

Diabetes without proper treatments can cause many complications, such as hypoglycemia, diabetic ketoacidosis and nonketotic hyperosmolar coma. Serious long-term complications include cardiovascular diseases, chronic renal failure and retinal damage. Providing adequate and suitable treatment for diabetes is important, hence it is essential to search for methods to measure glucose.

Platinum have been used as a sensing material for H\textsubscript{2}O\textsubscript{2} over many years, due to the catalytic property of platinum toward the oxidation and reduction of H\textsubscript{2}O\textsubscript{2}. For the sensing method based on reduction of H\textsubscript{2}O\textsubscript{2} by using platinum, we normally will need to take care of the reduction of O\textsubscript{2}. The O\textsubscript{2} will act as interferrent to the H\textsubscript{2}O\textsubscript{2} detection. For the sensing method based on oxidation of H\textsubscript{2}O\textsubscript{2} by using platinum, the oxidation of platinum itself resulted in decreasing of the signal is a problem. Moreover, the oxidation of other interferrent, such as ascorbic acid, become another problem, so both ways have their difficulties. In this chapter, we have chosen the oxidation of H\textsubscript{2}O\textsubscript{2} by platinum as our sensor development direction and considered ascorbic acid as a interferrent model to show how to reduce its interference from our targeted signal by using nano-technology and electrochemical techniques. We have also solved the oxidation of the platinum problem by carefully controlling the oxidation potential.
Finding ways to remove interference to targeting species is an everlasting problem that has long persecuted scientists. In the past, in the area of electrochemistry, we have two approaches to reduce the interference. One is using a specific catalyst to react with the target species and detect the signal by recovering the catalyst or changing the product. Another is using a membrane to allow the target molecule, but not the interferents, to pass through to the inner solution and detect the targeting molecule. Both of them work in certain situations and have their own advantages and disadvantages. The most important thing is that both of the strategies are not counteracting to each other, but can be coexist and functional together to synergistically enhance the selectivity. For our approach, we have combined these two strategies and developed a nano-structured platform that can not only immobilize both enzyme and filtering membrane, but also can choose the ideal analyte-specific catalyst without considering the compatible issue to synergistically reduce the interferences.

Nano-technology was widely used in increasing the catalytic activity of the catalyst, filtering the unwanted material and increasing the functional area of the electrode. In this chapter, by using nano-porous alumina as the nano-structured material, we have developed a nano-structured platform and enjoyed the benefits brought by nano-technology. By using the advanced nano-technology, we are targeting to develop a platform to measure $\text{H}_2\text{O}_2$ and glucose by electrochemical oxidation in a broad concentration range, and in a sensitive and quantitative, and selective manner.
2.2 Experimental section

2.2.1 Reagent preparation

Potassium hydroxide (KOH) and Potassium phosphate monobasic (KH$_2$PO$_4$) were purchased from “Riedel-deHaen”. Potassium chloride (KCl), 25% glutaraldehyde solution and L-ascorbic acid were purchased from “Sigma-Aldrich”. 27.9mg/mL Glucose oxidase solution from Aspergillus niger, 5% Nafion resin in ethanol and 99% Bovine serum albumin were purchased from “Sigma”. 20% Nafion resin in propanol/water was purchased from “Aldrich”. High-purity O$_2$ gas (99.7%) was obtained from “Zhonghong Industry Gas Company (Shenzhen)”. H$_2$O$_2$ solution (3%) was purchased from “Fisher Chem Alert Guide”. The molar concentration of 3% H$_2$O$_2$ is 0.882M. Ultra pure water was obtained from “Barnstead nanopure infinity ultrapure water system”

Standard saturated O$_2$ solution was prepared by purging pure O$_2$ in ultra pure water for 1 hour and using a septum and parafilm to keep it in a good condition. The saturated concentration of O$_2$ in water at 25°C and 1bar is 1.273mM. The atmospheric saturated O$_2$ solution was prepared by equilibrate PBS solution in normal atmosphere. The 0μM O$_2$ solution was prepared by purging N$_2$ to PBS solution for more than 1 hour.

The phosphate-buffered saline (PBS) containing 0.1M KCl, 0.05M KH$_2$PO$_4$ was prepared by adding 3.4023g KH$_2$PO$_4$ and 3.7275g KCl to 450 mL ultrapure water and adjusted the pH to 7.2 by adding about 8.5ml 2M NaOH. After that, 41.5mL ultrapure water was added to the solution. 0.5 M KCl solution was prepared by adding 0.1864g of KCl to 5mL ultra pure water.

2.07mg/mL aqueous glucose oxidase (GOx) with BSA solution was prepared by weighing 1.6 mg of bovine serum albumin (BSA) in a 5-mL brown vial and then dispense 0.5mL of PBS (pH=7.2) into it. After that, 40μL of 27.9 mg/mL GOx solution was added to the same solution. 5% aqueous glutaraldehyde (GA) solution was prepared by dispensing 0.4mL of PBS (pH=7.2) into a 5-mL brown vial, followed by 0.1mL of 25% GA solution.
dispensing to the same solution.

0.4M and 0.04M D-glucose solution was prepared by weighing 0.7206g and 0.0721g of β-D-Glucose and adding them to two 10mL volumetric flasks respectively which would then be filled with water until the mark. The glucose solution was then warmed and cooled for complete anomeration.

0.16M ascorbic acid solution was prepared by weighing 0.0282g of ascorbic acid into 1mL PBS solution.

2.2.2 Electrode fabrication

2.2.2.1 Pt nano-frame electrode

The 60 μm thick aluminum oxide nanoporous frame was purchased from Whatman Co. The frame was first sonicated in ethanol for 30 minutes and then in water for 30 minutes. The frame was then dried in a fume hood. After the cleaning step, 60μm-thickness nano-porous Al₂O₃ substrates were used as templates for plasma sputtering deposition of Pt nanoparticles on the 20nm side surfaces. The sputtering parameter we used here was the same as the deposition of 200nm Pt in a plain surface. The deposited substrate was cut in a suitable size (50mm²) and was connected to a piece of aluminum foil using copper adhesive band. The substrate part was then assembled onto the quick fit electrochemical cell and became a finished electrode.

The structure of the electrode is described as follows: The upper part was a 60 μm thick aluminum oxide (Al₂O₃) frame part. The lower part was a 200nm thick metal platinum film. There were a lot of inversely conical shaped tunnels in array form connect the upper part of the frame to the lower part of the frame and the terminal was sealed by the platinum film. The diameter of the upper outlet was 150nm and the diameter of the lower outlet was 20nm. The structure is shown in Fig. 1A.
2.2.2.2 GOx-Pt nano-frame electrode

The Pt nano-frame electrode was first wetted by 8μL of 0.5M KCl solution. Excess solution was removed and the least amount of remaining solution was left to evaporate to the atmosphere. The KCl adding and evaporating process was repeated for three times in order to form a layer of KCl saturated solution on the wall of the alumina hole. 8μL PBS solution was added to the electrode to fill the hole and remove the air. Excess solution was removed from the electrode.

The electrode was then prepared by dispensing 4μL of 2.07mg/mL GOx with BSA solution onto the wetted Pt nano-frame electrode surface and then 2μL of 5% GA solution was dispensed onto the same electrode surface. The electrode was shaked by the shaker and dried with dry N2 for 5 minutes. The BSA was used to provide a biological environment for GOx and increase the thermal stability of GOx. The immobilization solution adding and drying process was repeated for 4 times. The electrode was transferred to a refrigerator and was dried overnight (more than 10 hours) at 4°C. The electrode was rinsed with ultrapure water a couple of times to remove the weakly bounded glucose oxidase before use. The structure of GOx-Pt nano-frame electrode is shown in Fig. 1B.

2.2.2.3 Nafion-GOx-Pt nano-frame electrode

The Nafion-GOx-Pt nano-frame electrode was prepared by the same procedure as the GOx-Pt nano-frame electrode except that before transferring it into the refrigerator, 2μL of 5% Nafion in ethanol was added on to the electrode surface.

2.2.2.4 GOx-Pt nano-frame electrode in DPA experiment

The Pt nano-frame electrode was first wetted by adding 8μL of PBS solution onto it. The electrode was shaked on the shaker vigorously to remove the air bubbles in the alumina hole. Excess solution was removed from the electrode.
The electrode was then prepared by dispensing 6μL of 2.07mg/mL GOx with BSA solution onto the wetted Pt nano-frame electrode surface and then 3μL of 5% GA solution was dispensed onto the same electrode surface. The electrode was shaked on the shaker and dried with dry N₂ for 15 minutes. The immobilization solution adding and drying process were repeated for one more time. The electrode was transferred into a refrigerator and was left to dry overnight (more than 10 hours) at 4°C. The electrode was rinsed with ultrapure water a couple of times to remove the weakly bounded glucose oxidase before use.

2.2.2.5 Nafion-GOx-Pt nano-frame electrode in DPA experiment

The Nafion-GOx-Pt nano-frame electrode in DPA experiment was prepared by the same procedure as the GOx-Pt nano-frame electrode in DPA experiment except before transferring it into the refrigerator, 6μL of 20% Nafion in propanol was added on to the electrode surface.

2.2.3 Measurements

Electrochemical measurements were performed at a CHI 750A electrochemical workstation (USA). A three-electrode system was employed with Saturated calomel electrode (Hg/Hg₂Cl₂) as reference electrode, a platinum plate as counter electrode, and home-made nanoframe-Pt electrode (diameter= 3 mm, geometric area=7.065mm²) as working electrode for the measurements related to pt-nanoframe electrode.

For the measurements related to Gox-Pt-nanoframe electrode, Gox-Pt-nanoframe electrode (diameter= 3 mm, geometric area=7.065mm²) was used as a working electrode. For the measurements related to Nafion-Gox-Pt-nanoframe electrode, Nafion-Gox-Pt-nanoframe electrode (diameter= 3 mm, geometric area=7.065mm²) was used as a working electrode.

For the measurements related to Gox-Pt-nanoframe electrode in the DPA experiment, Gox-Pt-nanoframe electrode in DPA experiment (diameter= 3 mm, geometric area=7.065mm²) was used as a working electrode. For the measurements related to
Nafion-Gox-Pt-nanoframe electrode DPA experiment, Nafion-Gox-Pt-nanoframe electrode in DPA experiment (diameter= 3 mm, geometric area=7.065mm$^2$) was used as a working electrode.

All the potentials in this report were with respect to Saturated calomel electrode (Hg/Hg$_2$Cl$_2$) and all measurements were carried out at room temperature (25 ± 2°C) at 1atm. For those O$_2$ concentration unspecific experiments, the O$_2$ concentration is atmospheric was saturated (about 0.263mM). For amperometric i-t curve experiment, polarization of the electrode at the potential used in the later experiment for 400s was needed. For differential pulse amperometry experiment, stabilization of the electrode at the setting used in the later experiment for 400s was needed.

2.3 Results & discussion

2.3.1 Electrodes design and reasons behind

The structure of Pt nano-frame electrode and GOx-Pt nano-frame electrode are shown in Fig. 1A & Fig. 1B. Both of them contain an aluminum oxide porous layer. This layer has three functions. The first one is blocking or trapping of interference species, as shown in the Fig. 1C. The charged species, highly polar species or slow macromolecules are believed not to be detected due to slow diffusion rate in the alumina porous phase. The second function is to create a nano reacting site which adheres to high surface area compared with conventional pt electrode, as some of the platinum was stuck on the wall of aluminum oxide. The third function is providing a biological hydrated environment for glucose oxidase and thus increase the life time of the electrode. Both electrodes contain a platinum thin film. The platinum thin film is created by plasma sputtering method and it can be considered as the assembling of nano platinum particles which has high catalytic activities toward many species such as oxygen and hydrogen peroxide. The roughness (high area to volume ratio) of the film also further increases the surface area of the electrode.
In the GOx-Pt nano-frame electrode, there is a glucose oxidase with BSA cross linked layer more than the Pt nano-frame electrode. This layer is used to converse our target molecule glucose to gluconic acid and hydrogen peroxide, so that we can detect the H$_2$O$_2$ in the platinum thin film. The reaction equation of glucose is shown below:

\[
\text{GOD}_{\text{ox}} + \beta\text{-D-glucose} \rightarrow \text{GOD}_{\text{red}} + \text{gluconic acid}
\]

\[
\text{GOD}_{\text{red}} + \text{O}_2 \rightarrow \text{GOD}_{\text{ox}} + \text{H}_2\text{O}_2
\]

The cross linking agent we used was glutaraldehyde in which there are two carbonyl groups can react with the amino groups in the side chain of the protein (GOx & BSA). The corresponding dimension of the glucose oxidase (native dimer) was about 70 Å x 55 Å x 80 Å and the pore of the aluminum oxide near the platinum thin film was about 20nm. This means the enzyme can diffuse and crosslink inside the pore. The BSA has three functions. One is to provide a biological environment for GOx. Second is to increase the thermal stability of GOx. Third is to act as a filler to prevent the GOx stacking together to affecting the conformation and bio-activity.

Apart from detecting the glucose concentration, we can also detect the oxygen level in solution before we detect the glucose, as the concentration of oxygen will affect the rate of converting glucose to gluconic acid and H$_2$O$_2$. The possible half reaction of oxidation and reduction of O$_2$ and H$_2$O$_2$ is shown below:

Reduction at 0.1 V against SCE
\[
4\text{H}^+ + \text{O}_2 + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}
\]
\[
2\text{H}^+ + \text{O}_2 + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2
\]
\[
2\text{H}^+ + \text{H}_2\text{O}_2 + 2\text{e}^- \rightarrow 2\text{H}_2\text{O}
\]
Oxidation at 0.4V against SCE
\[
\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{e}^- + 2\text{H}^+
\]
2.3.2 Cyclic voltammetry experiments of $O_2$ and $H_2O_2$

The cyclic voltammograms of $H_2O_2$ and $O_2$ at different concentrations on Pt nano-frame electrode are shown in Fig. 2A and 3A respectively. The reduction peak current and oxidation peak current at different $H_2O_2$ concentration is plotted against the $H_2O_2$ concentration in Fig. 2B and 2C respectively. The reduction peak current at different $O_2$ concentrations is plotted against the $O_2$ concentration in Fig. 2B. The $O_2$ or $H_2O_2$ was added by injecting suitable amount of saturated $O_2$ solution or 3% $H_2O_2$ solution in a 4mL of $N_2$ purged PBS solution and then mixed it by pipette up and down 5 times. The solution was added to the cell by micro pipette carefully to prevent bubble formations. 3 minutes (counted from the start of adding the solution to the cell) was needed for the $O_2$ to diffuse to and equilibrate on the surface of the electrode.

The reduction peak of $O_2$ and $H_2O_2$ was around 0.12V against SCE. It is less negative than the conventional platinum electrode (around 0.05V). This means the platinum thin film prepared by plasma sputtering has catalytic effect toward $O_2$ and $H_2O_2$. For the oxidation peak of the $H_2O_2$, it is around 0.4V against SCE. The potential taken to generate the calibration curve and measure $O_2$ was 0.1V. For $H_2O_2$, it was 0.1V (reduction) and 0.4V (oxidation). As the calibration curves obtained from peak current of the $O_2$ and $H_2O_2$ cyclic voltammograms showed great linearity, we can detect the $O_2$ or $H_2O_2$ concentration with great confidence by using Pt nano-frame electrode. There is a high potential to develop it to a $H_2O_2$ concentration related glucose sensor. Furthermore, this sensor has high probability to detect the $O_2$ concentration before detecting the glucose content.

The Response factor for $H_2O_2$ oxidation against voltage taken graph is shown in Fig. 4A. The Response factor for $H_2O_2$ and $O_2$ reduction against voltage taken graph are shown in Fig. 4B and 4C respectively. The response factor was generated by first generating a base line in the CV (Fig. 2A & 3A) and then taking all the voltage to generate the calibration curve. After that, the slope of the calibration curve was taken to be response factor and was plotted against the voltage. The response factor gives us information about which potential taken to be
measured in CV will give us the highest response while keeping the concentration constant. For H$_2$O$_2$ oxidation, the maximum response factor was from 0.3 to 0.4 V. For O$_2$ reduction, the maximum response factor was around 0.1V. This factor also helps us to choose the potential used in amperometric i-t curve for the oxidation of H$_2$O$_2$. The potential we 0.4V, a little bit larger than the maximum to ensure the potential was high enough for the oxidation to effectively take place.

The cyclic voltammograms of O$_2$ at different concentrations on GOx-Pt nano-frame electrode is shown in Fig. 5A. The reduction peak current at different O$_2$ concentrations is plotted against O$_2$ concentration in Fig. 5B. After the electrode preparation, the electrode was equilibrated in atmospheric saturated O$_2$ PBS solution for 10 cycles. The potential window was the same as the later O$_2$ experiment. This process was conducted to ensure that the electrode was relatively stable within the potential window that would be used later. Atmospheric saturated O$_2$ PBS solution was used to simulate the experiment condition used later: as Platinum is oxygen sensitive metal, some changes may only occur under sufficient oxygen level. The electrode was then rinsed by O$_2$ free blank solution to remove the remaining O$_2$ in order to minimize the O$_2$ contamination in the later experiment. The oxygen adding method is the same as the experiment of O$_2$ on Pt nano-frame electrode (Fig. 2A).

The calibration curve obtained from peak current of O$_2$ shows great linearity ($R^2=0.9848$) and thus we can measure the O$_2$ concentration with confidence before detecting the glucose content. The potential taken to generate the calibration curve and measure O$_2$ was 0.025V in here. The reduction peak was shifted to 0.025V which means that the surface of the platinum was different from the Pt nano-frame electrode. The atmospheric saturated PBS oxygen level that we used in the later experiment was determined by using the peak current generated from the cyclic voltammograms of atmospheric saturated O$_2$ and the calibration curve. The concentration was 277.4μM (PBS) which was not far away from the theoretical value 262μM (water), considering the atmospheric saturated concentration of O$_2$ being inconsistent from place to place.
2.3.3 Enzyme binding constant recognition

The amperometric i-t curve of successive glucose addition is shown in the Fig. 6A. In the experiment, the successive addition was done by adding 0.1mL 0.4M glucose in a 4mL PBS with stirring per 60s. The concentration added was shown in the graph. The potential we used was 0.4V. Before the experiment, polarization at 0.4V for 400s was needed in order to clean the reactive materials that may affect the later experiment. The graph of Current against Glucose Concentration added is shown in Fig. 6B. This graph was used to eliminate the dilution effect shown in the amperometric i-t curve.

As the concentration increased, the slope of the curve decreased, which means the high concentration of the substrate shifted the equilibrium of most of enzymes toward the enzyme substrate bounded state and the reaction rate became independent of the substrate concentration. In our enzyme electrode situation, the current was directly proportional to H$_2$O$_2$ local concentration on the surface of the electrode. Under the H$_2$O$_2$ equilibrated condition (generated rate = lost rate), the concentration of H$_2$O$_2$ was directly proportional to the generated rate, because the final lost rate was directly proportional to the H$_2$O$_2$ equilibrated concentration. i.e. The current we measured was directly proportional to the rate of reaction and can be considered as rate with different units.

The Lineweaver-Burk plot (1/current against 1/concentration) is shown in Fig. 6C. The slope was 2.0197 mM/$\mu$A and the y intercept was 8.407x10$^{-2}$ $\mu$A$^{-1}$. In Lineweaver-Burk plot, slope = Km/Vmax and y intercept = 1/ Vmax. The calculated Km was 24.02 mM and the Vmax was 11.89 $\mu$A. The Km was smaller than the theoretical value of free glucose oxidase 33mM. The lower the Km the tighter the enzyme binds with the substrate.

2.3.4 Amperometry experiments of H$_2$O$_2$ on Pt nano-frame electrode

The amperometric i-t curves of addition of different H$_2$O$_2$ concentrations from 10uM to 100uM, 100uM to 1000uM and 1mM to 10 mM are plotted in Fig. 7A, 7B and 7C.
respectively. The potential used was 0.4V. The electrode used was Pt nano-frame electrode. These graphs can be used to generate the calibration curve to calculate the equilibrated H$_2$O$_2$ concentration on the GOx-Pt nano-frame electrode surface. The calibration curve also can be used to detect the H$_2$O$_2$ concentration. According to Fig. 7C, there is saturation occurred after adding 11 mM H$_2$O$_2$ accumulatively.

The calibration curve is shown in Fig. 8, which showed great linearity from 10μM to 2mM (R$^2$=0.9976). The amperometry i-t curve of successive addition of 1000μM H$_2$O$_2$ per 30s is shown in Fig. 9. Imposing the curve on Fig. 7A, 7B and 7C, we can find the linear range of H$_2$O$_2$ and it was from 10μM to 5mM by considering whether or not the current increase was equal when adding the same amount of H$_2$O$_2$ in different accumulative H$_2$O$_2$ concentrations. The reproducibility of H$_2$O$_2$ signal was great in the linear range. The saturation occurred after 10mM accumulative addition.

### 2.3.5 Amperometry experiments of glucose on Gox-Pt nano-frame electrode

The amperometric i-t curves of addition of different glucose concentrations from 20μM to 100μM, 100μM to 1000μM and 1mM to 10mM are shown in Fig. 10A, 10B, 10C respectively. The potential we used was 0.4V. As shown in Fig. 10A, the detection limit (S/N=3) was found to be 40μM and the 20μM glucose addition at 30s and 60s did not show any obvious response. Using the Fig. 10A, 10B and 10C, a calibration curve is generated and is shown in Fig. 11. The calibration curve showed great linearity from 50μM to 2mM (R$^2$=0.9947). By using the regression equation of H$_2$O$_2$, we can calculate the equilibrated local concentration of H$_2$O$_2$, when we know the current that obtained by the glucose addition. When the Gox-Pt nano-frame electrode was in a saturated condition ($V_{max} = 11.89$μA), the local equilibrated H$_2$O$_2$ concentration on the surface of the electrode was 1750μM. When the glucose concentration was 1mM, the response current was 0.2393μA and the local equilibrated H$_2$O$_2$ concentration on the surface of the electrode was 29.16μM.

The amperometry i-t curves of successive addition of 100μM and 1000μM per 30s are
shown in Fig. 12A and 12B respectively. The potential we used was also 0.4V. From Fig. 12A, it shows great linearity and reproducibility from 100μM to 1000μM as the current increase in each addition in different accumulative Glucose concentration is approximately the same. From Fig. 12B, we can see the current increase was the same at the initial part. After a couple of additions, the accumulative glucose concentration increase and the current increase became smaller. Combining Fig. 10A, 10B, 10C and 12B, the linear range is 50μM to 5mM and great reproducibility within the linear range.

2.3.6 Interference study of ascorbic acid

The amperometric i-t curve of addition of glucose, water and ascorbic acid is shown in Fig. 13A. First, 500μM glucose was added two times to see whether glucose can generate the signal in the Gox-Pt nano-frame electrode and then water was added two times with the same volume as the glucose addition to see whether the response was coming from water or contamination. After that, 500μM glucose was added two times again to see whether the glucose can regenerate the signal in the same electrode. Furthermore, we added 200μM ascorbic acid (the same volume) two times after the glucose addition to see whether the electrode can resist the ascorbic acid from detection, and finally 500μM glucose was added two times to test the reproducibility after addition of ascorbic acid.

The water test result was expectable and the signal is not directly related to water. The fidelity and confidence of the Gox-Pt nano-frame electrode was guaranteed. For the ascorbic acid interference test, the electrode cannot resist ascorbic acid from detection and the response was approximately 4 times more than when the glucose in the condition that glucose concentration was 2.5 times higher than the ascorbic concentration. this was unexpected based on our group members' previous result, as the ascorbic acid should be resisted by the alumina porous membrane. After the addition of ascorbic acid, the signal of glucose was blurred as the signals of the ascorbic acid was too high. However, fortunately, the ascorbic acid in our body was about 50 times less than the glucose concentration, so the problem induced by the ascorbic acid would not be large.
The interference experiment was repeated by using Nafion-Gox-Pt nano-frame electrode. The procedure was exactly the same as before (Fig. 13A) and the result is shown in Fig. 13B. Consider the response current with nafion and without nafion, glucose signal dramatically increased after nafion was added. It is due to previous weakly bound enzyme in the case without nafion was now trapped by the nafion resin. There was a small drop in the current after the first addition and an increase after the second addition. The current increase related to glucose addition was decreased after adding of ascorbic acid. This means the immobilization of nafion induced some anti-ascorbic acid effect, but the signal cannot be recovered after adding ascorbic acid as interference. The partial anti-ascorbic acid effect, but not total anti ascorbic acid effect was due to penetration of nafion into the glucose oxidase instead of covering it. The decrease of current related to the glucose addition was due to contamination of ascorbic acid with glucose oxidase and hence the rate of conversion decreased, reducing the surface concentration of H₂O₂.

2.3.7 Life time study of GOx-Pt nano-frame electrode

The life time of the electrode was investigated and the result is shown in Fig. 14. The life time was measured by adding 500μM glucose two times and detecting its signal. The increased current for each addition was then taken average. After the measurement the electrode was rinsed by ultra pure water a couple of times and 1 time PBS, and then it was stored in the refrigerator (4°C) until the next measurement. Before the measurement, the electrode was first rinsed with ultra pure water a couple of times and then polarization at 0.4V was needed to remove the reactive species that may affect the measurement. The % of day 0 current was defined by increased current on that day over the increased current on day 0.

From Fig. 14, at first the activity decreased dramatically and after one day, the current was relatively stable at around 70%. The stable current was kept for a couple of days more until day 4. The activity dramatically dropped on day 7. On the first 4 days of measurement, some PBS remained on the electrode when taken out from the refrigerator, so the enzyme was in aqueous biological environment. On day7, the electrode was found to be dry when taken
out from the refrigerator, as the enzyme was believed to have passed through a highly ionic state with the ionic strength is so high that the enzyme was denatured. Furthermore, the drying state was not suitable for enzyme as the solvent (water) was related to the conformation of the enzyme. The day 7 data was expectable and the drops of other days were believed to be caused by rinsing with PBS for every two days.

As the day 7 data in the original experiment was affected by drying, the dried electrode was unable to give us any useful information after day 7. Another life time experiment was conducted to fill in the missing data of the original life time experiment. The second life time experiment was conducted exactly the same way as the original experiment. The percentage dropping with respect to the second day in the second life time experiment was recorded. Assuming the percentage drop with respect to the second day in the second life time experiment was similar to that of the original experiment. The estimation curve was created. The estimation curve shows that the activity was still kept at around 70% after 2 weeks provided that we kept the electrode in the PBS solution properly (not dried) and stored it in the refrigerator (4°C).

### 2.3.8 Strong catalytic effect of Pt nano-frame electrode

The cyclic voltammogram of ascorbic acid at different concentrations on Pt nano-frame electrode (from 0μM to 3000μM) is shown in Fig. 15A. There was a peak at 0.05V corresponding to the oxidation of ascorbic acid. For the glass carbon electrode, the peak corresponding to the oxidation of ascorbic acid was above 0.4V. For the platinum electrode, the peak corresponding to the oxidation of ascorbic acid was above 0.2V. The oxidation peak of ascorbic acid shifted from high potential to 0.05V showing that our platinum electrode had significant catalytic effect toward ascorbic acid oxidation. By using the abnormal low oxidation potential of ascorbic acid, we hope we can separate the peak between ascorbic acid and H₂O₂ and reduce the interference caused by ascorbic acid.

The oxidation of H₂O₂ on Pt nano-frame electrode was then investigated. The cyclic
voltammogram of H$_2$O$_2$ at different concentrations on Pt nano-frame electrode (from 0µM to 1000µM) is shown in Fig. 15B. The cyclic voltammogram showed that the oxidation of H$_2$O$_2$ started at around 0.3V which was far from the oxidation peak of ascorbic acid (0.05V). This means theoretically we can calibrate out the interference caused by ascorbic acid when measuring H$_2$O$_2$ concentration by using the CV method in here or the DPA method to be discussed later.

To prove the idea, we added constant ascorbic acid to different H$_2$O$_2$ concentrations and performed Cyclic Voltammetry. The result is shown in Fig.15C. The shape of the cyclic voltammogram did not shift very much when ascorbic acid was added. The Ipa of H$_2$O$_2$ at 0.5V was similar to that of the Fig. 15B as the effect of ascorbic acid at baseline potential (0.3V) and at the Ipa measured potential (0.5V) was similar. When the current in the baseline potential and measuring potential both were affected by the ascorbic acid similarly, the ascorbic acid can be calibrated out as it affected the current only but not the change of current.

Apart from Cyclic Voltammetry, we decided to also use Differential pulse amperometry (DPA), which is another technique that also involves using a base line in the measurement. Traditional amperometry measures the current directly. In other words, it will measure the current caused by anything including the interference such as ascorbic acid. Baseline corresponding to another potential is not used to calibrate out the interference reaction that occurs in both baseline potential and measuring potential. On the other hand, Differential pulse amperometry measures the difference of the current between baseline potential and measuring potential. The reaction occurring in both baseline potential and measuring potential can be calibrated out.

2.3.9 Reduction of interference of ascorbic acid by using DPA

Fig.16 shows the differential pulse amperometry curve of adding different amounts of H$_2$O$_2$ and same amount of Ascorbic acid. These figures show that the interference caused by ascorbic acid cannot be totally calibrated out, but can be reduced significantly. The failure of
removing the interference of ascorbic acid totally can be explained by the fact that there is more ascorbic acid (about 1/6) oxidized at 0.5V compared to at 0.175V.

The 0.175V that we used as baseline voltage was optimized. At first, we tried to maximize the H₂O₂ response by increasing the voltage difference between the measuring voltage and baseline voltage, so that the “response of AA to response of H₂O₂” ratio can also be minimized. Finally, we found that the largest and safe measuring voltage was 0.5V. If we increased this voltage further, the platinum on the electrode would start to have some side reactions and would affect our measurement. Similarly, The smallest and safe baseline voltage that we found was 0.175V. If we decreased it further, the O₂ and H₂O₂ would be reduced and would affect our measurement. By applying the DPA technique and optimized parameter, the signal of 50μM H₂O₂ was larger than 100μM ascorbic acid (Fig. 16A).

From the experiments described in Fig. 16, We can calculate the “response of AA to response of H₂O₂ ratio” and the graph of the ratio against the concentration of H₂O₂ is shown in Fig. 17A. The AA concentration used was 100μM which was similar to the average AA concentration in normal human blood. The calibration curve of H₂O₂ by using differential pulse amperometry on Pt nano-frame electrode is shown in Fig. 17B. As shown in Fig. 17B, we can see the concentration of H₂O₂ from 50μM to 1mM was linear related to the signal response. From Fig. 17A, we can see the response ratio was below 20% when H₂O₂ was at 100μM. Thus, the range that can measure the H₂O₂ without AA significant interference was 100μM to 1000μM. This range really matched the physiological H₂O₂ concentration in normal human blood.

After finishing the H₂O₂ part, we proceeded to the glucose part. We immobilized the Glucose oxidase on the Pt nano-frame electrode by the linker (glutaraldehyde) with stabilizing agent (BSA) and repeated the Fig. 16 experiment. The result is shown in Fig. 18. The graph of the “response of AA to response of glucose ratio” against the concentration of glucose is shown in Fig. 18A. The calibration curve of glucose by using differential pluse amperometry on Gox-Pt nano-frame electrode is shown in Fig. 18B.
As shown in Fig. 18B, we can see the concentration of glucose from 200μM to 5mM was linear related to the signal response. From Fig. 18A, we can see the response ratio was below 20% when glucose was 1mM. Thus, the range that can measure the glucose without AA significant interference was 1mM to 5mM. The physiological level of glucose in blood was 4-15mM. Dilution would be needed if we wanted to measure the glucose concentration in the blood.

In addition, the response ratio becomes negative when glucose concentration is high. That is expectable and because of the contamination of the AA toward glucose oxidase. When the activity of the glucose oxidase decreases, the \( \text{H}_2\text{O}_2 \) local concentration and the signal response will also decrease. When the glucose concentration is high, the signal drop caused by the contamination overcome the signal increase corresponding to AA itself and thus causes such negative response ratio.

### 2.3.10 Reduction of interference of ascorbic acid by using DPA and nafion

In order to reduce the AA interference further, we then immobilized a negative charge polymer (nafion) on the Gox-Pt nano-frame electrode and become Nafion-Gox-pt nano-frame electrode. As at in the equilibrium state, AA at pH 7.2 is negative charge, so negative charge membrane was widely used to remove the negative charge interference by using electrostatic repulsion. Nafion is the one of the most common polymer that can be used to form such a membrane. The effect of nafion is well recognized and comparably effective.

Again, we repeated the experiment shown in Fig. 16 by using this electrode and the results are shown in Fig. 19. As shown in Fig. 19B, the concentration of glucose from 200μM to 5mM is linear related to the signal response. Fig. 19A shows that the response ratio was below 20% when glucose was at 500μM. Thus, the range that can measure the glucose without AA significant interference was 500μM to 5mM. Dilution would also needed, if we want to measure the glucose concentration in blood.
The comparison of the methods and electrode used is shown in Fig. 20. We used the “response of 100μM AA to response of 500μM glucose ratio” as a parameter to show the improvement of the methods and the electrode. As shown in Fig. 20, the DPA method used in our way had significant effect of reducing AA signal. The nafion worked well in our electrode and also significantly reduced the AA interference.

2.3.10 Interference coefficient of ascorbic acid with respect to H₂O₂ detection

In order to quantify the interference contribution of ascorbic acid with respect to the H₂O₂ detection, we need to determine the interference coefficient of ascorbic acid. The interference coefficient is defined by the slope of the calibration curve of the interferent divided by the slope of the calibration curve of the analyte. The interference coefficient can also act as a parameter to compare the selectivity of different methods.

In Fig. 21A, by using the DPA method and the parameters used previously, different concentrations of H₂O₂ added on the Pt nano-frame electrode resulted in the corresponding different increase of current. Using the data shown in Fig. 21A, we can plot the calibration curve of H₂O₂ shown in Fig. 21B. The slope of the calibration curve was 0.00854 μA/μM and the R² was 0.996 which means the calibration curve had a very high linear relationship. The differential current was linear related to the concentration of the H₂O₂ from 100μM to 1000μM.

After determining the slope of the calibration curve of H₂O₂, we determined that of ascorbic acid. Using the same parameters, the same electrode and the same condition, the DPA curve of ascorbic acid was calculated as shown in Fig. 22A and the calibration curve of ascorbic acid is shown in Fig. 22B. The slope of the calibration curve was 0.000974μA/μM and the R² was 0.986. The slope was much smaller than that of H₂O₂. The differential current was linear related to the concentration of the ascorbic acid from 200μM to 1000μM. The electrode started to saturate after the accumulative addition of 3400μM ascorbic acid, as shown in Fig. 22A, so the last two 2000μM ascorbic acid additions were not used as data.
points to plot the calibration curve shown in Fig. 22B.

Calculation of the slopes of the calibration curves of both H$_2$O$_2$ and ascorbic acid shows that the interference coefficient was 0.114 which means the signal of H$_2$O$_2$ was about 10 times bigger than that of ascorbic acid. The interference coefficient found was consistent with the result shown in Fig. 16.

The determination of interference coefficient of ascorbic acid with respect to Glucose detection on Nafion - GOx - Pt nano-frame electrode involved immobilization of nafion which would further increase the detection limit of ascorbic acid to being closer to the saturated concentration. Since the linear range would become too small and the linear relationship would not be good, the interference coefficient of ascorbic acid with respect to Glucose detection was not determined as the slope of calibration curve of ascorbic in here is unquantifiable. Even if we could found the value, it would not be reliable and cannot be used to compare with other results.
2.4 Conclusions

An amperometric glucose sensor and, GOx-Pt nano-frame electrode, was developed by immobilization of glucose oxidase on the surface of Pt nano-frame electrode by cross linking agent, glutaraldehyde, in the presence of BSA. The Pt nano-frame electrode was prepared by plasma sputtering on the porous alumina and has high catalytic activity toward reduction of O$_2$ and H$_2$O$_2$. The linear range of detecting O$_2$ was from 64μM to 320μM. The linear range of detecting H$_2$O$_2$ was from 10μM to 5mM. The interference and fidelity experiment of GOx-Pt nano-frame electrode and Nafion - GOx - Pt nano-frame electrode was conducted. The GOx-Pt nano-frame electrode cannot resist ascorbic acid interference. The Nafion-GOx-Pt nano-frame electrode can partially resist ascorbic acid interference. GOx-Pt nano-frame electrode demonstrated advantages of high sensitivity, good stability, great linearity and reproducibility. The linear range of detecting glucose was from 50μM to 5mM. The detection limit was 40μM. The Km of the glucose oxidase on GOx - Pt nano-frame electrode was 24.02mM. By using DPA method and nafion, we can significant reduce the ascorbic interference to an acceptable level. The measurable range of glucose without normal level (100μM) ascorbic acid significant interference (20%) was 500μM to 5mM. The interference coefficient of ascorbic acid with respect to H$_2$O$_2$ detection by using the DPA method on Au nano-frame electrode was 0.114.
2.5 References


2.6 Legends and Figures

Fig. 1: (A) Platinum nano-frame electrode structure. (B) Glucose oxidase immobilized platinum nano-frame electrode structure. (C) Illustration of separation of molecules in platinum nano-frame electrode.

Fig. 2: (A) Cyclic voltammogram of H$_2$O$_2$ at different concentrations on Pt nano-frame electrode (from 0μM to 1540μM). pH=7.2, Scan rate= 20mV/s, Potential window = 0.5 to -0.1 V. (B) Reduction peak current (μA) against H$_2$O$_2$ concentration (μM). Calibration curve of H$_2$O$_2$ (from 0μM to 1540μM) on Pt nano-frame electrode by taking reduction peak current at 0.1V. (C) Oxidation peak current (μA) against H$_2$O$_2$ concentration (μM). Calibration curve of H$_2$O$_2$ (from 0μM to 1540μM) on Pt nano-frame electrode by taking oxidation peak current at 0.4V.

Fig. 3: (A) Cyclic voltammogram of O$_2$ at different concentrations on Pt nano-frame electrode (from 0μM to 320μM). pH=7.2, Scan rate= 20mV/s, Potential window = 0.5 to -0.1 V. (B) Reduction peak current (μA) against O$_2$ concentration (μM). Calibration curve of O$_2$ (from 0μM to 320μM) on Pt nano-frame electrode by taking reduction peak current at 0.1V.

Fig. 4: (A) Response factor (μA/μM) for H$_2$O$_2$ oxidation against voltage taken (V). (B) Response factor (μA/μM) for H$_2$O$_2$ reduction against voltage taken (V). (C) Response factor (μA/μM) for O$_2$ reduction against voltage taken (V). The response factor is the slope of the calibration curve that is generated by taking the peak current at a specific voltage. For each specific voltage, we have a set of peak currents for a set of concentrations and we generate one calibration curve that has one slope specific for one voltage taken.
Fig. 5: (A) Cyclic voltammogram of O\textsubscript{2} at different concentrations on Gox-Pt nano-frame electrode (from 0μM to 320μM). pH=7.2, Scan rate= 20mV/s, Potential window = 0.35 to -0.1 V. (B) Reduction peak current (uA) against O\textsubscript{2} concentration (uM). Calibration curve of O\textsubscript{2} (from 0μM to 320μM) on Gox-Pt nano-frame electrode by taking reduction peak current at 0.025V.

Fig. 6: (A) Amperometric i-t curve of successive glucose addition (10mM per 60s) on Gox-Pt nano-frame electrode. Potential used = 0.4V. Stirring condition (B) Current (uA) against glucose concentration added. It is generated by Fig. 6A with the correction of dilution effect. (C) Lineweaver-Burk plot (1/current against 1/concentration) from 27.9mM to 92.3mM. Km was found to be 24.02mM. Vmax = 11.89uA

Fig. 7: (A) Amperometric i-t curve of addition of different H\textsubscript{2}O\textsubscript{2} concentrations (10μM to 100μM) on Pt nano-frame electrode. Potential used = 0.4V. Stirring condition. (B) Amperometric i-t curve of addition of different H\textsubscript{2}O\textsubscript{2} concentrations (100μM to 1000μM) on Pt nano-frame electrode. Potential used = 0.4V. Stirring condition. (C) Amperometric i-t curve of addition of different H\textsubscript{2}O\textsubscript{2} concentrations (1mM to 10mM) on Pt nano-frame electrode. Potential used = 0.4V. Stirring condition.

Fig. 8: Current increase (μA) against H\textsubscript{2}O\textsubscript{2} concentration added (μM) on Pt nano-frame electrode. Calibration curve of H\textsubscript{2}O\textsubscript{2} generated by Fig. 7A, B, C.

Fig. 9: Amperometry i-t curve of successive addition of 1000μM H\textsubscript{2}O\textsubscript{2} per 30s on Pt nano-frame electrode. Reproducibility test and linear range test. Linear response to H\textsubscript{2}O\textsubscript{2} concentration until 5mM. Potential used = 0.4V. Stirring condition.
Fig. 10:  (A) Amperometric i-t curve of addition of different glucose concentration (20μM to 100μM) on Gox-Pt nano-frame electrode. The inner graph is the amperometric i-t curve of addition of 40μM glucose showing the detection limit S/N=3 is 40μM. Potential used =0.4V. Stirring condition. (B) Amperometric i-t curve of addition of different glucose concentration (100μM to 1000μM) on Gox-Pt nano-frame electrode. Potential used = 0.4V. Stirring condition. (C) Amperometric i-t curve of addition of different H₂O₂ concentration (1mM to 10mM) on Gox-Pt nano-frame electrode. Potential used = 0.4V. Stirring condition.

Fig. 11: Current increase (μA) against glucose concentration added (μM) on Gox-Pt nano-frame electrode. Calibration curve of glucose generated by Fig. 10A, B, C.

Fig. 12: (A) Amperometry i-t curve of successive addition of 100μM glucose per 30s. Reproducibility test and linear range test. Potential used = 0.4V. Stirring condition. (B) Amperometry i-t curve of successive addition of 1000μM glucose per 30s. Reproducibility test and linear range test. Linear response to glucose concentration until 5mM. Potential used = 0.4V. Stirring condition.

Fig. 13: (A) Amperometric i-t curve of addition of glucose, water and ascorbic acid on Gox-Pt nano-frame electrode. Fidelity and interference test for Gox-Pt nano-frame electrode. (B) Amperometric i-t curve of addition of glucose, water and ascorbic acid on Nafion-Gox-Pt nano-frame electrode. Fidelity and interference test for Nafion-Gox-Pt nano-frame electrode.

Fig. 14: % of current increase with respect to day 0 current increase (%) against time (day). Life time test of GOx-Pt nano-frame electrode. The estimation curve was deduced from the percentage dropped with respect to second day in another experiment similar to the original experiment.
Fig. 15: (A) Cyclic voltammogram of ascorbic acid at different concentrations on Pt nano-frame electrode (from 0μM to 3000μM). pH=7.2, Scan rate= 50mV/s, Potential window = -0.2 to 0.5 V. (B) Cyclic voltammogram of H$_2$O$_2$ at different concentrations on Pt nano-frame electrode (from 0μM to 100μM). pH=7.2, Scan rate= 30mV/s, Potential window = -0.2 to 0.5 V. (C) Cyclic voltammogram of H$_2$O$_2$ at different concentrations (from 0μM to 1000μM) with constant ascorbic acid concentration (100μM) on Pt nano-frame electrode. pH=7.2, Scan rate= 30mV/s, Potential window = -0.2 to 0.5 V.

Fig. 16: (A) Differential pulse amperometry curve of addition of 50μM H$_2$O$_2$ and 100μM ascorbic acid on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.5V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s. (B) Differential pulse amperometry curve of addition of 100μM H$_2$O$_2$ and 100μM ascorbic acid on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.5V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s. (C) Differential pulse amperometry curve of addition of 200μM H$_2$O$_2$ and 100μM ascorbic acid on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.5V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s. (D) Differential pulse amperometry curve of addition of 500μM H$_2$O$_2$ and 100μM ascorbic acid on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.5V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s

Fig. 17: (A) 100μM ascorbic acid effect on the differential current response of different H$_2$O$_2$ concentrations (from 50μM to 1000μM) on Pt nano-frame electrode. (B) Differential current response (μA) against H$_2$O$_2$ concentration (μM). Calibration curve of H$_2$O$_2$ by using differential pulse amperometry on Pt nano-frame electrode.

Fig. 18: (A) 100μM ascorbic acid effect on the differential current response of different glucose concentrations (from 0.2mM to 10mM) on Gox-Pt nano-frame electrode. (B) Differential current response (μA) against glucose concentration (mM). Calibration curve of Glucose by using differential pulse amperometry on Gox-Pt nano-frame electrode.
Fig. 19: (A) 100μM ascorbic acid effect on the differential current response of different glucose concentration (from 0.2mM to 10mM) on Nafion-Gox-Pt nano-frame electrode. (B) Differential current response (μA) against glucose concentration (mM). Calibration curve of Glucose by using differential pulse amperometry on Nafion-Gox-Pt nano-frame electrode.

Fig. 20: 100μM ascorbic acid effect on 500μM glucose response using different methods and electrodes.

Fig. 21: (A) Differential pulse amperometry curve of addition of different H$_2$O$_2$ concentrations (100μM to 1000μM) on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.4V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s. (B) Differential current response (μA) against H$_2$O$_2$ concentration (μM). Calibration curve of H$_2$O$_2$ by using differential pulse amperometry on Pt nano-frame electrode.

Fig. 22: (A) Differential pulse amperometry curve of addition of different ascorbic acid concentrations (200μM to 2000μM) on Pt nano-frame electrode. E$_1$ = 0V, E$_2$ = 0.175V, E$_3$ = 0.4V, t$_1$ = 2s, t$_2$ = 0.1s, t$_3$ = 0.1s. (B) Differential current response (μA) against ascorbic acid concentration (μM). Calibration curve of ascorbic acid by using differential pulse amperometry on Pt nano-frame electrode.
Fig. 1
Fig. 4

(A) Graph showing response factor in units of 10^4 / µA·M^1 vs. E vs SCE / V.

(B) Graph showing response factor in units of 10^4 / µA·M^1 vs. E vs SCE / V.

(C) Graph showing response factor in units of 10^4 / µA·M^1 vs. E vs SCE / V.
Fig. 5

(A) Cyclic voltammetry plots for different oxygen concentrations. The curves are labeled with their respective oxygen concentrations: 320 μM (purple), 192 μM (blue), 128 μM (green), 64 μM (orange), 0 μM (red), and atmospheric saturated (brown).

(B) Plot of the peak current density vs. oxygen concentration. The data points indicate a linear relationship with oxygen concentration.

\[ i / \mu A \]

\[ E \text{ vs SCE} / V \]

\[ O_2 / \mu M \]

\[ I_{pc} / \mu A \]
Fig. 6

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Fig. 7

(A) Graph with concentrations of 10μM, 50μM, 100μM, 20μM, 500μM, 1000μM, and 1000μM.

(B) Graph with concentrations of 100μM, 200μM, 500μM, and 1000μM.

(C) Graph with concentrations of 2mM, 5mM, 10mM, and 1000μM.
Fig. 8

![Graph showing the relationship between \( \Delta i / \mu A \) and \([H_2O_2] / \mu M\).]
Fig. 9
Fig. 10

A

B

C

$\text{i / } \mu\text{A}$

$t / \text{s}$

$\text{i / } \mu\text{A}$

$t / \text{s}$

$\text{i / } \mu\text{A}$

$t / \text{s}$
Fig. 11

![Graph showing the relationship between [Glucose] and Δi/μA. The graph displays a linear correlation with increasing glucose concentration.]
Fig. 12

A

$\text{t} / \text{s}$

$\text{i} / \mu\text{A}$

B

$\text{t} / \text{s}$

$\text{i} / \mu\text{A}$
Fig. 13

A

B

i / µA

Glucose 500µM

Water

Glucose 500µM

Water

Glucose 500µM

AA 200µM

Glucose 500µM

AA 200µM

Glucose 500µM

Glucose 500µM

Glucose 500µM

Glucose 500µM

0 200 400 600

t / s

0 200 400 600

t / s
Fig. 14
Fig. 15
Fig. 16
Fig. 17

A

Response / %

[H₂O₂] / µM

B

Δi / µA

[H₂O₂] / µM
Chapter 2

Fig. 18

(A) Response (%) vs. [Glucose] (mM)

(B) \( \Delta i / \mu A \) vs. [Glucose] (mM)
Fig. 19

A

Response / %

[Glucose] / mM

B

$\Delta i / \mu A$

[Glucose] / mM
Fig. 20
Fig. 21

\[ \Delta i \ (\mu A) = -0.340 + 0.00854 \times [H_2O_2] \ (\mu M) \]

\[ R^2 = 0.996 \]
Fig. 22

[A graph showing the relationship between current (i) and time (t) with concentrations of ascorbic acid.]

\[ \Delta i (\mu A) = 0.0966 - 0.000974 \times \text{[Ascorbic acid]}(\mu M) \]

\[ R^2 = 0.986 \]
Chapter 3

Nano-structured Platform for Highly Selective, Sensitive and Quantitative
Electrochemical Detection of Carbon Monoxide (CO) in aqueous buffer solution with
biological interferents.
3.1 Introduction

Nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H\textsubscript{2}S) together make up a family of biologically active gases (gaseous triumvirate) with an increasingly well-defined range of physiological effects plus roles to play in a number of disease states. It is becoming increasingly clear that both the synthesis and the biological activity of each gas are regulated by the presence of the others, and as such it is necessary to consider these molecules not in isolation but acting together to control cell functions.

All three gases are synthesized naturally in the body, often by the same cells within the same organs, and exert essentially similar biological effects albeit via different mechanisms. Within the cardiovascular system, all are vasodilators and are protective towards tissue damage in the heart. On the other hand, all exhibit complex effects in inflammation with both pro- and anti-inflammatory effects. The complete physiological functions of all three gases are still under investigation. There is an urgent demand on the sensing technique of all three gases in solution phase, so that we can apply the technique in vivo system to investigate the physiological function of all three gases and their potential medical use in the future.

In this chapter, we are going to show you the development of a CO sensing method in solution phase. Traditionally, direct measurement of CO only focuses on gas phase and the methods are well developed, including infrared spectroscopy (IR), gas chromatography (GC), electrochemical oxidation and semiconductor resistance change. The gas phase targeted technology obviously does not work for solution phase. However, in the biological area, detection in solution phase is extremely important.

In order to detect CO in biological samples, some people tried to transform CO from solution phase to gas phase. The most widely used way to measure CO in tissues or biological samples is purging the carrier gas into the sample solution and connecting the headspace of the vial to GC system or IR absorption cell, but the CO concentration
measured could only be considered as relative concentration, not real concentration, although the CO production rate can be measured by this methods.

Another popular way to measure CO in biological sample is trapping the CO using hemoglobin (Hb) or myoglobin (Mb) and the CO-Hb or CO-Mb are measured by CO-oximeter which is based on ultra violet visible (UV-V) and IR absorption. In this method, we can get the real concentration, but we cannot measure it at real time, as complex formation is required. Real time measurement is especially important for the detection of exocytosis of CO and investigation of the cell signalling system.

To measure CO in biological aqueous phase in real time, electrochemical method is the most suitable one, as it is fast, sensitive, flexible, selective and have been well developed in detecting CO in gas phase. However, the traditional electrochemical method to detect CO in gas phase would not work for biological aqueous phase without any modification, as the catalytic reaction to convert CO to CO₂ is relatively difficult and have to be conducted under extreme conditions, such as pH=1 or 398K (working temperature of common low temperature water gas shift reaction catalyst), and water will act as a poison in the catalytic reaction. As CO is iso-electronic to N₂ and inert, it is extremely difficult to detect CO in mild solution phase by using oxidation.

Besides catalytic reactions, absorption is also another possible way. Previously, ZnO and Cu (I) ions have already been used to detect CO in gas phase using the change before and after the absorption of CO in a relatively mild condition. Both of them have similar electron configuration. For the CO detection experiment using Cu (I) ion that was done previously by P. Richard G, the conversion of copper ions is actually conducted in solution phase and Cu (I) act as a core to detect CO in gas phase by diffusion. However, in their experiment, Cu (II) solution would have to be used in the system at pH below 5. In such condition, the biological sample will be damaged. despite this, from their experiment, we know copper is one of the potential materials that may fulfil our requirement.
To our best knowledge, there is no report on direct measurement of CO in biological solution by electrochemical method without basic or acidic internal solution. There are some reports using gas permeable membrane with acidic or basic internal solution to directly measure CO by oxidation.

In this section, we demonstrated a new way to measure CO by using the coordination behaviour of CO to Cu$_2$O in relative mild conditions, pH 8 and room temperature. In our experiment, two different fabrication methods of the sensor were developed. The morphology was characterized by Scanning Electron Microscopy (SEM) and performances of the two methods were compared.

3.2 Experimental section

3.2.1 Reagent preparation

Potassium Sulfate (K$_2$SO$_4$) and Potassium Nitrate (KNO$_3$) were purchased from Nacalai Tesque. Copper (II) sulfate (CuSO$_4$), Potassium hydroxide (KOH), Potassium phosphate monobasic (KH$_2$PO$_4$) and Acetic acid (CH$_3$COOH) 99.8% were purchased from Riedel-deHaen. Carbon Monoxide (CO) 99.5% was purchased from Hong Kong Specialty Gases CO. LTD. Ultra pure water was obtained from “Barnstead nanopure infinity ultrapure water system”.

Standard saturated CO solution was prepared by purging pure CO in ultra pure water for 1 hour and using a septum and parafilm to keep it in a good condition. The saturated concentration of O$_2$ in water at 298.15 K and 1bar was 0.982mM. The supporting solution contained 0.05M KH$_2$PO$_4$ and 0.1M KNO$_3$. KOH was added to the supporting solution to adjust the pH of the solution to 8.00. The CO saturated supporting solution was prepared by purging pure CO in the supporting solution for 30 minutes. The Cu deposition solution contained 20mM CuSO$_4$, 0.1M K$_2$SO$_4$ and 0.05M acetic acid. KOH was added to the Cu deposition solution to adjust the pH of the solution to 4.91.
Chapter 3

3.2.2 Electrode fabrication

3.2.2.1 Cu sputtering electrode

The 60 um thick aluminum oxide nanoporous frame was purchased from Whatman Co. The upper surface contained 20nm diameter pores and the lower surface contained 150nm diameter pores. The frame first was sonicated in ethanol for 30 minutes and then in water for 30 minutes. Then, the frame was dried in fume hood. After the treatment, the cleaned frame was used as a template for plasma sputtering deposition of Cu on the upper surface. The deposition was carried out using a Thin Film Sputtering System-Denton Discovery 18 instrument (Denton Vacuum). The thickness of Cu deposited was about 200nm.

3.2.2.2 Cu deposition electrode

To prepare a Cu deposition electrode, an Au sputtering electrode was required. The Au sputtering electrode was prepared in a similarly way as the Cu sputtering electrode, but this time Au was sputtered on the surface instead of Cu. A Cu deposition solution was used. Chronopotentiometry was used as a deposition method to deposit Cu on the Au sputtering electrode. The diameter of the electrode was 3 mm (geometric area=7.065mm$^2$). The time duration we used was 800s. The setting of the constant current finally chosen by us was 80μA.

3.2.3 Pre-treatment

Before the measurement of CO, some pre-treatment and equilibrate processes had been done. The electrode was first applied -0.4V for 400s to convert all the surface Cu species (Cu$_2$O and CuO) into Cu. The electrode was then applied 0V for 400s to convert all surface Cu into Cu$_2$O. Secondly, the electrode was equilibrated by using CV technique in blank supporting solution for 40 cycles. Scan Rate was 50mV/s and Potential Window was from 0V to -0.25V. Thirdly, the electrode was stabilized by CV for 20 cycles in the CO saturated
supporting solution. Finally, the electrode was cleaned by CV for 20 cycles in the blank solution to remove excess CO.

### 3.2.4 Measurements

Electrochemical measurements were performed at a CHI 660C electrochemical workstation (USA). A three-electrode system was employed with Saturated calomel electrode (Hg/Hg$_2$Cl$_2$) as reference electrode, a platinum plate as counter electrode, and Cu sputtering electrode (diameter= 3 mm, geometric area=7.065mm$^2$) or Cu deposition electrode (diameter= 3 mm, geometric area=7.065mm$^2$) as working electrode for the measurements of CO.

All the potentials in this chapter were with respect to Saturated calomel electrode (Hg/Hg$_2$Cl$_2$) and all measurements were carried out at room temperature (25 ± 2°C) at 1atm.

### 3.3 Results & discussion

#### 3.3.1 Preparation & structure

##### 3.3.1.1 Effect of pre-treatment and reason behind

The SEM photos of the Cu sputtering electrode before and after the pre-treatment are shown in Fig. 1A & 1C respectively. The amperogram of the pre-treatments of the Cu sputtering electrode, applying -0.4V for 400s and 0V for 400s, are shown in Fig. 1B & 1D respectively. The pre-treatment of applying -0.4V for 400s aimed at converting Cu species (Cu$_2$O, CuO) into Cu. The pre-treatment of applying 0V aimed at converting Cu into Cu$_2$O. Cu$_2$O is a good binder for CO. Converting all the Cu species into Cu$_2$O can enhance the binding of the CO, and thus increase the sensitivity of the measurement of CO.

The SEM photos of the Cu deposition electrode before and after the pre-treatment are shown in Fig. 4A & 4C respectively. The amperogram of the pre-treatments of the Cu
sputtering electrode, applying -0.4V for 400s and 0V for 400s, are shown in Fig. 4B & 4D respectively. Comparing Fig. 1A and 4A, the Cu sputtering electrode had a very smooth surface, while the Cu deposition electrode had a very rough surface and the particle size in the Cu deposition electrode was around 500nm. Comparing Fig 1B with 4B, the shapes were similar and both of them contained a peak of negative current showing that Cu$_2$O and CuO was converted into Cu. By contrast, we could see a positive current peak in Fig. 1D and 4D, which representing the conversion of Cu to Cu$_2$O.

The potential (-0.4V and 0V) we chose was based on the CV of Cu that we had done before. Comparing 1A with 1C, 4A with 4C, we could not see a significant difference between them, that means the pre-treatment step in here changed only the nature (electron cloud) of the surface, but not the shape (atomic arrangement) of the surface. The solution we used was the supporting solution which contained KNO$_3$ 0.1M and KH$_2$PO$_4$ 0.05M at pH 8.00. KNO$_3$ acted as supporting electrolyte and KH$_2$PO$_4$ acted as buffer. pH 8.00 was chosen taking into consideration the Cu$_2$O stability and the Cu ions dissolving problem: Cu(I) and Cu(II) will dissolve in the solution in acidic environment, but Cu$_2$O and CuO will be stabilized in basic or neutral environment.

3.3.1.2 Effect of equilibrate and reason behind

After the pre-treatment step shown in Fig. 1B, 1D, 4B & 4D, the electrode underwent a couple of equilibrate processes. The SEM photo of the Cu sputtering electrode after equilibrate step is shown in Fig 2A and the CV of the equilibrate step, 40 cycles in supporting solution, 20 cycles in CO saturated supporting solution and 20 cycles in supporting solution again are shown in Fig. 2B,2C and 2D respectively. For the Cu deposition electrode, the SEM photo after equilibrate step is shown in Fig 5A and the CV of the equilibrate step, 40 cycles in supporting solution, 20 cycles in CO saturated supporting solution and 20 cycles in supporting solution again are shown in Fig. 5B,5C and 5D respectively.

The first 40 cycles in supporting solution aimed at equilibrating the ratio between Cu and
Cu₂O and simulating a situation that was similar to the measurement to minimize the change of the electrode and to acquire consistency during the real measurement at later stage. The second 20 cycles in CO saturated supporting solution aimed at stabilizing the electrode. Comparing Fig. 2B with 2D, 5B with 5D, the current change during scan was actually minimized by the process in between which are shown in Fig. 2C and 5C. The third 20 cycles in supporting solution aimed at removing the excess CO from second 20 cycles. As we would measure CO in the later part of the experiment, Excessive CO was cleared to acquire confident result. Without equilibrating in saturated CO (second 20 cycles), the electrode changing during CO measurement was a problem and caused a problem of reproducibility.

The Scan Rate was 50mV/s and the Potential Window was from 0 V to -0.25V. The Scan rate was chosen by considering the Cu converting and dissolving rate. If it was too fast the electrode would be damaged, whereas if it was too slow, current would not be large enough due to the sensitivity of the electrode. The range here aimed to converting Cu₂O to Cu and Cu to Cu₂O only and minimizing all other reactions were out of the potential window, for example, Cu₂O to CuO, CuO to Cu and O₂ to H₂O.

3.3.1.3 Deposition parameter consideration

For the Cu deposition electrode, chronopotentiometry was used as a electrochemistry technique to deposit Cu on the Au sputtering electrode. The constant current setting, 20uA, 40uA and 80uA for 800s was done in our experiment and the corresponding chronopotentiograms are shown in Fig. 3B, 3D and 3F respectively. The SEM photos of the Cu deposition electrode corresponding to 20uA, 40uA and 80uA for 800s are shown in Fig. 3A, 3C and 3E.

The chronopotentiograms in Fig. 3B, 3D with 3F were very similar. The potential required to maintain the constant current 20uA, 40uA and 80uA were all in the range of 0V to -0.1V. In this range, a more negative potential was required to maintain a more positive cathodic current. The more positive current setting for the same deposition time would result
in deposition of larger particles and larger particle population per unit area, as shown in Fig. 3B, 3D and 3E.

Larger particles formed in larger current settings because the total charge corresponding to the deposition of the copper in the same time duration increased with the current setting. The larger population of particle per unit area resulted in larger current setting because larger potential was needed to maintain the larger constant current. At the same time, larger potential increased the surface potential of the substrate leading to more sites having sufficient surface energy to generate a stable nucleus that could grow continuously. Finally, constant current 80μA for 800s was used as the parameter for later experiment considering the Cu surface covering ratio was the best among other parameters. The solution we used in deposition was Cu deposition solution which contained CuSO₄ 20mM as a source of copper, K₂SO₄ 0.1M as supporting electrolyte and acetic acid 0.05M as buffer at pH 4.91. The size of the electrode was 3 mm diameter.

3.3.2 Detection of CO

3.3.2.1 Peak illustration

The CV of CO from 2μM to 100μM and 100μM to 1000μM on Cu sputtering electrode are shown in Fig. 6 and 7 respectively. The CV of CO from 2μM to 100μM and 100μM to 1000μM on Cu deposition electrode are shown in Fig. 11 and 12 respectively. For Fig. 6, 7, 11 and 12, the oxidation peaks around -0.1V were related to the oxidation from Cu to Cu₂O and the oxidation peak would increase with CO concentration. The reduction peaks around -0.18V for Fig. 6, 7 and -0.16V for Fig. 11, 12 were related to the reduction from Cu₂O to Cu and the reduction peak would decrease with increasing CO concentration.

3.3.2.2 Basic principle

Before the measurement, equilibrate step was performed in order to get both of the peaks
stabilized. In other words, the conversion from Cu to Cu$_2$O and from Cu$_2$O to Cu were the same for each CV cycle, and thus the Cu to Cu$_2$O ratio of both electrodes was in a steady state. The steady state here was the equilibrium state for particular experiment conditions (e.g. CO concentration) and particular experiment parameters (e.g. Scan Rate and potential windows). During the measurement, CO was added to the electrode surface. The increase of oxidation peaks and the decrease of reduction peaks showing the number of moles of Cu oxidation to Cu$_2$O were larger than the number of moles of Cu$_2$O to Cu reduction, due to the fact that initially, in blank condition, they were the same. This means that the Cu to Cu$_2$O steady state ratio decreased with increasing CO concentration. The equilibrium shifted to Cu$_2$O from Cu after adding CO. The thermodynamic change before and after adding CO was expressed as follows:

\[
\begin{align*}
2\text{Cu} & \overset{+2\text{CO}}{\rightleftharpoons} \text{Cu}_2\text{O} \overset{+2\text{CO}}{\rightleftharpoons} 2\text{CuO} \\
2\text{CuCO(ads)} & \overset{+\text{O}}{\rightleftharpoons} \text{Cu}_2\text{O(CO)}_x(\text{lig}) \overset{+\text{O}}{\rightleftharpoons} 2\text{CuO} + 2\text{CO}
\end{align*}
\]

The interaction between CO and Cu$_2$O was strong and corresponded to ligation (lig). The interaction between CO and Cu was weak and corresponded to adsorption (ads). The interaction between CO and CuO was very weak and could be ignored. The ligation would stabilize the Cu$_2$O more and shift the equilibrium to Cu$_2$O from Cu and CuO. The tendency of the original Cu to Cu$_2$O ratio changing to a new Cu to Cu$_2$O steady state ratio drove the increase of the oxidation peak and the decrease of the reduction peak. Using this property, we could use the peak change to detect CO. Note that, the changing of the Cu to Cu$_2$O ratio itself during experiment was small compared to the difference between the original Cu to Cu$_2$O steady state ratio and the new Cu to Cu$_2$O steady state ratio, as the percentage of Cu species involved in the conversion during experiment was small compared to the total Cu species on the surface.
### 3.3.2.3 Surface effect in our experiment

Comparing Fig. 7 with 12, the oxidation peaks of Cu to Cu$_2$O on different substrate were located at a very similar potential: -0.1V against SCE and the peak shift of the oxidation peaks related to different CO concentration were negligible. By contract, the reduction peaks of Cu$_2$O to Cu on different substrate were located at different potential: -0.18V against SCE for Cu sputtering electrode and -0.16V against SCE for Cu deposition electrode. It was because work function of the smooth surface of the Cu sputtering electrode was larger than that of the rough surface of the Cu deposition electrode. In other words, due to the point charge effect of the rough surface, the electrons could more easily to escape from the surface and consumed less energy than the smooth surface.

Both of the electrodes had peak shift of the reduction peaks from more negative to less negative potential when CO concentration was increased. Comparing the peak when 1000uM CO was added with that in the blank condition, the peak shift of reduction peaks on Cu deposition electrode (from -0.165V to -0.145V) was larger than that on Cu sputtering electrode (from -0.18V to -0.17V). The peak shifted because some energy was needed to activate the comparatively inert reagent or place to carry out the reaction, since the comparatively active reagent or place was not enough to fulfil the reaction rate or the tendency of change in the given experiment conditions or parameters. In other words, the work function diversity of the surface affected the peak shifts here. For the Cu sputtering electrode, the surface was smooth and the surface conditions were similar leading to small work function diversity, thus small peak shift. For the Cu deposition electrode, the surface was rough and the surface conditions were different from one place to another leading to large work function diversity, thus large peak shift.

### 3.3.2.3 Calibration curve & detection limit

The calibration curve of CO from 5μM to 50μM and 50μM to 1000μM on Cu sputtering electrode by using $\Delta$Ipc were shown in Fig. 8 and 9 respectively. The calibration curve of CO
from 50μM to 1000μM on Cu sputtering electrode by using ΔIpa is shown in Fig. 10. The calibration curve of CO from 5μM to 100μM and 100μM to 1000μM on Cu deposition electrode by using ΔIpc are shown in Fig.13 and 14 respectively. The calibration curve of CO from 100μM to 1000μM on Cu deposition electrode by using ΔIpa is shown in Fig. 15.

The limiting factor of detection of CO of our method is the technique itself, so method detection limit was considered here. The detection limit of CO on Cu sputtering electrode was 4.60μM by using ΔIpc and 19.54 μM by using ΔIpa. The detection limit of CO on Cu deposition electrode was 4.95μM by using ΔIpc and 21.27μM by using ΔIpa. The detection limit was calculated by scanning the blank solution for 10 times and acquiring the real random error and taking the standard deviation as a noise. The detection limit was calculated using 3 time of noise as requirement and using the corresponding slope in the calibration. The performance of both electrodes in terms of detection limit was very similar. That means the structure of the electrode would not affect the detection limit in our case. One possible reason for this was the method itself being the limiting factor of the detection limit so on different surface, but using same technique, CV, would give a very close or similar detection limit. The similarity of the detection limit of different electrodes was the supporting evidence of this idea.

Comparing to the CO detection limit of P. Richard G experiment which was 3μM, it was found that the signal was two times of the noise. It is comparable to our detection limit as we used 3 times of noise as requirement and our detection limit was 4.60μM.

The slope, linear range and R² value of different calibration curves of different electrodes were compared in the table below.
### 3.3.2.4 Performance comparison

Comparing among different electrode types, all of them had a very good linearity as shown in the $R^2$ values. The negative sign of the slope means the peak decreased with increasing CO concentration. The performance (sensitivity) of all of them were very similar, except for in the low concentration range, 5 to 50 μM, the slope corresponding to the Cu sputtering electrode was about 3 time higher than that of the Cu deposition electrode. The amount of Cu$_2$O on the surface was one of the factors that affected the sensitivity, as the CO absorption to the Cu$_2$O was the major triggering event that shifted the equilibrium between Cu and Cu$_2$O and which was the core of our CO detection method. Considering the high concentration linear range of both electrodes in both $\Delta I_{pa}$ and $\Delta I_{pc}$ curves, the lower limit of the linear range for Cu sputtering electrode was lower than that of Cu deposition electrode. Combining all the data in the table, all of the electrodes were good for CO detection, but for low concentration CO detection, Cu sputtering electrode was better than Cu deposition electrode.

<table>
<thead>
<tr>
<th>Electrode type</th>
<th>$\Delta I_{pa} / \Delta I_{pc}$</th>
<th>Linear range (μM)</th>
<th>Slope (μA/μM)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu sputtering</td>
<td>$\Delta I_{pc}$</td>
<td>5 to 50</td>
<td>-0.0375</td>
<td>0.991</td>
</tr>
<tr>
<td>Cu deposition</td>
<td>$\Delta I_{pc}$</td>
<td>5 to 100</td>
<td>-0.0132</td>
<td>0.991</td>
</tr>
<tr>
<td>Cu sputtering</td>
<td>$\Delta I_{pc}$</td>
<td>50 to 1000</td>
<td>-0.00953</td>
<td>0.982</td>
</tr>
<tr>
<td>Cu deposition</td>
<td>$\Delta I_{pc}$</td>
<td>100 to 1000</td>
<td>-0.00871</td>
<td>0.973</td>
</tr>
<tr>
<td>Cu sputtering</td>
<td>$\Delta I_{pa}$</td>
<td>50 to 1000</td>
<td>0.00303</td>
<td>0.963</td>
</tr>
<tr>
<td>Cu deposition</td>
<td>$\Delta I_{pa}$</td>
<td>100 to 1000</td>
<td>0.00306</td>
<td>0.984</td>
</tr>
</tbody>
</table>
3.3.2.5 Interference consideration

The potential window used in our experiment, i.e. -0.25 to 0V against SCE, was relatively mild and narrow. The major possible interference was reduction peaks of O$_2$, but according to our experiment, no oxygen reduction peaks were observed in our potential window. From the other experiments in our lab, we knew that the oxygen reduction peak was at a the potential more negative than -0.25V, out of our potential window. Furthermore, all the experiments shown in this report was used the supporting electrolyte without purging N$_2$. That means about 260μM relatively high concentration of O$_2$ was in the solution for all the experiments when the CO concentration was below 100μM. Furthermore, varied concentration of O$_2$ was in the solution for all the experiments when the CO concentration was above 100μM, as we needed to acquire such high concentration of CO by purging CO into the supporting electrolyte, so some oxygen may be removed. As a result, we found that even though the O$_2$ concentration was high and varied in our experiment, we saw no effect of O$_2$ given in our experiment or our experiment was not affected by O$_2$ concentration, showing that the major possible interference, O$_2$, could be ignored.

Apart from O$_2$, Cl$^-$ was also a potential interference. As the Cl$^-$ would compete with CO for the binding site on Cu$_2$O. A gas permeable membrane should be used to separate Cl$^-$ from Cu$_2$O surface. (Development of such gas membrane would not be covered in this chapter.) Based on my knowledge, silicone is a well-developed high performance gas permeable membrane and has been widely used in reducing the charged interference, so in principle, the Cl$^-$ interference problem could be solved.
3.4 Conclusions

A new method to measure CO in aqueous buffer solution was developed. Two different fabrication methods of the sensor (Cu sputtering electrode and Cu deposition electrode) were developed. The morphology was characterized by Scanning Electron Microscopy (SEM) and performances of two methods were compared. Both of the fabrication methods of CO sensor were successful and workable. The detection limit of CO on Cu sputtering electrode was 4.60 μM by using $\Delta I_{pc}$ and 19.54 μM by using $\Delta I_{pa}$. The detection limit of CO on Cu deposition electrode was 4.95 μM by using $\Delta I_{pc}$ and 21.27 μM by using $\Delta I_{pa}$. They bore similar detection limit. For the low concentration range, 5 to 50 μM, by using $\Delta I_{pc}$, the sensitivity of Cu sputtering electrode was about 3 times higher than that of the Cu deposition electrode. Both of them had great linear relationship (good $R^2$). Although the Cu deposition electrode had small work function resulted in lower kinetic potential of the $\text{Cu}_2\text{O}$ reduction peak, it was not critical for our detection methods. As the preparation of the Cu sputtering electrode was easier and the sensitivity for low CO concentration range was better, we concluded that the Cu sputtering was the better fabrication method of the sensor between the two (sputtering and deposition).
3.5 References


[34] G. S. Marks, Antioxidants & Redox Signaling, 2002, 4, 271

3.6 Legends and Figures

Fig. 1: SEM photo of the Cu sputtering electrode before (A) and after (C) the pre-treatment and the amperogram of the pre-treatments (B: -0.4V vs SCE for 400s, D: 0V vs SCE for 400s). pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M

Fig. 2: SEM photo of the Cu sputtering electrode after equilibrate step (A) and the CV of the equilibrate step (B: 40 cycles in supporting solution, C: 20 cycles in CO saturated supporting solution, D: 20 cycles in supporting solution again after C). pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V

Fig. 3: SEM photo of the Cu deposition electrode (A,C,E) and the corresponding chronopotentiogram (B = 20μA, D = 40μA, F = 80μA). pH = 4.91, CuSO$_4$ = 20mM, K$_2$SO$_4$ = 0.1M, acetic acid = 0.05M, r = 3mm, Time = 800s

Fig. 4: SEM photo of the Cu deposition electrode before (A) and after (C) the pre-treatment and the amperogram of the pre-treatments (B: -0.4V vs SCE for 400s, D: 0V vs SCE for 400s). pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M

Fig. 5: SEM photo of the Cu deposition electrode after equilibrate step (A) and the CV of the equilibrate step (B: 40 cycles in supporting solution, C: 20 cycles in CO saturated supporting solution, D: 20 cycles in supporting solution again after C). pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V

Fig. 6: CV of CO from 2μM to 100μM on Cu sputtering electrode. pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V

Fig. 7: CV of CO from 100μM to 1000μM on Cu sputtering electrode. pH = 8, KNO$_3$ = 0.1M, KH$_2$PO$_4$ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V
Fig. 8: Calibration curve of CO from 5μM to 50μM on Cu sputtering electrode by using ΔIpc. Reference line potential = -0.08V to -0.09V. Potential taken = -0.18V

Fig. 9: Calibration curve of CO from 50μM to 1000μM on Cu sputtering electrode by using ΔIpc. Reference line potential = -0.08V to -0.09V. Potential taken = -0.18V

Fig. 10: Calibration curve of CO from 50μM to 1000μM on Cu sputtering electrode by using ΔIpa. Reference line potential = -0.19V to -0.2V. Potential taken = -0.1V

Fig. 11: CV of CO from 2μM to 100μM on Cu deposition electrode. pH = 8, KNO₃ = 0.1M, KH₂PO₄ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V

Fig. 12: CV of CO from 100μM to 1000μM on Cu deposition electrode. pH = 8, KNO₃ = 0.1M, KH₂PO₄ = 0.05M, Scan Rate = 50mV/s, Potential Window = 0 V to -0.25V

Fig. 13: Calibration curve of CO from 5μM to 100μM on Cu deposition electrode by using ΔIpc. Reference line potential = -0.08V to -0.09V. Potential taken = -0.16V

Fig. 14: Calibration curve of CO from 100μM to 1000μM on Cu deposition electrode by using ΔIpc. Reference line potential = -0.08V to -0.09V. Potential taken = -0.16V

Fig. 15: Calibration curve of CO from 100μM to 1000μM on Cu deposition electrode by using ΔIpa. Reference line potential = -0.19V to -0.2V. Potential taken = -0.1V
Fig. 1
Fig. 2
Fig 3
Fig 4

A

B

C

D

\( i / \mu A \)

\( t / s \)

\( 0 \quad 100 \quad 200 \quad 300 \quad 400 \quad 500 \)
Fig 5
Fig 6
Fig 7
\[ \Delta I_{pc} (\mu A) = -2.093 - 0.0375 \times [CO] (\mu M) \]

\[ R^2 = 0.991 \]
Fig 9

\[ \Delta \text{ipc(\text{uA})} = -3.838 - 0.00953 \times [\text{CO}](\text{\mu M}) \]

\[ R^2 = 0.982 \]
Fig 10

\[ \Delta I_{pa}(\mu A) = -0.553 + 0.00303 \times [\text{CO}](\mu M) \]

\[ R^2 = 0.963 \]
Fig 12

The graph shows a cyclic voltammetry plot with the x-axis representing potential (E vs SCE / V) and the y-axis representing current (i / μA). The plot includes multiple curves labeled as blank, 100uM, 200uM, 500uM, and 1000uM, indicating different concentrations of a solution.
Fig 13

\[ \Delta I_{pc} (\mu A) = 0.837 - 0.0132 \times [CO] (\mu M) \]

\[ R^2 = 0.991 \]
Fig 14

\[ \Delta I_{pc} (\mu A) = -0.888 - 0.00871 \cdot [CO] (\mu M) \]

\[ R^2 = 0.973 \]
Fig 15

\[ \Delta i_{pa} (\mu A) = -0.257 + 0.00306 \times [CO] (\mu M) \]

\[ R^2 = 0.984 \]
Chapter 4

Nano-structured Platform for Highly Selective, Sensitive and Quantitative Electrochemical Detection of Hydrogen Sulphide (H2S) in aqueous buffer solution with biological interferrents
4.1 Introduction

Hydrogen sulphide (H$_2$S) is famous for its unpleasant rotten egg smell and was traditionally considered as a toxic gas. After the discovery that nitric oxide (NO) can act as a signalling molecule to control a variety of biological processes, a similar role of H$_2$S has also been revealed. H$_2$S is endogenously generated in many cell types inside human body and has been associated with many important physiological functions. H$_2$S can act as a vasodilator by opening the K-ATP channel [1]. H$_2$S can mediate cardioprotection through increasing the nuclear localization of Nrf2 [2], which is a transcription factor that regulates the gene expression of a number of antioxidants and activate the antiapoptotic signalling pathway. H$_2$S is both pro and anti inflammatory depending on the concentration level, changeable by activating or deactivating stress stimulated proteins NF-κB and MAPKs [3]. Normally, high concentration of H$_2$S results in pro inflammatory response and low concentration results in anti inflammatory response. H$_2$S can also selectively enhance NMDA receptor-mediated responses and alter the induction of hippocampal long-term potentiation (LTP) [4], which is related to memory consolidation.

H$_2$S has shown a double-faced and double-edged biological identity, being both a toxin and a signalling messenger depending on the concentration and location of its endogenous production. While the demand for its in situ detection in a selective, quantitative, and sensitive manner is increasing, any effort for this goal has to overcome a serious challenge posed by the interference of many co-existing thiol-containing species such as cysteine, methionine and glutathione among others.

Conventionally, we can detect H$_2$S by using spectrophotometry, chromatography and electrochemistry. For spectrophotometry, we can use UV/Visible spectrometry, molecular absorption spectrometry and fluorescence. UV/Visible spectrometry and molecular absorption spectrometry are not instantaneous detection technique. As for fluorescence, the quenching effect of the fluorophore causing the loss of signal over time is the main problem.
For chromatography, we can use ion chromatography, gas chromatography and high performance liquid chromatography. All three techniques need sophisticated preparation of the sample, such as head space gas detection. Hence, fast response is not possible as the analyte takes time to run through the column.

For electrochemistry, we can use potentiometry, amperometry, galvanic and cathodic stripping. Except for cathodic stripping, all of them are real time, quantitative, sensitive, selective and miniaturizable. Electrochemistry appears to be an attractive method for monitoring H₂S in vivo. Despite these advantages of electrochemistry, the design and fabrication of electrode targeting a particular analyte in the presence of other interferences is extremely difficult and challenging.

Traditionally, several approaches have been developed for the electrochemical detection of H₂S in aqueous solution. For example, Ag/AgS electrode [5] can be used to detect H₂S, but long response time, deviation from ideal Nernstian behaviour and poisoning of the reference electrode component by sulfide are some of the problems that have limited the use of conventional sulfide selective electrodes.

Another approach is that we can use mediators with gas permeable membrane (GPM) (e.g. silicone, PTFE) to detect H₂S. Those mediators include inorganic mediators consisting of [Fe(CN)6]³⁻ [6], organometallic mediators consisting of ferrocene carboxylates [7] and organic mediators consisting of catechol, dopamine, hydroquinone and N,N-dimethyl phenylene-1,4-diamine (DMPD) [8,9], as well as diphenylamine (DPA) derivatives[9–11]. Current GPM materials are not sufficient for imparting sensor selectivity towards gaseous interferences or small molecule interferences. The major frequently appearing gas interference is O₂ which is an oxidizing agent and it may affect the redox behavior of the mediators and cause some interference during detection of H₂S. Those mediators are normally toxic and not biocompatible, so a mediator reservoir is needed between the working electrode and the bulk analyte solution. It may cause the loss of miniaturization capability and loss of sensitivity.
The final approach is that we can use a catalyst that particularly targets our analyte H$_2$S. Vanadium (V) oxide (V$_2$O$_5$) is a good example for this approach as shown by other groups [12]. For this approach, normally, we have to take care of the sulphur, the oxidation product of the H$_2$S, forming on the electrode surface. It may cause the inability of high concentration detection of H$_2$S and loss of the signal during detection. According to Jason A. Bennett experiment [12], by applying V$_2$O$_5$ in neutral pH, the linear range was found to be narrow, from 0.5 to 15μM, and did not fit the physiological concentration range of H$_2$S from 30 to 160 μM. It also suffered from the interference from NO and CO and lacked of the experiments related to other sulfur containing interferences, such as cysteine, methionine and glutathione.

For our approach, we selected nano-gold as our H$_2$S targeting catalyst. The absorption and desorption behaviors of sulfur containing molecule on nano-gold surface are well known and it was traditionally used to immobilize functional group on to the nano-gold surface. By using the absorption and desorption behaviors of H$_2$S on nano-gold, we could narrow down our detection of interference to sulphur containing molecules, as the potential of this absorption and desorption was so special. After that, by immobilizing the nafion, a negative polymer, on the electrode surface, we reduced the signal contributed from the remaining sulfur containing molecules and were able to detect H$_2$S specifically. Notice that by using the absorption and desorption of H$_2$S on the nano-gold surface instead of direct oxidation, we avoided the sulfur formation problem which commonly occurred in the detection methods involving direct oxidation of H$_2$S.
4.2 Experimental section

4.2.1 Reagent preparation

Potassium hydroxide (KOH) and Potassium phosphate monobasic (KH$_2$PO$_4$) were purchased from “Riedel-deHaen”. Potassium chloride (KCl) was purchased from “Sigma-Aldrich”. 20% Nafion resin in propanol/water was purchased from “Aldrich”. Hydrogen sulfide (H$_2$S) 99.5% was purchased from Hong Kong Specialty Gases CO. LTD. Ultra pure water was obtained from “Barnstead nanopure infinity ultrapure water system”.

Standard saturated H$_2$S solution was prepared by purging pure N$_2$ in ultra pure water for 30 minutes first, then purging pure H$_2$S for 30 minutes and using a septum and parafilm to keep it in good condition. The saturated concentration of H$_2$S in water at 298.15 K and 1bar was 0.103 M. The supporting solution contained 0.05M KH$_2$PO$_4$ and 0.1M KCl. KOH was adding to the supporting solution to adjust the pH of the solution to 7.2.

4.2.2 Electrode fabrication

4.2.2.1 Au nano-frame electrode

The 60 μm thick aluminum oxide nanoporous frame was purchased from Whatman Co. The upper surface contained 20nm diameter pores and the lower surface contained 150nm diameter pores. The frame first was sonicated in ethanol for 30 minutes and then in water for 30 minutes. Then, the frame was dried in fume hood. After the treatment, the cleaned frame was used as a template for plasma sputtering deposition of Au on the upper surface. The deposition was carried out using a Thin Film Sputtering System-Denton Discovery 18 instrument (Denton Vacuum). The sputtering parameters were set as the same as the deposition of 200nm Au on plain surface.
4.2.2.2 Nafion- Au nano-frame electrode

The Nafion- Au nano-frame electrode in DPA experiment was prepared by adding 6uL of 20% Nafion in propanol onto the Au nano-frame electrode and then dried by dry N\textsubscript{2} for 30 minutes.

4.2.3 Pre-treatment

Before the measurement of H\textsubscript{2}S, some pre-treatments had been done. The electrode was first applied -0.8V for 800s to convert all the surface oxidized Au species into Au and to establish a stable solution metal contact surface for later measurement. The electrode was then stabilized by the later used differential pulse amperometry (DPA) measurement profile, adsorption method (-0.8V for 0.9s, -0.75V for 0.05s and -0.65V for 0.05s) or desorption method (-0.5V for 0.9s, -0.65V for 0.05s and -0.8V for 0.05s) for 800s in the 50mM H\textsubscript{2}S supporting solution. Before starting those CV experiments, the electrode was equilibrated by using the later used CV profile 20cycles first to make sure the electrode was stable enough and the condition of the electrode would not change during the experiment in order to ensure the data quality.

4.2.4 Measurements

Electrochemical measurements were performed at a CHI 660C electrochemical workstation (USA). A three-electrode system was employed with Saturated calomel electrode (Hg/Hg\textsubscript{2}Cl\textsubscript{2}) as reference electrode, a platinum plate as counter electrode (diameter= 2 mm, geometric area= 4 mm\textsuperscript{2}), and Au nano-frame electrode (diameter= 3 mm, geometric area= 7.065mm\textsuperscript{2}) or Au plate (diameter= 2 mm, geometric area= 4 mm\textsuperscript{2}), as working electrode for the measurements of H\textsubscript{2}S.
All the potentials in this chapter were with respect to Saturated calomel electrode (Hg/Hg₂Cl₂) and all measurements were carried out at room temperature (25 ± 2°C) at 1atm. For differential pulse amperometry experiment, stabilization of the electrode at the setting used in the later experiment for 200s was needed.

4.3 Results & discussion

4.3.1 Structure of our sensors

The structure of Au nano-frame electrode is shown in Fig 1A. The nano-porous membrane had two sides. One side contained 20nm holes and another side contained 150nm holes. The Au was sputtering onto the 20nm hole side. The nano-porous membrane here was vital. It had three functions. The first function was to create Au nano reacting sites which adhere to high surface area compared with conventional Au electrode, as some of the platinum was stuck on the wall of the aluminum oxide. The second function was to provide a biocompatible substrate surface to immobilize macromolecules onto it, so that we could expand the functions of our electrode and strengthen the interference resistance. The third function was to act as an interference resistance membrane to separate those adhesive large size interferents from the metal surface to avoid the blockage and toxication of the electrode.

The SEM picture of the 20nm holes side of the nano-porous membrane after sputtering is shown in Fig 1B. The picture shows that the 20nm size holes were sealed and a semi-sphere nano surface was formed. The SEM pictures, top view and side view, of the 150nm holes side of the nano-porous membrane before sputtering are shown in Fig 1C and 1D respectively. The SEM pictures, top view and side view, of the 20nm hole side of the nano-porous membrane are shown in Fig 1E and 1F respectively.
4.3.2 Peak illustration and strategy determination

The cyclic voltammograms of 1mM H\textsubscript{2}S on Au nano-frame electrode and Au plate electrode are shown in Fig. 2A and 2B respectively. There were 4 peaks in the CV of Au nano-frame electrode (Fig. 2A). They were H\textsubscript{2}S oxidation peak (-0.15V), Sulfur reduction peak (-0.64V), H\textsubscript{2}S adsorption peak (-0.66V) and H\textsubscript{2}S desorption peak (-0.75V). The reaction equation for each peak is shown below [13].

\[
\begin{align*}
\text{H}_2\text{S}_{(aq)} & \longrightarrow \text{HS}_{(ads)} + \text{H}^+ + \text{e}^- & \text{Adsorption peak} \\
\text{HS}_{(ads)} + \text{H}^+ + \text{e}^- & \longrightarrow \text{H}_2\text{S}_{(aq)} & \text{Desorption peak} \\
\text{HS}_{(ads)} & \longrightarrow \text{S}_{(s)} + \text{H}^+ + \text{e}^- & \text{H}_2\text{S oxidation peak} \\
\text{S}_{(s)} + \text{H}^+ + \text{e}^- & \longrightarrow \text{HS}_{(ads)} & \text{Sulfur reduction peak}
\end{align*}
\]

On the other hand, we could only find 3 peaks in the CV of Au plate electrode (Fig. 2B). They were H\textsubscript{2}S oxidation peak (-0.1V), Sulfur reduction peak (-0.7V) and H\textsubscript{2}S adsorption peak (-0.68V). Desorption peak of H\textsubscript{2}S on Au plate electrode was missing. We changed the potential window of the CV on Au plate electrode, so that the sulfur reduction peak was suppressed by limiting the oxidation of H\textsubscript{2}S. The cyclic voltammogram of 1mM H\textsubscript{2}S on Au plate electrode with the changed potential window was shown in Fig. 2C. After suppressing the sulfur reduction peak, we could then find 4 peaks, hence we deduced that the reason behind the missing peak in Fig. 2B was due to the overlapping of the strong sulphur reduction peak and H\textsubscript{2}S desorption peak. Comparing the potential and the relative size of the peaks of Au nano-frame electrode with that of Au plate electrode, we found that the Au nano-frame electrode could enhance the oxidation of H\textsubscript{2}S and suppress the reduction of the sulfur. From Fig. 2A and 2B, we could see the sulfur formed during the first cycle blocking the surface and reducing the current of the second cycle on Au nano-frame electrode, but not on Au plate electrode.
In order to avoid the sulfur blockage problem, we decided to use the desorption and adsorption peaks instead of the reduction and oxidation peaks. The cyclic voltammograms before and after adding 1mM H$_2$S on Au nano-frame electrode are shown in Fig. 3A and 3B respectively. The cyclic voltammograms of blank solution after H$_2$S measurement and after applying -0.9V for 400s in blank solution on Au nano-frame electrode are shown in Fig. 3C and 3D respectively. By comparing Fig. 3A with 3C, we found that there was permanent modification of the nano-Au surface after the treatment of H$_2$S. By comparing Fig.3C and 3D, we knew that the modification caused by H$_2$S cannot be cleaned by applying a negative potential -0.9V for 400s. By comparing Fig. 3B with 3C, we knew the concentration increase of H$_2$S was related to the desorption and absorption peaks current increase, so by measuring the current change, theoretically we could measure the H$_2$S concentration.

4.3.3 Desorption method by using DPA on Au nano-frame electrode

Since the current change related to the concentration change of H$_2$S was small and the potential we used was relatively negative compared to other reducible interferents, we applied differential pulse amperometry (DPA) technique to measure our analyte, H2S, in order to increase the sensitive and resistance to interference. We developed two profiles to measure H$_2$S. One was desorption method and the other was adsorption method. The DPV setting of desorption method was first applying -0.5V 0.9s to cause H$_2$S adsorption on the nano-Au surface and then applying -0.65V 0.05s to take a reference current to screen out those reducible interferents with H$_2$S still adsorbed on the nano-Au surface in this potential. Finally, we applied -0.8V 0.05s to make the H$_2$S desorbed from the nano-Au surface and the current associated with the desorption of H$_2$S was measured. After one cycle of the potential change, we started another cycle by changing the potential back to -0.5V to adsorb H$_2$S again and closed the loop. By taking the differential current between the reference potential and the desorption potential, we could measure H$_2$S in a sensitive, quantitative and selective manner.

The DPA graphs of addition of different H$_2$S concentration 1μM to 10μM and 10μM to 100μM on Au nano-frame electrode by desorption method are shown in Fig. 4A and 4B.
respectively. From Fig. 4A, we found that the detection limit of our H$_2$S sensor by desorption method was 2$\mu$M. From Fig. 4A and 4B, we could see the current was stable and reproducible from 2$\mu$M to 100$\mu$M. We tried to test the upper bound of the linear range of our sensor by successive addition of 100$\mu$M H$_2$S. The DPA graphs of successive addition of 100$\mu$M H$_2$S and the successive addition curve of 100$\mu$M H$_2$S on Au nano-frame electrode by desorption method are shown in Fig. 5A and 5B. From Fig. 3B, we could see the Au nano-frame electrode was started to saturate after the addition of 200$\mu$M H$_2$S, so the upper bound of the linear range of our H$_2$S sensor by desorption method was 200$\mu$M.

By plotting the differential current change of Fig. 4A and 4B against the H$_2$S concentration we added, we obtained the calibration curve of H$_2$S. The calibration curves of H$_2$S of different concentration range 5$\mu$M to 200$\mu$M and 5$\mu$M to 50$\mu$M on Au nano-frame electrode by desorption method are shown in Fig. 6A and 6B respectively. From Fig. 6A, we found that the calibration equation from 5$\mu$M to 200$\mu$M and the corresponding $R^2$ were

$$\Delta i (\mu A) = -0.165 - 0.234 \times [H_2S] (\mu M)$$

$$R^2 = 0.996.$$  

From Fig. 6B, we found that the calibration equation from 5$\mu$M to 50$\mu$M and the corresponding $R^2$ were

$$\Delta i (\mu A) = -0.775 - 0.210 \times [H_2S] (\mu M)$$

$$R^2 = 0.990.$$  

Combining Fig. 6A and 6B, we found that the linear range of our H$_2$S sensor by desorption method was from 5$\mu$M to 200$\mu$M. It really fitted the physiological concentration range of H$_2$S in human blood from 30$\mu$M to 160$\mu$M.
4.3.4 Adsorption method by using DPA on Au nano-frame electrode

Similar to desorption method, we could also use adsorption method to measure H$_2$S. The DPV setting of adsorption method was first applying -0.8V 0.9s to cause H$_2$S desorption from the nano-Au surface and then applying -0.75V 0.05s to take a reference current to screen out those reducible interferents with H$_2$S still desorbed from the nano-Au surface in this potential. Finally, we applied -0.65V 0.05s to make the H$_2$S adsorbed on the nano-Au surface and the current associated with the adsorption of H$_2$S was measured. After one cycle of the potential change, we started another cycle by changing the potential back to -0.8V to desorb H$_2$S again and closed the loop. By taking the differential current between the reference potential and the adsorption potential, we could also measure H$_2$S in a sensitive, quantitative and selective manner.

The DPA graphs of addition of different H$_2$S concentration 10μM to 100μM and 100μM to 1000μM on Au nano-frame electrode by adsorption method are shown in Fig. 7A and 7B respectively. From Fig. 7A, we found that the detection limit of our H$_2$S sensor by desorption method was 10μM. From Fig. 7A and 7B, we could see the current was stable and reproducible from 10μM to 1000μM. We tried to test the upper bound of the linear range of our sensor by successive addition of 1000μM H$_2$S. The DPA graphs of successive addition of 1000μM H$_2$S and the successive addition curve of 1000μM H$_2$S on Au nano-frame electrode by adsorption method are shown in Fig. 8A and 8B. From Fig. 8B, we could see the Au nano-frame electrode was started to saturate after the addition of 3000μM H$_2$S, so the upper bound of the linear range of our H$_2$S sensor by adsorption method was 3000μM.

By plotting the differential current change of Fig. 7A and 7B against the H$_2$S concentration we added, we obtained the calibration curve of H$_2$S. The calibration curves of H$_2$S of different concentration range 10μM to 3000μM and 10μM to 200μM on Au nano-frame electrode by adsorption method are shown in Fig. 9A and 9B respectively. From Fig. 9A, we found that the calibration equation from 10μM to 3000μM and the corresponding $R^2$ were
\[ \Delta i (\mu A) = 0.0458 + 0.0109 \cdot [H_2S](\mu M) \]

\[ R^2 = 0.996. \]

From Fig. 9B, we found that the calibration equation from 10\(\mu M\) to 200\(\mu M\) and the corresponding \(R^2\) were

\[ \Delta i (\mu A) = 0.134 + 0.00862 \cdot [H_2S](\mu M) \]

\[ R^2 = 0.996. \]

Combining Fig. 9A and 9B, we found that the linear range of our \(H_2S\) sensor by desorption method was from 10\(\mu M\) to 3000\(\mu M\). It exceeded the physiological concentration range of \(H_2S\) in human blood from 30\(\mu M\) to 160\(\mu M\). By considering this wide linear range, the absorption method had a very high potential to be developed as a \(H_2S\) gas sensor.

### 4.3.5 Interference comparison between desorption and adsorption method

After the use of DPA approach, many of the reducible interferents were screened out by the potential window we used and for such a negative potential window we used, nearly no oxidizable interferent could be found. The remaining potential interferents were those sulfur containing compounds, including cysteine, methionine and glutathione, which would also undergo desorption and adsorption on nano-Au surface.

We tried to compare the interferences on Au nano-frame electrode by using desorption and adsorption method. The DPA curves of interferences on Au nano-frame electrode by using adsorption and desorption method are shown on Fig. 10A and 10B respectively. A constant concentration 50\(\mu M\) for both interferents and analyte was used to test the interference resistance of each method. From Fig. 10A, the interferences caused by all three interferents, cysteine, methionine and glutathione, were strong using adsorption method,
especially for methionine and glutathione. Comparing Fig. 10B with 10A, the interferences caused by methionine and glutathione were weak using desorption method, especially for glutathione. For desorption method, the current drop significantly after adding cysteine, so the interference of cysteine was strong using desorption method. In summary, the interference resistant of desorption method was stronger than adsorption method, so desorption method was chosen to have a further interference resistant improvement development.

4.3.6 Reducing interference by using nafion

By immobilizing nafion on the 100nm holes side surface, we had successfully reduced the interference. The DPA curves of interferences before and after immobilization of nafion are shown in Fig. 14A and 14B. Comparing Fig. 14B with 14A, the current drop caused by cysteine was significantly reduced by the immobilization of nafion. The pulse current curves (blue = -0.65V and red = -0.8V) before and after immobilization of nafion are shown in Fig. 14C and 14D. Comparing the blue line of Fig.14C with 14D, there was an increase of negative current after the addition of cysteine and it means cysteine was desorbed in -0.65V before the immobilization of nafion, but was still absorbed in -0.65V or even -0.8V (highlighted as the red line).

The nafion changed the nano-Au surface and thus changed the desorption potential of cysteine contributing the major interference resistant increase of Au nano-frame electrode. Another contributing factor was the negative charge of nafion repulsing the negative charge of the carboxyl group of all three interferents, including cysteine, methionine and glutathione. The DPA curve of control of nafion- Au nano-frame electrode is shown in Fig. 15. From Fig. 15, there was no increase of differential current after adding 100μL of water and the increase of differential current caused by addition of H₂S was stable, reproducible and valid.
4.3.7 Desorption method by using DPA on Nafion- Au nano-frame electrode

After the immobilization of nafion, we retested the detection limit, linear range and the sensitivity of our nafion – Au nano-frame electrode. The DPA graphs of addition of different 

$\mathrm{H}_2\mathrm{S}$ concentration 1$\mu$M to 10$\mu$M and 10$\mu$M to 100$\mu$M on nafion - Au nano-frame electrode by desorption method are shown in Fig. 11A and 11B respectively. From Fig. 11A, we found that the detection limit of our $\mathrm{H}_2\mathrm{S}$ sensor by desorption method was 2$\mu$M same as that of the electrode without nafion. From, Fig. 11A and 11B, we could see the current was stable and reproducible from 2$\mu$M to 100$\mu$M. We tried to test the upper bound of the linear range of our sensor by successive addition of 50$\mu$M $\mathrm{H}_2\mathrm{S}$. The DPA graphs of successive addition of 50$\mu$M $\mathrm{H}_2\mathrm{S}$ and the successive addition curve of 50$\mu$M $\mathrm{H}_2\mathrm{S}$ on nafion - Au nano-frame electrode by desorption method are shown in Fig. 12A and 12B. From Fig. 12B, we could see the Au nano-frame electrode started to saturate after the addition of 150$\mu$M $\mathrm{H}_2\mathrm{S}$, so the upper bound of the linear range of our $\mathrm{H}_2\mathrm{S}$ sensor by desorption method was 150$\mu$M. Compared to Au nano-frame electrode, the upper limit of the linear range of nafion – Au nano-frame electrode was 50$\mu$M lower.

By plotting the differential current change of Fig. 11A and 11B against the $\mathrm{H}_2\mathrm{S}$ concentration we added, we could get the calibration curve of $\mathrm{H}_2\mathrm{S}$. The calibration curves of $\mathrm{H}_2\mathrm{S}$ of different concentration range 2$\mu$M to 150$\mu$M and 2$\mu$M to 20$\mu$M on nafion - Au nano-frame electrode by desorption method are shown in Fig. 13A and 13B respectively. From Fig. 13A, we found that the calibration equation from 2$\mu$M to 150$\mu$M and the corresponding $R^2$ were

$$\Delta i \ (\mu\text{A}) = -0.0830 - 0.295 \times [\mathrm{H}_2\mathrm{S}] (\mu\text{M})$$

$$R^2 = 0.992.$$  

From Fig. 13B, we found that the calibration equation from 2$\mu$M to 20$\mu$M and the corresponding $R^2$ were
\[ \Delta i (\mu A) = 0.791 - 0.391 \times [H_2S](\mu M) \]

\[ R^2 = 0.990. \]

Combining Fig. 13A and 13B, we found that the linear range of our H$_2$S sensor by desorption method was from 2\(\mu\)M to 150\(\mu\)M. It quite fitted the physiological concentration range of H$_2$S in human blood which is from 30\(\mu\)M to 160\(\mu\)M.

Comparing Nafion – Au nano-frame electrode with Au nano-frame electrode, the sensitivity of Nafion – Au nano-frame electrode was slightly higher. Comparing the slopes of the calibration curves, it was -0.295\(\mu\)A/\(\mu\)M for Nafion – Au nano-frame electrode from 2\(\mu\)M to 150\(\mu\)M and -0.234\(\mu\)A/\(\mu\)M for Au nano-frame electrode from 5\(\mu\)M to 200\(\mu\)M. It might due to the modification of the nano-Au surface by nafion and this new surface adsorbed and desorbed more H$_2$S for each potential changing profile causing the delta differential current increase and thus the slope. Comparing the upper bounds of the linear ranges, it was 150\(\mu\)M for Nafion – Au nano-frame electrode and 200\(\mu\)M for Au nano-frame electrode. Au nano-frame electrode had wider linear range than the Nafion – Au nano-frame electrode. It might due to the presence of less active sites in Nafion – Au nano-frame electrode than in Au nano-frame electrode, since the nafion might also block the active sites for H$_2$S.

4.3.8 Interference coefficient of cysteine, methionine and glutathione with respect to H$_2$S detection

In order to quantify the interference contribution of cysteine, methionine and glutathione with respect to the H$_2$S detection, we need to determine their interference coefficients. The interference coefficient is defined by the slope of the calibration curve of the interferrent divided by the slope of the calibration curve of the analyte, as defined in Chapter 2 previously. The interference coefficient can act as a parameter to compare the selectivity of different methods.
In Fig. 16A, by using the DPA method and the parameters used previously, different concentrations of cysteine added on the nafion-Au nano-frame electrode resulted in the corresponding increase of the differential current. Using the data shown in Fig. 16A, we can plot the calibration curve of cysteine shown in Fig. 16B. The slope of the calibration curve was 0.0027 μA/μM and the $R^2$ was 0.981 which means the calibration curve has a linear relationship. The differential current was linear related to the concentration of the cysteine from 125μM to 375μM. The two 500μM cysteine additions were not used to plot the calibration curve, because after the accumulative addition of 1500μM cysteine, the electrode started to saturate.

After the determination of the slope of the calibration curve of cysteine, we calculated its interference coefficient. From Fig. 13A, which is the calibration curve of $H_2S$ produced by the same method and parameters as that of cysteine, the slope of the calibration curve of $H_2S$ was 0.295μA/μM and the $R^2$ was 0.992. The slope was much larger than that of cysteine. The slope of calibration curve of $H_2S$ we posted here corresponded to the linear range from 2μM to 150μM.

Using the slopes of the calibration curves of $H_2S$ and cysteine, we calculated that the interference coefficient was 0.00915 which means the signal of $H_2S$ was about 100 times larger than that of cysteine. The interference coefficient found was consistent with the result shown in Fig. 14B, which shows that the signal corresponding to $H_2S$ was much larger than that of cysteine.

After the determination of the interference coefficient of cysteine, we moved on to that of methionine and glutathione. Using the same method and parameters except the concentration added in cysteine experiment, we produced the DPA curves of methionine and glutathione shown in Fig. 17 and 18 respectively. In both curves, the signal caused by addition of interferents was negligible, especially in the case of methionine. Even though there was a small drop of the baseline after the first addition of glutathione, there was no signal correlated to the concentration of glutathione we added, as no signal other than the
baseline could be seen when we increased the concentration of glutathione added. By analysing the results shown in Fig. 17 and 18, we concluded that there was no interference contributed by methionine and glutathione with respect to the detection of H₂S using our DPA desorption method on nafion - Au nano-frame electrode, so the interference coefficients of methionine and glutathione with respect to the H₂S detection were 0.

4.4 Conclusions

A differential amperometric H₂S sensor, Au nano-frame electrode, was successfully developed by using desorption and adsorption property of H₂S on the nano-Au surface. The Au nano-frame electrode was prepared by plasma sputtering on the porous alumina and had flexible controllable adsorption and desorption state toward H₂S by controlling the potential applied to surface. By immobilizing nafion on the surface of Au nano-frame electrode, the interference caused by cysteine, methionine and glutathione was significantly reduced. The fidelity experiment of nafion - Au nano-frame electrode was conducted.

The nafion - Au nano-frame electrode and Au nano-frame electrode demonstrated a number of advantages including high sensitivity, good stability, great linearity and reproducibility. The linear range and detection limit of detecting H₂S by desorption method were from 5μM to 200μM and 2μM respectively. The linear range and detection limit of detecting H₂S by adsorption method were from 10μM to 3mM and 10μM respectively. After the immobilization of nafion, we retested the detection limit, linear range and sensitivity of the electrode by using desorption method. The sensitivity of the electrode was higher and the detection limit was the same, but the upper bound of the linear range was decreased from 200μM to 150μM. Both methods, desorption and adsorption, fitted the physiological concentration range in human blood from 30μM to 160μM. The interference coefficient of cysteine with respect to H₂S detection by using the DPA desorption method on nafion - Au nano-frame electrode was 0.00915. There was no interference found from methionine and glutathione by using the DPA desorption method on nafion - Au nano-frame electrode.
4.5 References


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4.6 Legends and Figures

Fig. 1:  (A) Gold nano-frame electrode structure. (B) SEM picture (side view) on 20nm hole side of alumina porous membrane after sputtering. (C) SEM picture (top view) on 150nm hole side of alumina porous membrane before sputtering. (D) SEM picture (side view) on 150nm hole side of alumina porous membrane before sputtering. (E) SEM picture (top view) on 20nm hole side of alumina porous membrane before sputtering. (F) SEM picture (side view) on 20nm hole side of alumina porous membrane before sputtering.

Fig. 2:  (A) The cyclic voltammograms of 1mM H$_2$S on Au nano-frame electrode. pH=7.2. Scan rate=100mV/s. Potential window = -0.9 to 0.4V. 2 cycles. (B) The cyclic voltammograms of 1mM H$_2$S on Au plate electrode. pH=7.2. Scan rate=100mV/s. Potential window = -0.9 to 0.4V. 2 cycles. (C) The cyclic voltammograms of 1mM H$_2$S on Au plate electrode. pH=7.2. Scan rate=100mV/s. Potential window = -0.8 to -0.2V. 2 cycles.

Fig. 3:  (A) The cyclic voltammograms before adding H$_2$S on Au nano-frame electrode. pH=7.2. Scan rate=100mV/s. Potential window = -0.9 to -0.5V. 2 cycles. (B) The cyclic voltammograms after adding 1mM H$_2$S on Au nano-frame electrode. pH=7.2. Scan rate=100mV/s. Potential window = -0.9 to -0.5V. 2 cycles. (C) The cyclic voltammograms of blank solution after H$_2$S measurement on Au nano-frame electrode. pH=7.2. Scan rate=100mV/s. Potential Window = -0.9 to -0.5V. 2 cycles. (D) The cyclic voltammograms of blank solution after applying -0.9V for 400s in blank solution on Au nano-frame electrode. pH=7.2. Scan rate=100mV/s. Potential Window = -0.9 to -0.5V. 2 cycles.
Fig. 4: (A) The DPA graphs of addition of different H$_2$S concentration 1μM to 10μM on Au nano-frame electrode by desorption method. $E_1$ = -0.5V, $E_2$ = -0.65V, $E_3$= -0.8V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s. (B) The DPA graphs of addition of different H$_2$S concentrations 10μM to 100μM on Au nano-frame electrode by desorption method. $E_1$ = -0.5V, $E_2$ = -0.65V, $E_3$= -0.8V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s.

Fig. 5: (A) The DPA graphs of successive addition of H$_2$S (100μM per 100s) on Au nano-frame electrode by desorption method. $E_1$ = -0.5V, $E_2$ = -0.65V, $E_3$= -0.8V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s. (B) The successive addition curve of 100μM H$_2$S on Au nano-frame electrode by desorption method. Showing the upper bound of linear range=200μM. $E_1$ = -0.5V, $E_2$ = -0.65V, $E_3$= -0.8V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s.

Fig. 6: (A) The calibration curves of H$_2$S of different concentrations ranging from 5μM to 200μM on Au nano-frame electrode by desorption method. (B) The calibration curves of H$_2$S of different concentrations from 5μM to 50μM on Au nano-frame electrode by desorption method.

Fig. 7: (A) The DPA graphs of addition of different H$_2$S concentrations from 10μM to 100μM on Au nano-frame electrode by adsorption method. $E_1$ = -0.8V, $E_2$ = -0.75V, $E_3$= -0.65V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s. (B) The DPA graphs of addition of different H$_2$S concentrations from 100μM to 1000μM on Au nano-frame electrode by adsorption method. $E_1$ = -0.8V, $E_2$ = -0.75V, $E_3$= -0.65V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s.

Fig. 8: (A) The DPA graphs of successive addition of H$_2$S (1mM per 100s) on Au nano-frame electrode by adsorption method. $E_1$ = -0.8V, $E_2$ = -0.75V, $E_3$= -0.65V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s. (B) The successive addition curve of 1mM H$_2$S on Au nano-frame electrode by adsorption method. Showing the upper bound of linear range= 3000μM. $E_1$ = -0.8V, $E_2$ = -0.75V, $E_3$= -0.65V, $t_1$ = 0.9s, $t_2$ = 0.05s, $t_3$ = 0.05s.
Fig. 9:  (A) The calibration curves of H$_2$S of different concentrations ranging from 10μM to 3000μM on Au nano-frame electrode by adsorption method. (B) The calibration curves of H$_2$S of different concentrations ranging from 10μM to 200μM on Au nano-frame electrode by adsorption method.

Fig. 10:  (A) The DPA curves of interferences (50μM cysteine, 50μM methionine and 50μM glutathione) on Au nano-frame electrode by using adsorption method. $E_1 = -0.8$V, $E_2 = -0.75$V, $E_3= -0.65$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s. (B) The DPA curves of interferences (50μM cysteine, 50μM methionine and 50μM glutathione) on Au nano-frame electrode by using desorption method. $E_1 = -0.5$V, $E_2 = -0.65$V, $E_3= -0.8$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s.

Fig. 11:  (A) The DPA graphs of addition of different H$_2$S concentrations 1μM to 10μM on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5$V, $E_2 = -0.65$V, $E_3= -0.8$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s. (B) The DPA graphs of addition of different H$_2$S concentrations 10μM to 100μM on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5$V, $E_2 = -0.65$V, $E_3= -0.8$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s.

Fig. 12:  (A) The DPA graphs of successive addition of H$_2$S (50μM per 100s) on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5$V, $E_2 = -0.65$V, $E_3= -0.8$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s. (B) The successive addition curve of 50μM H$_2$S on nafion - Au nano-frame electrode by desorption method. Showing the upper bound of linear range=150μM. $E_1 = -0.5$V, $E_2 = -0.65$V, $E_3= -0.8$V, $t_1 = 0.9$s, $t_2 = 0.05$s, $t_3 = 0.05$s.

Fig. 13:  (A)The calibration curves of H$_2$S of different concentrations ranging from 2μM to 150μM on nafion - Au nano-frame electrode by desorption method. (B) The calibration curves of H$_2$S of different concentrations ranging from 2μM to 20μM on nafion - Au nano-frame electrode by desorption method.
Fig. 14: (A) The DPA curves of interferences (250μM cysteine, 50μM methionine and 1mM glutathione) before immobilization of nafion on Au nano-frame electrode. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$. (B) The DPA curves of interferences (250μM cysteine, 50μM methionine and 1mM glutathione) after immobilization of nafion on Au nano-frame electrode. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$. (C) The pulse current curves (blue = -0.65V and red = -0.8V) before immobilization of nafion on Au nano-frame electrode. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$. (D) The pulse current curves (blue = -0.65V and red = -0.8V) after immobilization of nafion on Au nano-frame electrode. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$.

Fig. 15: The DPA curve of control of nafion- Au nano-frame electrode. For testing the fidelity. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$.

Fig. 16: (A) The DPA graphs of addition of different cysteine concentrations from 125μM to 500μM on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$. (B) The calibration curves of cysteine of different concentrations ranging from 125μM to 375μM on nafion - Au nano-frame electrode by desorption method.

Fig. 17: The DPA graphs of addition of different methionine concentrations from 125μM to 500μM on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$.

Fig. 18: The DPA graphs of addition of different glutathione concentrations from 500μM to 2000μM on nafion - Au nano-frame electrode by desorption method. $E_1 = -0.5V, E_2 = -0.65V, E_3 = -0.8V, t_1 = 0.9s, t_2 = 0.05s, t_3 = 0.05s$. 

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Fig. 1
Fig. 2
Fig 3

(A) 1 2

(B) 1 2

(C) 1 2

(D) 1 2

E vs SCE / V

i / μA

Cycle 1 Cycle 2

Cycle 1 Cycle 2

Cycle 1 Cycle 2

Cycle 1 Cycle 2
Fig 4
Chapter 4

Fig 6

\[ \Delta i (\mu A) = -0.165 - 0.234 \times [H_2S](\mu M) \]
\[ R^2 = 0.996 \]

\[ \Delta i (\mu A) = -0.775 - 0.210 \times [H_2S](\mu M) \]
\[ R^2 = 0.990 \]
Fig 7

(A) Graph showing the relationship between time (t/s) and current (i/μA) with concentrations of 10μM, 20μM, 50μM, and 100μM. The current increases with time.

(B) Graph showing the relationship between time (t/s) and current (i/μA) with concentrations of 100μM, 200μM, 500μM, 1000μM. The current increases with time.
Fig 9

**A**

\[ \Delta i (\mu A) = 0.0458 + 0.0109 \times [H_2S](\mu M) \]

\[ R^2 = 0.996 \]

**B**

\[ \Delta i (\mu A) = 0.134 + 0.00862 \times [H_2S](\mu M) \]

\[ R^2 = 0.996 \]
Fig 10
Fig 11
Fig 12
Fig 13

\[ \Delta i (\mu A) = -0.0830 - 0.295 \times [H_2S](\mu M) \]

\[ R^2 = 0.992 \]

\[ \Delta i (\mu A) = 0.791 - 0.391 \times [H_2S](\mu M) \]

\[ R^2 = 0.990 \]
Fig 14
Fig 15
Fig 16

\[ \Delta i (\mu A) = -1.5130 - 0.0027 \times [\text{Cys}] (\mu M) \]

\[ R^2 = 0.981 \]
Fig 17
Fig 18
Chapter 5

Summary and Perspective
5.1 Summary

We have achieved the main objective of this project by successfully develop a robust and flexible and versatile nano-structured platform for the detection of small redox-active biomessengers and demonstrate the platform by applying it to the detection of H$_2$O$_2$, CO and H$_2$S.

The main achievements of this project are (1) the prove-of-concept demonstration of a bio-compatible platform which can cooperate the bio-macromolecule and inorganic catalyst without direct contact, (2) detecting different small bio-molecules by using analyte-specific catalysts, (3) selective detection of different small endogenously generated bio-molecule by employing different types of reactions.

5.2 Perspective

The work reported in this thesis opened up the new way to measure our targeted analytes. The successful developed techniques reported in this thesis can apply to the research field and help the researcher to explore the unknown area related to H$_2$S, CO and H$_2$O$_2$. As the technique of measuring H$_2$S and CO become more and more mature, more finding and application related to H$_2$S and CO will appear in the future. But still, more and more effort is still needed to improve sensitivity, linear range and detection limit of the sensor of small cellular messengers.
List of publication

*Journal articles*


Conference presentations

