Detection of thiols by o-quinone electrocatalytic sensors

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A Thesis

entitled

Detection of Thiols by o-Quinone Electrocatalytic Sensors

by

Tianxia Zhu

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Chemistry

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August 2012
In biological systems, thiols play very important roles as components of protein structures and as metabolic intermediates. Thiols are widely distributed in living cells as important antioxidants that can protect cells from any oxidative damage. Thiols are associated with some diseases such as cardiovascular disease, therefore it is necessary to develop new biosensors that can accurately detect and quantify thiols that are considered important biomarkers.

In this thesis, new immobilization membranes, co-electrocatalysts and immobilization methods were evaluated using pyrroloquinoline quinone (PQQ) as the primary electrocatalyst. Previous studies have shown that the o-quinone moiety of PQQ exhibits a reversible 2-electron, 2-proton oxidation and reduction reaction. The electron transfer between its oxidized and reduced form is also known to catalyze reactions of the oxidation of thiols to disulfides. Thiols can be determined at lower overpotentials by amperometric detection during the oxidation of the reduced form of PQQH$_2$ to PQQ. Nanoparticles, such as gold (Au NP) and copper (Cu NP), and especially single wall carbon nanotubes (SWNTs) exhibit novel electronic properties making them suitable for enhancing electrochemical sensitivity. Cysteine and homocysteine were detected at
micromolar concentration by incorporation of coenzyme PQQ and a co-catalyst of SWNTs-COOH into a Au-PPy nanocomposite conducting polymer film. Cysteine and homocysteine are quantitatively detected by monitoring the amperometric current resulting from oxidation of PQQH$_2$ back to PQQ at a glassy carbon electrode surface.

Substituents attached to the quinone moiety are very important in determining the catalytic activity of an electrocatalyst. They can affect reduction of the quinone and oxidation of the hydroquinone. Therefore changing the substituents and tuning the structure of PQQ can change the redox potential and electrocatalytic power of the resulting compound. Modification of the detection overpotential can also be adjusted through changing the molecule, therefore increasing the sensitivity for oxidizable biological compounds such as thiols.

A benzimidazole analogue of PQQ was also synthesized and the electrochemical properties of this compound were evaluated. Similar reversible cyclic voltammetric behavior was observed as in PQQ, which is due to the two-electron redox reaction of the $o$-quinone moiety. However, the benzimidazole analogue exhibited limited electrocatalytic capabilities.
This thesis is dedicated to my husband, thank you for your support, and understanding.
Acknowledgements

I would like to thank my research advisor, Dr. Jon R. Kirchhoff for his support, advice, patience and graduate education throughout the years. Thanks him for allowing me to work in the laboratory, he is the best advisor.

I would also like to thank my committee members Dr. L. M. V. Tillekeratne for the organic synthesis part. I would also like to thank my other committee member Dr. Jared Anderson, Dr. Dragan Isailovic for their time and valuable suggestions. I would like thank Charlene Hansen and Pam Samples at the Chemistry department office, Dr. Kim for help with NMR spectroscopy and Pannee for help with SEM images. Thanks to my current and previous lab members, Maria, Kristi, Josh, Jessie. Thanks you guys for sharing your experience and knowledge with me. I really had a good time with all of you for last three years.

Special thanks go to my family members. I would like to thank my dear husband Baoyu Wang, for his advice, care, understanding and encouragement; my kids for their cooperation.

Finally I want to thank Department of Chemistry for the financial assistantship.
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List of Abbreviations

$\Delta E_p$..................Peak Separation
Au NP..................Gold Nanoparticles
Cu NP..................Copper Nanoparticles
CV.....................Cyclic Voltammetry
Cys...................Cysteine
$E^0$..................Formal Potential
EC Au..................Electrodeposition of Gold Nanoparticles
EC Cu..................Electrodeposition of Copper Nanoparticles
GCE...................Glassy Carbon Electrode
HCYS................Homocysteine
$i_p$....................Anodic peak current
$i_c$....................Cathodic peak current
PPy...................PolyPrrole
PQQ...................Pyrroloquinoline Quinone
SEM..................Scanning Electron Microscopy
SEM-EDS...............Scanning Electron Microscope-Energy Dispersive Spectroscopy
SWNTs................Single Wall Carbon Nanotubes
SWNTs-COOH........Single Wall Carbon Nanotubes containing Carboxylic acid
Chapter 1

Introduction

1.1 Thiols

In organic chemistry, a thiol is an organosulfur compound that contains a carbon-bonded sulfhydryl (−C−SH or R−SH) group where R represents an alkane, alkene, or other carbon-containing group of atoms. Thiols are sulfur analogues of alcohols. So thiols and alcohols have similar structures and properties. Thiols can form thioethers, thioacetals and thioesters, which are analogous to ethers, acetals and esters for the reactions of alcohols. Due to the lower electronegativity difference between sulfur and hydrogen compared to oxygen and hydrogen, the S-H bond is less polar compared to an O-H bond, so thiols have lower boiling points and solubility compared to alcohols.

Usually, low molecular weight thiols have strong and repulsive odors, resembling that of garlic. Most low molecular weight thiols can be found in the spray of skunks. For example, (E)-2-butene-1-thiol, S-(E)-2-butenyl thioacetate, S-3-methylbutenyl thioacetate, 2-quinolinemethanethiol, and S-2-quinolinemethyl thioacetate can be found from striped skunk secretion.

Thiols are easily oxidized to disulfides by oxidation reagents, such as bromine and iodine. Disulfides can be reduced back to thiols by reduction reagents, such as metallic zinc. And thiols can undergo a reversible oxidation-reduction reaction (Equation 1-1).

\[
2 \text{R-SH} \rightleftharpoons \text{R-S-S-R} + 2 \text{H}^+ + 2 \text{e}^-(1-1)
\]
This reversible oxidation-reduction reaction is a very important process in many biological and chemical systems. In biological systems, thiols play very important roles as components of protein structures and as metabolic intermediates. For example, reduced glutathione plays an important role in transport, protein synthesis, catabolism, and metabolism.\textsuperscript{6} High levels of homocysteine have been proposed as a risk factor for Alzheimers and cardiovascular diseases.\textsuperscript{7}

Thiols are widely distributed in living cells as important antioxidants that can protect cells from any kind of oxidative damage. The most common and most studied biothiols are glutathione (L-glutamyl-L-cysteinyl-glycine, GSH), homocysteine (HCYS), and cysteine (CYS).\textsuperscript{8} (Figure 1-1)

![Figure 1-1. Structures of common thiols.](image)

**1.1.1 Glutathione (GSH)**

Glutathione is a tripeptide containing glutamic acid, cysteine, and glycine. It is an antioxidant, which can prevent the damage of reactive oxygen species, such as free radicals, to important cellular components.\textsuperscript{9} In the human body, glutathione does not have
to be obtained via food. It can be synthesized. Glutathione exists in reduced (GSH) and oxidized (GSSG) states. Thiol groups are reducing agents, so glutathione can be oxidized to glutathione disulfide (GSSG), also called L(-)-glutathione. Glutathione disulfide can be reduced back to GSH by glutathione reductase, using NADPH. In healthy cells and tissue, more than 90% of glutathione exists in the reduced form, and less than 10% exists as glutathione disulfide.

1.1.2. Homocysteine (HCYS)

Homocysteine is a non protein sulfur amino acid. It differs from cysteine by the additional methylene (-CH₂-) group, whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration (Figure 1-2). In remethylation, homocysteine forms methionine by obtaining a methyl group from N-5-methyltetrahydrofolate or from betaine. This reaction with N-5-methyltetrahydrofolate exists in all tissues and is vitamin B12 dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B12 independent. A considerable proportion of methionine is then activated by ATP to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors such as S-adenosylhomocysteine.
Homocysteine can be converted to cysteine in presence of B-vitamins. Homocysteine is not obtained via food; it can be biosynthesized from methionine via a multi-step process. Detection of high levels of homocysteine ("Homocystinemia") is due to the disruption of homocysteine metabolism, which has been associated with increased risk of vascular heart disease. Severe hyperhomocysteinemia results from rare genetic defects that causes deficiencies in cystathionine beta synthase, methylenetetrahydrofolate reductase, or in enzymes involved in methyl-B12-synthesis and homocysteine methylation. Mild hyperhomocysteinemia results from mild impairment in the methylation pathway.

1.1.3 Cysteine (Cys)

Cysteine is an α-amino acid and can be biosynthesized in humans (see Figure 1-3). The synthesis begins from the amino acid serine and homocysteine to form the
asymmetrical thioether cystathionine. Then cystathionine converts into cysteine and alpha-ketobutyrate in the presence of the enzyme cystathionine gamma-lyase.\textsuperscript{14}

\[
\text{Homocysteine} + \text{Serine} \rightarrow \text{Cystathionine} + \text{Alpha-Ketobutyrate}
\]

Figure 1-3. Cysteine synthesis.

The thiol group is very easy to oxidize to the disulfide derivative cystine, which plays a very important structural role in many proteins. Disulfide bridges are often vital for the stability of a final protein structure, and the incorrect pairing of cysteine residues usually prevents the folding of a protein into its native conformation.\textsuperscript{15}

Cysteine is an important source of sulfide in human metabolism. In human cysteine metabolism, L-cysteine is consumed in several ways as shown below (Figure 1-4).\textsuperscript{16}

Figure 1-4. Metabolic flow chart of cysteine.
1.2 Thiol detection

Thiols play a very important role in biochemistry, both as components of protein structures and as metabolic intermediates. Almost 30 years ago, McCully reported the markedly elevated plasma homocysteine concentrations found in persons with homocystinuria were responsible for the development of premature occlusive vascular disease.\(^{17}\) In 1976, Wilcken and Wilcken found homocysteine-cysteine mixed disulfide after a methionine load was significantly higher in patients with coronary artery disease (CAD) than in respective control subjects.\(^{18}\) These landmark findings provided the basis for subsequent studies, which since 1990 have increased exponentially. Techniques that are able to quantify and detect biological thiols, and their related redox status are crucial tools to investigate their roles in various diseases and determine the efficacy of potential therapies. Many techniques have been developed to detect the concentration of thiols, such as spectrofluorimetry\(^{19-21}\) and HPLC,\(^{22}\) however, these methods have some limitations, such as need for derivatization, sample processing and long run time. Other mass methods, such as trap-and-release membrane introduction mass spectrometry (T&R–MIMS), gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC-MS) have been used for thiol detection.\(^{23}\) Although mass methods decreased the sample preparation process, the need for derivatization, and run time, some problems still exist, such as complex procedures and bad reproducibility.

Electrochemical techniques provide a simple method for thiol detection with the main challenge for this method being its selectivity. It is difficult to directly oxidize thiols on conventional solid electrode surfaces due to the requirement of large overpotentials
(>1.0 V vs Ag/AgCl for cysteine) to proceed.\textsuperscript{24,25} Large overpotentials can oxidize some other biological substances, thus effecting its selectivity. This problem can be overcome by indirect detection of thiols using mercury or mercury amalgam electrodes,\textsuperscript{26,27} but toxicity prevents further application of this method. Current organic and inorganic electroactive indicators and electrocatalysts for thiol detection were also used, which can improve sensitivity and selectivity for the electrode response.\textsuperscript{28} Inoue and Kirchhoff modified a glassy carbon electrode using pyrroloquinoline quinone (PQQ) in a polypyrrole (PPy) film to detect thiols. This type of thiol sensor can detect thiols at low potentials due to the electrocatalytic reaction between thiols and PQQ.\textsuperscript{29}

1.3. Chemically Modified Electrode

Chemically modified electrodes have attracted considerable interest during the past decade. Chemically modified electrodes provide a wide range of analytical applications and powerful opportunities for electroanalysis. Most commonly modified electrodes include gold, platinum and glassy carbon. The electrode surface can be modified by introducing a selected reagent (monomeric or polymeric) that can govern its electrochemical properties. Reagents can be immobilized on the electrode surface through the approaches noted below.

(1) chemisorption (monolayer formed on the surface of electrode).\textsuperscript{30}

(2) covalent bonding\textsuperscript{31} (monolayer or multilayer), a compound immobilized on the surface of an electrode through covalent bonds, such as amidization, esterification or etherification.
(3) polymer film coating,\textsuperscript{32} conductive or nonconductive polymer film coated on the electrode surface, which has low solubility in the contacting solution.

Conducting polymer films have become popular modifiers. Polymer coated electrodes can be achieved by different processes.\textsuperscript{33}

(1) Dip-coating, this can be done by immersing the electrode into a polymer solution for a certain time, film formation by adsorption.

(2) Solvent evaporation, after the polymer solution applied on the electrode surface, the solvent is evaporated.

(3) Spin coating, a diluted polymer solution is applied to a rotating electrode surface.

(4) Electrochemical deposition, a polymer is formed based on the variable solubility at different oxidation states.

(5) Electrochemical polymerization, a polymer film directly formed on the electrode surface by oxidization or reduction of a monomer solution.

(6) Cross linking, a cross linking film formed by copolymerization of bifunctional and polyfunctional monomers.

At modified electrodes, electrons transfer between the electrode and some solution substrate, and this process is mediated by an immobilized redox couple (mediator). The redox reaction proceeds at a low overpotential compared to the bare electrode. The substrate, $S$, is transported across the polymer film-solution interface and diffuses into the polymer film membrane. The electrocatalyst or mediator, $R/O$, undergoes heterogeneous electron transfer at the electrode surface. The mediator undergoes homogeneous electron transfer with the substrate in the polymer film.\textsuperscript{33} This type of electrocatalysis process can be seen in Figure 1-5.
1.4 Nafion

Nafion is a sulfonated tetrafluoroethylene based fluoropolymer-copolymer with superior conductive properties that make it widely used for sensor fabrication. Protons on the SO$_3$H groups are very active, and "hop" from one acid site to another leaving pores, which can accept only cations into the membranes and reject anions or electrons. So Nafion can be fabricated for various cationic conductivities.$^{34,35}$ It offers the advantage of high conductivity, ease of fabrication, and high partition coefficients for many redox compounds.$^{36}$ So many papers have been published using Nafion in the modification of carbon paste electrodes (CPE),$^{37}$ glassy carbon electrodes,$^{38}$ carbon fiber microelectrodes,$^{39}$ and mercury film electrodes.$^{40}$ A very thin film of Nafion is enough to help the diffusion of the analyte to the electrode, while preventing other interferences to approach the electrode.$^{41}$ The most important reason for the widespread application of Nafion modified electrodes in electroanalytical chemistry is their ability to preconcentrate positively charged molecules, due to its cationic exchange properties, which can increase the sensitivity of the method.$^{42-44}$ The structure of Nafion is shown in Figure 1-6.
Polypyrrole is another polymer widely used for sensor fabrication due to its high conductivity (up to 600 S/cm), redox stability, and better electrochromic properties compared to other polymers. Polypyrrole is a chemical compound formed from polymerization of a number of connected pyrrole rings. The structure is shown below (Figure 1-7).

Figure 1-7. Structure of Polypyrrole.

Polypyrrole films generally are created at a fixed potential or repetitive potential scans during cyclic voltammetry. However, it is difficult to control the film thickness by these methods. This can be overcome by potentiostatic methods. The process of electropolymerization of pyrrole is shown in Figure 1-8. First a pyrrole monomer is electrochemically oxidized to a radical cation (1) at a positive potential. Then there are two options for this radical cation. One is the reaction with another monomer radical cation to form a dimeric radical cation, and another way is the reaction with a neutral monomer to form a dimeric diradical cation. The dimer can be created from a dimeric

![Figure 1-6. Structure of Nafion.](image)
cation losing protons and electrons. Then, the dimer undergoes electrochemical oxidization and the process is repeated until the charge on the chain is incorporated by a counterion.\textsuperscript{47,48}

Figure 1-8. Electropolymerization of pyrrole.
1.6 Nanoparticle Modified Electrodes in the Development of Biosensors

1.6.1 Single Wall Carbon Nanotubes (SWNTs)

Carbon nanotubes have been used in chemical and biochemical sensing and nanoscale electrodevices during the past decade due to their novel electronic properties, which can be metallic or semiconducting, depending on their radius or chiralities.\textsuperscript{49,50} Carbon nanotubes are one family of fullerene structures. There are two types of nanotubes: single-walled carbon nanotubes (SWNTs) and multiwalled carbon nanotubes (MWNTs). SWNTs are one-atom-thick layers of graphite rolled up into a seamless cylinder, with a diameter of about several nanometers and length of 1 to 100 microns.\textsuperscript{51} MWNTs are considered as multiple layers of graphite wrapped up together to form a tube shape, sharing the same central axis.

SWNTs possess a large length-to-diameter ratio (large electroactive surface) with good conductivity that make it possible to form highly porous three-dimensional networks suitable for anchoring numerous biomolecules, leading to an increase of binding events and high electrochemical sensitivity. More importantly, SWNTs exhibit direct electron transfer with those biomacromolecules\textsuperscript{52} and anenhanced Faradaic response.\textsuperscript{53} Therefore, SWNTs are ideal candidates to get closer to the active site of biomolecules and achieve the electrical wiring between active sites of the biomolecules and the electrode. Furthermore, SWNTs can undergo organic functionalization bringing new properties to nanostructured electrodes. Many biosensors have been fabricated using SWNTs, such as a DNA sensor,\textsuperscript{54} and a glucose sensor using glucose oxidase.\textsuperscript{55}

In SWNTs based biosensors, biomolecules can be immobilized through several paths,\textsuperscript{56} see Figure 1-9.
Figure 1-9. Immobilization strategies of enzymes on SWNTs: (a) covalent binding via amide coupling with the carboxylic acid groups of oxidized nanotubes; (b) electrochemical coating of nanotubes with affinity partners and subsequent immobilization of affinity counter part modified enzymes; (c) adsorption of enzymes on SWNTs via hydrophobic or electrostatic ineractions; (d) entrapment of enzymes in a polymer matrix formed around SWNTs; and (e) immobilization via affinity interactions onto functionalized nanotubes. Reproduced by permission of The Royal Society of Chemistry.

As mentioned earlier, conducting polymer films attracted wide attention as interfaces between the electrode and biomacromolecules. The most important reason for the use of conducting polymers is the possibility to simply entrap biomolecules inside the polymer matrix during its electrochemical deposition or after incubating the modified electrode into a biomolecule solution. Recently, more research has focused on the combination of SWNTs with polymers for designing a new generation of
The easiest way to combine polymer–SWNT–biomolecules is the simple adsorption of the biomolecule on a SWNT, then modification with a conducting polymer film. A more complex configuration was done by successive deposition of a SWNT coating and polymer film providing a layer-by-layer assembled composite.

There are two different kind of biosensors based on application of SWNTs, one is a direct electronic transfer (DET) between the active sites of biomolecules and the bulk electrode, and another is based on mediated electron transfer (MET), which the electrons transfer between active sites of biomolecules and the electrodes through a redox mediator. These processes are represented in Figure 1-10.

![Figure 1-10](image)

Figure 1-10.(A) Anodic direct electron transfer and (B) mediated electron transfer.
1.6.2. Gold Nanoparticles (Au NP)

Gold is the most stable metal in the group 8 elements. Gold nanoparticles typically have sizes ranging from 1-100 nm, and exhibit particular optical and electronic properties. Usually, gold nanoparticles are prepared by reducing chloroauric acid (HAuCl₄) in a liquid. During this process, Au³⁺ was reduced to a neutral gold atom. Gold metal is a very good conductor because the electrons are not bonded to individual atoms, instead they form an electron cloud around an atomic core. The electron cloud is moving, so gold metal transports electrons very easily. Gold has many advantages compared to other metals. It is biocompatible, nontoxic, easily functionalized with other ligands, and very stable in a biological matrix,⁶⁹ which contribute to various applications in biosensors, such as a DNA⁷⁰ and glucose sensors.⁷¹

It was found that the conductivity and stability of sensors generated by metal nanoparticles can be improved by forming a metal-polymer composite. The nanoparticles can penetrate into the pore structure of a conducting polymer, and improve electrocatalytic activity. Polypyrrole is the most widely used conducting polymer to support metal nanoparticles. Polypyrrole/Au nanocomposites have been reported⁷² (Figure 1-11).
Figure 1-11. (a) TEM micrographs of PPy/Au nanocomposite films. (b) Detail of Au nanoparticles embedded in polypyrrole after electrochemical polymerization. (c) SEM image of pure PPy film. (d) SEM image of PPy/Au nanocomposite. Reprinted from Electrochimica Acta, Vol 52, Wei Chen, Chang Ming Li, Peng Chen, C.Q. Sun, Electrosynthesis and characterization of polypyrrole/Au nanocomposite, Pages No. 5, Copyright (2007), with permission from Elsevier.

1.6.3. Copper Nanoparticles

Cu nanoparticles have attracted much more attention in the last two decades. Similar to Au nanoparticles, they have amazing optical, electronic, catalytic, and magnetic properties, and are inexpensive relative to gold. Chemical reduction is the most widely used method to synthesize Cu nanoparticles, due to its simplicity and ease of
control of the nanoparticle size and shape.\textsuperscript{73} As mentioned above, metal nanoparticles are not very stable directly attached to an electrode surface. This issue can be solved by using a conductive stabilizing material, such as polymer\textsuperscript{74} (Figure 1-12).

Figure 1-12. AFM images of the (A) bare GCE, (B) PPy/GCE, and (C) nano-Cu/PPy/GCE surfaces as three-dimensional views. Reprinted from Talanta, Vol 80, Şükriye Ulubay, Zekerya Dursun, Cu nanoparticles incorporated polypyrrole modified GCE for sensitive simultaneous determination of dopamine and uric acid, Pages No. 6, Copyright (2010), with permission from Elsevier
1.7. Pyrroloquinoline Quinone (PQQ)

Pyrroloquinoline quinone (PQQ, 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid) was first discovered by J.G. Hauge, as the third redox cofactor in bacteria.⁶¹ Its structure was identified in 1979, as a coenzyme redox factor from bacterial primary alcohol dehydrogenases.⁶² Usually, it exists in the form of a quinoprotein, such as glucose dehydrogenase, one of the quinoproteins, used in a glucose sensor.

PQQ has a tricyclic aromatic ring structure, a quinoline ring, a pyridine ring (electron-withdrawing), and a pyrrole ring (electron donating) (see Figure 1-13). The \( o \)-quinone moiety exists at the 4 and 5 position of the quinoline ring, which is the most important active site of the molecule for chemical and electrochemical reactions.⁶³ PQQ is a powerful free radical–scavenger, which makes it a superior antioxidant due to its high molecular stability and the role it plays in energy transfer directly within the mitochondria. PQQ can carry out thousands of electron transfers without undergoing molecular breakdown.

![Figure 1-13. Structure of PQQ.](image)

The \( o \)-quinone moiety of PQQ exhibits an efficient, pH dependent and reversible two-electron, two-proton transfer between the quinone and the hydroquinone forms (Figure 1-14).⁶⁴,⁶⁵
1.8. PQQ Analogues as Electrocatalysts

In order to fully understand the role of pyrroloquinoline quinone in biological systems, a significant amount of research has been done. Several PQQ analogues have been prepared in order to understand the relationship between its structure and mechanism of the catalytic activity of PQQ. In Zhang’s paper, they synthesized three isomeric analogues with different orientations. Through further studies of these PQQ analogues they found these analogues exhibited useful catalytic properties as PQQ, but they easily lost their catalytic ability during the catalytic cycles.

Fouchard, et al, synthesized imidazole derivatives of PQQ (Figure 1-16) with the pyrrole ring replaced by imidazole to assess the role of the pyrrole ring in the catalytic behavior of PQQ. But the catalytic abilities of these three derivatives were not as good as that of PQQ.
Both PQQ and the PQQ analogues are electroactive compounds, due to the presence of the \( o \)-quinone moiety. Replacement of the pyrrole ring of PQQ using an imidazole ring effected the catalytic power of these quinones and improved the catalytic potential. Variations in the redox capabilities of these quinones will be very useful in the development of electrochemical sensors.

In the Kirchhoff laboratory, one research project is focused on the development of chemically modified electrodes for the detection of biological compounds, such as thiols. Inoue developed a biosensor for detection of thiols by incorporation of PQQ into a conducting polypyrrole film on a glassy carbon electrode surface.\(^{29}\) Figure 1-17 represents this biosensor. In this biosensor, PQQ is a mediator. First, PQQ is reduced to PQQH\(_2\), while the thiol is oxidized to disulfide. The amount of thiol in a sample is proportional to the amperometric current resulting from the reversible oxidation of PQQH\(_2\) back to PQQ at the electrode. Homocysteine, glutathione, and cysteine were successfully detected by this biosensor. Furthermore, the analysis of cysteine in dietary supplements and human urine were performed.

Some limitations of this biosensor are stability and cost. The stability of this biosensor mostly depends on how well the PQQ molecules are entrapped into the
polypyrrole layer during the electropolymerization process.\textsuperscript{29} PQQ is expensive; it cost $90.70 for 1 mg from Sigma-Aldrich.

![Figure 1-17. Schematic diagram of the biosensor based on PQQ.](image1)

The goals of this thesis were two folds. First, improved electrocatalytic activity for the PQQ-based sensor was investigated. The benefits of new membrane materials, co-electrocatalysts and immobilization methods were evaluated. Second, the simpler synthetic o-quinone analogue of PQQ was synthesized and evaluated as an electrocatalyst for thiol detection.

![Figure 1-18. Structure of benzimidazole derivative of PQQ.](image2)
Chapter 2

Evaluation of New Immobilization Membrane Materials, co-Electrocatalysts and Immobilization Methods

2.1. Experimental

2.1.1 Reagents

All solutions were prepared using deionized water with resistivity greater than 17 MΩ-cm from a Barnstead B-pure water purification system. PQQ, Nafion, chitosan, L-cysteine (98%), trisodium citrate dihydrate, chloroauric acid, and pyrrole were purchased from Sigma-Aldrich. Single Wall Carbon Nanotubes (SWNTs) were purchased from Nano Lab. Sulfuric acid, potassium chloride, potassium hydroxide, citric acid and sodium phosphate were obtained from Fisher (Fair Lawn, NJ). Aluminum oxide sanding gel was purchased from Nicsand Inc (Cleveland, OH).

2.1.2 Instrumentation

Electrochemical measurements were conducted using a Bioanalytical Systems (BAS, West Lafayette, IN) 100B electrochemical analyzer interfaced to a Gateway GX270 computer with a conventional three electrode system. A bare glassy carbon electrode (GCE) or a modified glassy carbon electrode (3 mm in diameter) (BAS, MF-2012) was used as the working electrode. Glassy carbon electrode was first polished with aluminum oxide polishing gel, then sonicated for 5 mins in deionized water before using.
A platinum wire was used as the auxiliary electrode, and a Ag/AgCl (3M NaCl) was used as the reference electrode. When the reference electrode was not in use, it was stored in a 3 M NaCl solution. A 1210 Branson Ultrasonic Cleaner from Branson Corp (Danbury, CT) was used to sonicate the glassy carbon electrode. A pH meter from Denver Instruments Co. (Denver, Co) was used to measure the pH for all the buffer solutions. Methods used in this thesis included cyclic voltammetry, square wave voltammetry, amperometry, and scanning electron microscopy (JEOL JSM-7500F).

2.2. Electrode Preparation Methods

All modified electrodes were abbreviated according to the order of deposition. For example, PQQ/Chitosan/SWNTs/GCE indicates a glassy carbon electrode was coated with SWNTs first then Chitosan and finally PQQ.

2.2.1. Chitosan as a Matrix Combination for Single Wall Carbon Nanotubes

2.2.1.1. Preparation of PQQ/Chitosan/SWNTs/GCE

The natural polymer chitosan was explored as a matrix for designing a biosensor. A chitosan solution was prepared by dispersing 3 mg of chitosan into 10 mL of 3% acetic acid with magnetic stirring for about 1 h. Then a chitosan-PQQ solution was prepared by adding PQQ to the chitosan solution to get a 1.55 mM PQQ solution. A few drops of KOH were added to help PQQ dissolve.

A solution of carbon nanotubes, chitosan, and PQQ was prepared by adding 3 mg of nanotubes to 1 mL of chitosan PQQ stock solution, sonicated for 2 h. The bare glassy carbon electrode was coated by 5 μL of the above solution, drying at room temperature.
A PQQ and nanotube solution was prepared by dissolving 0.5 mg of PQQ into 1 mL of 0.1 M KCl solution. Adding a few drops of KOH helps PQQ dissolve completely. 0.6 mg carbon nanotubes was then added and the solution was sonicated for about 1 h to obtain a homogeneously dispersed solution. The bare glassy carbon electrode was coated by 5 μL of the above solution. Then the PQQ/SWNTs/GCE was coated by 5 μL of chitosan solution and the electrode dried at room temperature.

2.2.2. Nafion as a Matrix Combination for Single Wall Carbon Nanotubes and Gold Nanoparticles

2.2.2.1. Preparation of PQQ/Nafion/SWNTs-COOH/GCE

A single wall carbon nanotube solution was prepared. 0.6 mg of SWNTs was dispersed in 1 mL of dimethylformamide (DMF) to give a 0.6 mg/mL black suspension, which also contained 5 μL of Nafion (0.5%, v/v). Glassy carbon electrodes were modified, according to Tachikawa’s produre.\(^{68}\) 5 μL of the SWNTs solution was placed on a clean glassy carbon electrode surface. The electrode was dried at room temperature. Then 3 μL of the PQQ solution was added to the surface of the GC electrode, and allowed to dry again. Single wall carbon nanotubes containing carboxylic groups were also used to modify GC electrodes using the same procedure as above.

2.2.2.2. Preparation of PQQ/Nafion/Au NP/GCE

20 mL of 1.0 mM H\(_{2}\)AuCl\(_4\) was added to a 50 mL Erlenmeyer flask on a stirring hot plate and boiled for a few minutes. Then 2 mL of a 1% solution of trisodium citrate dihydrate, Na\(_3\)C\(_6\)H\(_5\)O\(_7\)·2H\(_2\)O was quickly added. The gold nanoparticles were formed as
the citrate reduced the gold (III). Once the solution turned deep red, the flask was removed from the heat, and the solvent was removed by evaporating at room temperature. A 2 mM Au nanoparticle solution was then prepared.

This electrode was prepared by two methods: a layer-by-layer approach and a single layer approach. 5 μL of the 2 mM Au nanoparticle solution was placed on a clean glassy carbon electrode surface and dried at room temperature. Then 5 μL Nafion solution (in DMF, 0.5% v%) was added and allowed to dry again. Finally, 3 μL of 1.55 mM PQQ solution was added and allowed to dry for around 20 hours.

A Au nanoparticle-Nafion-PQQ solution was prepared with the same concentrations as above. This mixture was deposited as a single layer on a clean glassy carbon electrode surface and dried at room temperature for around 20 hours.

2.2.2.3. Preparation of PQQ/Nafion/EC Au/GCE

Gold nanoparticles were electrochemically deposited on the surface of a bare glassy carbon electrode according to the procedure of Finot. A solution of 0.2 μM of HAuCl₄ in 0.5 M H₂SO₄ was prepared. To this solution a glassy carbon electrode was immersed and the potential was scanned from 1100 to 0 mV. Different film thicknesses were achieved by varying the scan rate and the number of cycles. 5 μL of Nafion in DMF solution was deposited on the Au NP, allowed to dry at room temperature. Finally 3 μL of 1.55 mM PQQ solution was deposited, and allowed to dry again.
2.2.3. Pyrrole as a Matrix Combination for Single Wall Carbon Nanotubes, Gold, or Copper Nanoparticles

2.2.3.1. Preparation of PQQ/PPy/Au NP/GCE

Electropolymerization of pyrrole was performed on a clean GC electrode according to Inoue’s method. Generally, 1 mL of PQQ solution was bubbled with Argon for 30 min. Then 7 μL of freshly distilled pyrrole was added with 7 mg KCl and 2 mM (volume from 10 mL to 30 mL) of Au NP. The potential was fixed at 630 mV. The charge was monitored until the desired thickness was achieved. Different electrodes were modified by varying the amount of nanoparticles added into solution.

2.2.3.2. Preparation of PQQ/PPy/SWNTs-COOH/GCE

Electropolymerization was performed as above, except adding 0.6 mg/mL single wall carbon nanotubes into the PQQ solution replacing the Au nanoparticles. Different electrodes were modified by varying the amount of SWNTs-COOH from 6 μg to 18 μg.

2.2.3.3. Preparation of EC Cu/PQQ/PPy/GCE

First, pyrrole was electropolymerized with PQQ on the clean bare glassy carbon electrode surface according to Inoue’s method. Then Cu nanoparticles were electrochemically deposited on the surface of PQQ/PPy/GCE by cycling the electrode in 1 mM CuSO₄ in 0.5 M H₂SO₄. The potential was scanned from 1100 to 0 mV, scan rate 50 mV/s, 10 cycles.
Over oxidation of polypyrrole was performed in order to compare the electrode stability. The polypyrrole film was electrochemically over oxidized at 1000 mV for 300 s in KOH solution.

2.2.3.4. Preparation of PQQ/SWNTs-COOH/ Au-PPy Nanocomposite/ GCE

Single wall carbon nanotubes and PQQ were entrapped after and before polymerization. In the first strategy, chemically synthesized gold polypyrrole (Au-PPy) nanocomposite was prepared according to Njagi. 77 In general, 2.5 μL of 0.0125 M HAuCl₄ in 0.1 M phosphate buffer (PB) at pH 6.8, and 2.5 μL of 0.0925 M pyrrole in 0.1 M PB at pH 6.8 were deposited on the surface of a glassy carbon electrode. The solution was allowed to react and dry in air at room temperature for 30 mins. 5 μL of a 0.6 mg/mL SWNTs-COOH solution and 3 μL of a 1.55 mM PQQ solution were then added onto the already modified Au-PPy-GCE. Finally the electrode was allowed to dry again in air at room temperature for 15 h. In the second strategy, 2.5 μL of 0.0125 M HAuCl₄ were deposited on the GC electrode surface, followed by 5 μL 0.6 mg/mL of SWNTs-COOH solution, 3 μL 1.55 mM of PQQ solution. The electrode was allowed to dry for 30 mins and then, 2.5 μL of 0.0925 M pyrrole were deposited to allow polymerization. Finally the electrode was allowed to dry for around 15 h at room temperature.

The thickness of PPy-Au nanocomposites film was controlled by monitoring the amount of reagents during the polymerization reaction. Control experiments were done with or without PQQ and with or without SWNTs-COOH.
2.3. Amperometric Detection of Thiols

Cysteine was detected at the modified glassy carbon electrode by measuring the current response as a function of time under anaerobic and hydrodynamic conditions. 3 mL of citrate phosphate buffer was purged in argon gas for 15 mins. 25 μL of cysteine solution was injected successively, the solution was stirred and kept under anaerobic conditions during the detection.

2.4. Results and Discussion

2.4.1. Chitosan as a Matrix with Single Wall Carbon Nanotubes

A PQQ-chitosan solution was deposited on the surface of clean GCE. Electrodes prepared in this way were not very stable. The peaks disappeared after two days when left in the air or kept in the buffer solution (Figure 2-1).

![Figure 2-1. Cyclic voltammetry of PQQ/Chitosan/GCE (pH 7.0 citrate phosphate buffer, 11.63 nanomoles of PQQ) First run (pink); After one day (blue), scan rate 100 mV/s.](image-url)
Single wall carbon nanotubes were incorporated into the matrix. This electrode shows high sensitivity and a fast response. Figure 2-2 shows typical cyclic voltammogram of GCE modified by SWNTs, chitosan and PQQ. The voltammogram shows the best formed redox couple when 4.65 nanomoles of PQQ and 3.0 μg of SWNTs were deposited as compared to no SWNTs and 11.63 nanomoles of PQQ. The ratio between $i_{pa}/i_{pc}$ is 1.29, the peak separation is $\Delta E_P = E_{pc} - E_{pa} = 21-(202) = 223$ mV, and formal potential is $E^{0'} = (E_{pc} + E_{pa})/2 = -91$ mV for SWNTs/Chitosan-PQQ/GCE. The ratio between $i_{pa}/i_{pc}$ is 1.27, the peak separation is $\Delta E_P = E_{pa} - E_{pc} = -57-(136) = 79$ mV, and formal potential is $E^{0'} = (E_{pc} + E_{pa})/2 = -97$ mV for Chitosan-PQQ/GCE. SWNTs clearly enhanced the response from PQQ when incorporated into the chitosan matrix.
Figure 2-2. Cyclic voltammetry of (A) PQQ/Chitosan/SWNTs/GCE (pH 7.0 citrate phosphate, SWNTs 3.0 μg, PQQ 4.65 nanomoles); (B) PQQ/Chitosan/GCE (pH 7.0 citrate phosphate, 11.63 nanomoles of PQQ), scan rate 100 mV/s.

The stability of this electrode was evaluated when stored in 0.1 M citrate phosphate buffer solution (pH 7.0) in room temperature. The peak decreased slowly with time (see Figure 2-3), and eventually leveled off.

Figure 2-3. Stability of PQQ/Chitosan/SWNTs/GCE (pH 7.0 citrate phosphate, SWNTs 3.0 μg, PQQ 4.65 nanomoles), first run (black), second day (yellow), third day (pink), fourth day (blue), fifth day (purple), scan rate 100 mV/s.

The effect of scan rate on the peak current was studied by cyclic voltammetry for PQQ/Chitosan/SWNTs/GCE electrode. As Figure 2-4 shows as the scan rate increases, the peak current also increases. A linear relationship was obtained between the square root of the scan rate (mV/s) and cathodic current ($i_{pc}$) and also a linear relationship was found between scan rate and current. This indicates that both a diffusion controlled process and surface controlled process are exhibited in this system.\textsuperscript{78}
Figure 2-4. (a) Plot of cathodic peak current vs scan rate (b) Plot of cathodic peak current vs square root of scan rate
2.4.2. Nafion as a Matrix for Single Wall Carbon Nanotubes and Gold Nanoparticles

2.4.2.1. PQQ/Nafion/SWNTs-COOH/GCE

Nafion is highly conductive to cations, making it suitable for many membrane applications. Figure 2-5 shows typical cyclic voltammograms of PQQ/ Nafion/ SWNTs/ GCE. The ratio between $i_{pa}/i_{pc}$ is approximately equal to 0.98, the peak separation is $\Delta E_p = E_{pa} - E_{pc} = -38 - (-220) = 182$ mV, and formal potential is $E^0 = \frac{E_{pc} + E_{pa}}{2} = -129$ mV for PQQ/ Nafion/ SWNTs/ GCE. The stability was studied as a function of time (Figure 2-6). After an initial loss in signal, a stabilized residual signal is still observable after 3 weeks, similar result was reported by Tachikawa and coworker.\cite{68}

Figure 2-5. Cyclic voltammetry of PQQ/ Nafion/ SWNTs/ GCE (pH 7.0 citrate phosphate, SWNTs 3.0 μg, PQQ 4.65 nanomoles ), scan rate 100 mV/s.
Another chitosan layer was added to the outside of PQQ/ Nafion/ SWNTs/ GCE, in an attempt to increase the stability. The stability was monitored by cyclic voltammetry, and showed no improvement. Electron transfer between the solution and electrode was hindered (see Figure 2-7). The ratio between $i_{pc}/i_{pa}$ is 1.38, the peak separation is $\Delta E_p = E_{pa} - E_{pc} = -9 - (-196) = 187$ mV, and formal potential is $E^{0'} = (E_{pc} + E_{pa})/2 = -103$ mV for Chitosan/PQQ/ Nafion/ SWNTs/ GCE.
Figure 2-7. Cyclic voltammogram of Chitosan / PQQ/ Nafion/ SWNTs/ GCE (pH 7.0 citric phosphate buffer, SWNTs 3.0 μg, PQQ 4.65 nanomoles), scan rate 100 mV/s.

Figure 2-8 shows a typical cyclic voltammetry of PQQ/ Nafion/SWNTs-COOH/ GCE. Voltammograms were sharper than those of PQQ/ Nafion/ SWNTs/GCE. That shows the carboxylic groups in SWNTs promote the electron transfer between electrochemical active molecules and the electrode. The ratio between $i_{pa}/i_{pc}$ is 1.02, the peak separation is $\Delta E_p = E_{pa} - E_{pc} = 45 - (-193) = 148$ mV, and formal potential is $E^0 = (E_{pc} + E_{pa})/2 = -119$ mV for PQQ/Nafion/SWNTs-COOH/GCE.
Figure 2-8. Cyclic voltammogram of PQQ/Nafion/SWCNTs-COOH/GCE (0.1 M citrate phosphate, pH 4.16 citric phosphate buffer, SWNTs 3.0 μg, PQQ 4.65 nanomoles), scan rate 100 mV/s.

The stability of PQQ/Nafion/SWCNTs-COOH/GCE electrodes was studied, at beginning, the peak current decrease a little bit, after that, it stabilized (Figure 2-9). The relative steady state response is 65% after 1 day in drying storage and 84% after 1 day in buffer for cathodic peaks.
Figure 2-9. Current change as function of time for PQQ/ Nafion/SWNTs-COOH/ GCE (a) dry storage in refrigerator (b) in 0.1 M citrate phosphate buffer solution.

As the scan rate increases, the peak current also increases. The linear relationship was shown in Figure 2-10 between the square root of the scan rate (mV/s) and cathodic peak current ($i_{pc}$), and between scan rate and cathodic peak current. This indicates a diffusion controlled process and surface controlled process are both exhibited in this system. $^78$
2.4.2.2. PQQ/Nafion/Au NP/GCE

Cyclic voltammograms of both modified PQQ/Nafion/Au NP/GCE and PQQ/Nafion/GCE were performed in 0.1 M (pH 4.53) citrate phosphate buffer solution. Figure 2-11 shows a typical cyclic voltammogram of the electrode modified by PQQ/Au NP/Nafion/GCE (pink) and PQQ/Nafion/GCE. The electron transfer was enhanced by gold nanoparticles, due to their high surface area and conductivity, compared to the electrode modified by PQQ/Nafion/GCE. The ratio between $i_{pa}/i_{pc}$ is approximately equal to 1.02, the peak separation is $\Delta E_P = E_{pa} - E_{pc} = -45 - (-193) = 148$ mV, and formal potential is $E^0' = (E_{pc} + E_{pa})/2 = -119$ mV for PQQ/Nafion/Au NP/GCE.

Figure 2-11. Comparison of electrodes PQQ/Au NP/Nafion/GCE (pink), containing Au NP 10 nM, PQQ 4.65 nanomoles; PQQ/Nafion/GCE (blue), containing PQQ 4.65 nanomoles in 0.1 M (pH 4.53) citrate phosphate buffer solution, scan rate 100 mV/s.
A linear relationship was obtained between peak current vs scan rate and peak current vs square root of scan rate in the range 10 to 70 mV/s. So diffusion controlled process and surface controlled process are found in this system in the range of below 70 mV/s. 

(a)

(b)
Figure 2-12. (a) Plot of cathodic peak current vs scan rate; (b) Plot of cathodic peak current vs square root of scan rate for PQQ/Au NP/Nafion/GCE in 0.1 M pH 4.53 citrate phosphate buffer.

2.4.2.3. Preparation of PQQ/Nafion/EC Au/GCE

Electrodeposition is one of the immobilization techniques to introduce Au NP to the electrode surface. According to Finot,\textsuperscript{76} the thickness of Au NP deposited onto the electrode surface decreases as the scan rate increases and the number of scans decreases. Figure 2-13 shows a typical cyclic voltammogram for electrodes modified by electrodeposition of gold nanoparticles and followed by layered deposition of PQQ and Nafion. From Figure 2-13, the thinner film of EC Au NP shows well defined reduction-oxidation peaks compared to a thicker film (10 mV/s). Electrodeposition of Au NP shows no advantage over simple deposition described in section 2.4.2.2.
2.4.3. Pyrrole as a Matrix Combination for Single Wall Carbon Nanotubes, Gold or Copper Nanoparticles

2.4.3.1. Preparation of PQQ/Au NP/PPy/GCE

The electrodes were modified by incorporation of PQQ into a polypyrrole film on the surface of glassy carbon electrodes, in the presence or absence of nanoparticles. Figure 2-14 shows the comparison of electrodes modified by PQQ/PPy/GCE and PQQ/Au NP/PPy/GCE. The peak current decreases with the inclusion of the Au nanoparticles. The peak separation also increases indicating slower electron transfer: $\Delta E$
\[ E_{pa} - E_{pc} = 188 - (-1) = 189 \text{ mV for PQQ/PPy/GCE, compared to } \Delta E = 213 - (-41) = 254 \text{ mV for PQQ/Au NP/PPy/GCE.} \]

Figure 2-14. Comparison of electrode modified by (A) PQQ/PPy/GCE; (B) PQQ/Au NP/PPy/GCE in 0.1 M pH 4.53 citrate phosphate buffer, scan rate 100 mV/s.

The peak current decreases more by increasing the amount of Au NP into the testing solution. Presumably the Au NP competes with PQQ, so as more Au NP was added, less PQQ was entrapped into the PPy film. The same result was obtained for SWNTs-COOH, the peak current decreased as more SWNTs-COOH was added into the testing solution.

**2.4.3.2. EC Cu/PQQ/PPy/GCE**
According to Ulubay et al., modified electrodes had very good stability by electrochemical deposition of Cu metal nanoparticles in an over oxidized polypyrrole film. But in my study, once the polypyrrole film was over oxidized, the peak current for PQQ decreased (PQQ was lost in this procedure). Although, after electrodeposited Cu metal nanoparticle, the peak current for PQQ increased a little bit. Figure 2-15 shows the cyclic voltammogram of an electrode prepared by electropolymerization of pyrrole in the presence of PQQ (A), then after the polypyrrole film was overoxidized (B), and finally after copper nanoparticles were electrochemically deposited on the surface of electrode modified by PQQ, PPy (C).

Figure 2-15. Comparison of cyclic voltammograms of electrodes modified by (A) PQQ/PPy/GCE; (B) PQQ/PPy (overoxidation of polypyrrole)/GCE; (C) EC Cu/PQQ/PPy/GCE (overoxidation of polypyrrole), containing PQQ 4.65 nanomoles in 0.1 M (pH 4.53) citrate phosphate buffer solution, scan rate 100 mV/s.
Figure 2-16 shows a typical cyclic voltammogram of an electrode after electropolymerization of pyrrole in presence of PQQ (B) and then after electrodeposition of copper nanoparticles (A) After electrodeposition of Cu NP, the peak current was found to increase.

![Cyclic Voltammogram](image)

Figure 2-16. Comparison of cyclic voltammograms of electrodes modified by (A) EC Cu/PQQ/PPy/GCE; (B) PQQ/PPy/GCE (pink), containing PQQ 4.65 nanomoles in 0.1 M (pH 4.53) citrate phosphate buffer solution, scan rate 100 mV/s.

2.4.3.3 Preparation of PQQ/SWNTs-COOH/Au-PPy Nanocomposite/GCE

The electron transfer was enhanced by adding SWNTs-COOH, due to its amazing electronic properties. Figure 2-17 is typical cyclic voltammogram of electrodes modified by PQQ/SWNTs-COOH/Au-PPy Nanocomposite/GCE. This electrode is very stable, same peak current was obtained for 20 cycles. The ratio between $i_{pa}/i_{pc}$ is approximately equal to 1.07, the peak separation is $\Delta E_p = E_{pa} - E_{pc} = 269-11 = 258$ mV, and formal
potential is $E^0 = \frac{(E_{pc} + E_{pa})}{2} = 140$ mV for PQQ/SWNTs-COOH/Au-PPy Nanocomposite /GCE.

Figure 2-17. 20 cycles of cyclic voltammograms of electrode modified by PQQ/SWNTs-COOH/Au-PPy nanocomposite /GCE (0.1 M, pH 4.53 citrate phosphate buffer, SWNTs-COOH 3.0 μg, PQQ 4.65 nanomoles ), scan rate 100 mV/s.

Figure 2-18 is typical cyclic voltammogram of electrodes modified by (A) PQQ/SWNTs-COOH/Au-PPy Nanocomposite/GCE, SWNTs-COOH and PQQ entrapped after polymerization (B) no PQQ and (C) no SWNTs-COOH. Broad redox peaks were observed from electrode modified by SWNTs-COOH/Au-PPy Nanoposite/GCE, these peaks should correspond to the redox of carboxylic acid on the surface of SWNTs.68
Figure 2-18. Comparison of cyclic voltammograms of electrodes modified by (A) PQQ/SWNTs-COOH/Au-PPy nanocomposite/GCE; (B) SWNTs-COOH/Au-PPy nanocomposite/GCE; (C) PQQ/Au-PPy nanocomposite/GCE, (0.1 M, pH 4.53 citrate phosphate buffer, SWNTs-COOH 3.0 μg, PQQ 4.65 nanomoles), scan rate 100 mV/s.

Figure 2-19 shows the linear relationship between cathodic peak current, scan rate and square root of scan rate. This indicates that diffusion controlled process and surface controlled process exhibit in this system, which is also confirmed by the slope of log iₚ vs log v 0.715 is larger than theoretical value 0.53 for purely diffusion controlled system.⁷⁸
Figure 2-19. (a) Plot of cathodic peak current vs scan rate; (b) Plot of cathodic peak current vs square root of scan rate (c) Variation of the logarithm of peak current with the logarithm of the scan rate for PQQ/SWNTs-COOH/Au-PPy nanocomposite /GCE in 0.1 M pH 4.53 citrate phosphate.

The stability of PQQ/SWNTs-COOH/ Au-PPy/GCE electrodes was studied. Initially, the peak current decreased before stabilizing (Figure 2-20). The relative steady state response after removal of loosely bound PQQ is 47% after 4 h under dry storage condition and 50% after 4 h when stored in buffer.
Figure 2-20. Plot of peak current as function of time for PQQ/SWNTs-COOH/Au-PPy nanocomposite /GCE (a) dry storage in refrigerator (b) in 0.1 M citrate phosphate buffer solution.

The results of SEM images are shown in Figure 2-21. Figures A, B, C were obtained from a Au-PPy Nanocomposite/GCE modified electrode, and Figures D, E, F were obtained from a SWNTs-COOH/Au-PPy nanocomposite/GC modified electrode.
Figures A and D showed low magnification of the Au-PPy nanocomposite /GCE and SWNTs-COOH/Au-PPy nanocomposite/GCE. Figures B and E showed larger scaled morphology of Au-PPy nanocomposite film and SWNTs-COOH/Au-PPy nanocomposite film, respectively. The surface of GC electrode is very rough after deposition of Au-PPy nanocomposite film or SWNTs-COOH/Au-PPy nanocomposite film (Figure B and E). The detail of Au-PPy nanocomposites was shown in Figure C and F, the size of Au-PPy nanoparticles in Figure C is much smaller than that of Figure F, this might be formed by overlapping of SWNTs-COOH. Also, Figure C demonstrates the pure Au-PPy nanocomposites are uniformly distributed in the film, and confirmed by SEM-EDS and SEM-Mapping (Figure 2-22).
Figure 2-21. (A) SEM micrographs of Au-PPy/GCE; (B) SEM image of Au-PPy film nanocomposite; (C) Detail of Au-PPy nanocomposites; (D) SEM micrographs of PQQ/SWNTs-COOH/Au–PPy nanocomposite /GCE; (E) SEM image of PQQ/SWNTs-COOH/Au-PPy nanocomposite film; (F) Detail of Au-PPy nanocomposites embedded in SWNTs-COOH and PQQ.
Figure 2-22. (A) SEM-EDS spectra of Au-PPy nanocomposite film; (B) SEM-EDS-Mapping of Au-PPy nanocomposite film (red: Au, green: C).
Table 2.1 summarizes the peak currents for the PQQ redox reaction varied with the amount of reagents (HAuCl₄ and pyrrole 1:1 by v/v) used to prepare the Au-PPy nanocomposite when the same amount of PQQ and SWNTs-COOH was entrapped into the film. From Table 2-1, the peak current for PQQ increases as the film becomes thicker when the reagent volume was larger than 2.0 μL. Although for $i_{pa}$ of 3.5 μL is a little larger than that of 3.0 μL, $i_{pc}$ still generally decreases after 2.5μL.

<table>
<thead>
<tr>
<th>Volume (μL)</th>
<th>$i_{pa}$ (μA)</th>
<th>$i_{pc}$ (μA)</th>
<th>$E^{0'}$ (mV)</th>
<th>$\Delta E_p$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>89.6</td>
<td>100.2</td>
<td>121.9</td>
<td>165.9</td>
</tr>
<tr>
<td>2.0</td>
<td>123.0</td>
<td>118.7</td>
<td>130.2</td>
<td>168.3</td>
</tr>
<tr>
<td>2.5</td>
<td>88.2</td>
<td>81.7</td>
<td>140.0</td>
<td>258.0</td>
</tr>
<tr>
<td>3.0</td>
<td>4.4</td>
<td>6.99</td>
<td>115.2</td>
<td>63.7</td>
</tr>
<tr>
<td>3.5</td>
<td>4.8</td>
<td>4.5</td>
<td>74.35</td>
<td>126.3</td>
</tr>
<tr>
<td>4.0</td>
<td>2.8</td>
<td>3.4</td>
<td>115.2</td>
<td>58.9</td>
</tr>
</tbody>
</table>

From cyclic voltammetry measurements at a scan rate of 100 mV/s. $E^{0'}=(E_{pa} + E_{pc})/2$; $\Delta E_p= E_{pa} - E_{pc}$

The advantage of this method is that the procedure is easy, and a very small amount of biomolecule and monomer are needed for polymerization and immobilization.
2.4.4. Amperometric Detection of Thiols

Figure 2-23 shows the schematic diagram of the cysteine sensor. The immobilized quinone compound was used as a biocatalyst to mediate the detection of cysteine. First, the quinone oxidized cysteine to cystine, and the quinone is reduced to hydroquinone. Then cysteine was quantitatively detected by monitoring the amperometric current resulting from the oxidation of hydroquinone back to quinone at the glassy carbon electrode.

![Schematic diagram of modified glassy carbon electrode for cysteine detection.](image)

2.4.4.1. Amperometric Response for Cysteine using Electrode Modified by Chitosan/PQQ/ SWNTs-COOH/GCE

The Figure 2-24 shows the amperometric response by each injection of 25 μL of 4.95 mM cysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 6.80).
The electrode modified by Chitosan/PQQ/SWNTs/GCE responded rapidly to the change of concentration of cysteine, but the signal decreased as the concentration of cysteine increased. This might be caused by adsorption of cysteine on the surface of modified electrode.

2.4.4.2. Amperometric Response for Cysteine using Electrode Modified by PQQ/Nafion/ SWNTs-COOH/ GCE

Figure 2-25 shows the amperometric response by each injection of 25 μL of 4.95 mM cysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 3.53). The electrode modified by PQQ/Nafion/SWNTs-COOH/GCE responded rapidly to the change of concentration of cysteine, producing a steady state signal.
2.4.4.3. Amperometric Response for Cysteine using Electrode Modified by PQQ/Nafion/Au NP/ GCE

Figure 2-26 shows the amperometric response by each injection of 25 μL of 4.95 mM cysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 3.53). The electrode modified by PQQ/Nafion/Au NP/GCE responded rapidly to the change of concentration of cysteine, however this system is not stable as shown in the lack of a steady state response.
2.4.4.4. Amperometric Response for Cysteine using Electrode Modified by EC Cu/PQQ/PPy/GCE

Figure 2-27 shows the amperometric response by each injection of 25 μL of 4.95 mM cysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 3.53). The electrode modified by EC Cu/PQQ/PPy/GCE responded rapidly to the change of concentration of cysteine, similar to the electrode modified by Au NP. This system is not stable, as shown in the lack of a steady state response.
2.4.4.5. Amperometric Response for Thiols using Electrode Modified by PQQ/SWNTs-COOH/Au-PPy Nanocomposite/GCE

Figure 2-28 shows the amperometric response by each injection of 25 μL of 4.95 mM cysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 3.08). Amperometric detection was performed after the electrode was stabilized. The electrode modified by PQQ/SWNTs-COOH/Au-PPy nanocomposite/GCE responded rapidly to the change of concentration of cysteine, producing a steady state signal. A calibration curve shows a linear relationship between peak current and concentration of cysteine. The limit of detection (S/N=3) for cysteine was 0.41 μM, sensitivity was 0.007 A/M (average values given for three electrodes).
Figure 2-28. (a). Amperometric response for cysteine (4.95 mM, 25 μL each injection) electrode modified by PQQ/SWNTs-COOH/Au-PPy nanocomposite/GCE (applied potential, 450 mV); (b). Calibration curve for cysteine at PQQ/SWNTs-COOH/Au-PPy nanocomposite/ GCE.
Amperometric detection was performed in pH 3.45 phthalate buffer solution and similar results were obtained. Also another Nafion layer was added on the surface of PQQ/SWNTs-COOH/Au-PPy nanocomposite/GCE, in order to block analytes sticking onto the electrode surface. No improvement was obtained.

Figure 2-29 shows the amperometric response by each injection of 10 μL of 30.46 mM homocysteine into 3 mL of citrate phosphate buffer (0.1 M, pH 3.08). Amperometric detection was performed after the electrode was stabilized. A calibration curve shows linear relationship between peak current and concentration of homocysteine. The limit of detection (S/N=3) for homocysteine was 0.22 μM, sensitivity was 0.013 A/M (average values given for three electrodes).
Figure 2-29. (a) Amperometric response for homocysteine (10.00 mM, 10 μL each injection) electrode modified by PQQ/SWNTs-COOH/Au-PPy nanocomposite/GCE (applied potential, 500 mV); (b) Calibration curve for homocysteine at PQQ/SWNTs-COOH/Au-PPy nanocomposite/ GCE.

2.5. Conclusion

Different new immobilization materials, co-catalysts and immobilization methods were evaluated in this thesis. Indirect detection of cysteine and homocysteine at micromolar concentration is achieved by incorporation of coenzyme PQQ, and co-catalysts, SWNTs-COOH, into a Au-PPy nanocomposites conducting polymer film. Cysteine and homocysteine are quantitatively detected by monitoring the amperometric current resulting from oxidation of PQQH$_2$ back to PQQ catalyst at the GC electrode surface.

The main advantage is the simplicity of the sensor design that deposited coenzyme, PQQ, and co-catalyst, SWNTs-COOH in to Au-PPy nanocomposites conducting
polymer. This polymer can be easily synthesized by one step. And another advantage is very less coenzyme, co-catalysts and monomer needed to design sensor
Chapter 3

Synthesis of Benzimidazole Quinone Analogue of PQQ

3.1. Experimental

3.1.1 Reagents

All solutions were prepared using deionized water with resistivity greater than 17 MΩ-cm from a Barnstead B-pure water purification system. 2,3-dimethoxytoluene and anhydrous methanol were purchased from Sigma-Aldrich. Acetonitrile, ethanol, methylene chloride, hexane, ethyl acetate, acetone, anhydrous ethyl ether, concentrated nitric acid (69.0%-70.0%), sodium bicarbonate, anhydrous sodium sulfate, and ammonium chloride were obtained from Fisher (Fair Lawn, NJ). Silica gel and sand for chromatography were also obtained from Fisher. N-bromosuccinimide, purified before use, and anhydrous THF, n-butyl lithium, methyl iodide, formic acid (99%), 10% Pd/C, 33% HBr in glacial acetic acid, molecular sieves, manganese oxide were obtained from Dr. Tillekeratne’s group.

3.1.2 Instrumentation

A 1210 Branson Ultrasonic Cleaner from Branson Corp (Danbury, CT) was used to sonicate the glassy carbon electrode. A pH meter from Denver Instruments Co.
(Denver, Co) was used to measure the pH for all the buffer solutions. Characterization methods used for this work included NMR (Varian 200, 400 and 600 MHz) and TLC.

3.2 Synthesis

3.2.1 1-Bromo-3, 4-dimethoxy-2-methylbenzene (8)

To a stirred solution of 2,3-dimethoxytolueul compound 7 (0.3 g, 1.97 mmol) was added a solution of N-bromosuccinimide (0.36 g, 2.05 mmole) in acetonitrile (3.5 mL). The reaction mixture was stirred for 3 h at room temperature. The reaction was monitored by TLC. The solvent was then removed under reduced pressure and the residue was dissolved in CH$_2$Cl$_2$ and washed with saturated aqueous sodium bicarbonate solution, followed by water several times. The organic extract was dried over anhydrous sodium sulfate and concentrated in vacuum to obtain 8 as yellow oil (0.45 g, 95%). $^1$H NMR (200 MHz, CDCl$_3$) δ 7.27-7.23 (d, 1H, $J = 12.0$ Hz), 6.69-6.64 (d, 1H, $J = 12.0$ Hz), 3.85 (s, 3H), 3.79 (s, 3H), 2.35 (s, 3H).

3.2.2 1, 2-Dimethoxy-3, 4-dimethylbenzene (9).

Compound 8 (0.36 g, 0.016 mmol) was dissolved in anhydrous THF (4 mL) at -78 °C (dry ice in acetone) in a two neck round bottom flask. When the temperature was stable, n-butyl lithium (0.7 mL of a 2.5 M solution in hexanes, 0.017 mmol) was added. The reaction mixture was stirred 15 mins. Methyl iodide (0.2 mL) was added and the mixture was stirred at -78 °C for 30 mins. The reaction was quenched by saturated aqueous ammonium chloride solution and extracted with ethyl ether. The combined organic extract was dried over anhydrous Na$_2$SO$_4$ to obtain 9 (2.3 g, 90%). $^1$H NMR (400
MHz, CDCl$_3$) δ 6.87-6.85 (d, 1H, $J = 12.0$ Hz), 6.69-6.67 (d, 1H, $J = 12.0$ Hz), 3.84 (s, 3H), 3.79 (s, 3H), 2.21-2.19 (d, 6H, $J = 12.0$ Hz).

3.2.3. 1, 2-Dimethoxy-3, 4-dimethyl-5, 6-dinitrobenzene (6).

Compound 9 (0.83 g, 5 mmol) was added very slowly to concentrated nitric acid (2.8 mL) in an ice bath. The mixture was stirred at 0 °C overnight, and was poured into ice cold water. It was extracted with methylene chloride and the combined organic extract was dried over anhydrous Na$_2$SO$_4$, concentrated and purified by flash chromatography on silica (20% hexanes/CH$_2$Cl$_2$) to give 6 as yellow oil (0.44 g, 35%). TLC $R_f = 0.45$ (silica gel 230-400 Mesh, 50% EtOAc/hexanes). $^1$H NMR (200 MHz, CDCl$_3$) δ 3.90 (s, 1H), 3.85 (s, 1H), 2.37 (s, 1H), 2.28 (s, 1H).

3.2.4. 4, 5-Dimethoxy-6, 7-dimethyl-1H-benzo[d]imidazole (10).

Compound 6 (1.2 g, 4.69 mmol) was dissolved in formic acid (12 mL) to which was added 10% Pd/C (0.6 g). The resulting reaction mixture was refluxed for 2 h, the catalyst was filtered off and the filtrate was concentrated in vacuum. Purification by flash column chromatography on silica (5% methanol/CH$_2$Cl$_2$) afforded 10 (0.68 g, 70%) as a colorless oil. $^1$H NMR (600 MHz, CD$_3$OD) δ 8.03 (s, 1H), 4.04 (s, 3H), 3.82 (s, 3H), 2.43 (s, 3H), 2.28 (s, 3H).

3.2.5. 6, 7-Dimethyl-1H-benzo[d]imidazole-4, 5-diol (5).

A solution of 10 (1 g, 4.85 mmol) in 33% HBr in glacial acetic acid (187 mL) was refluxed overnight. The reaction mixture was concentrated under vacuum to give
compound 5 (0.77 g, 90%) as an orangish solid. $^1$H NMR (400 MHz, CD$_3$OD) δ 9.15 (s, 1H), 2.45 (s, 3H), 2.32 (s, 3H).

3.2.6. 6, 7-Dimethyl-1H-benzo[d]imidazole-4, 5-dione (1).

To a stirred solution of 5 (0.078 g, 0.438 mmol) in anhydrous acetone (4.38 mL) and anhydrous methanol (0.5 mL) with molecular sieves (0.219 g) was added manganese oxide (0.380 g, 10 equiv) and the resulting mixture was stirred at room temperature for 3 h. The catalyst was filtered off and the filtrate was concentrated in vacuum. Purification by flash column chromatography on silica (5% methanol/CH$_2$Cl$_2$) afforded 1 (0.05 g, 70%) as an orange solid. $^1$H NMR (200 MHz, CD$_3$OD) δ 7.84 (s, 1H), 2.27 (s, 1H), 1.93 (s, 1H).

3.3. Electrochemical Evaluation of 1

3.3.1 In solution

The reversibility of electron transfer and the magnitude of the redox potential for compound 1 were studied by evaluating the electrochemical behavior in solution. A 2.8 x 10$^{-4}$ M solution of compound 1 was prepared by adding 0.49 mg of compound 1 into 10 mL of DI water. A few drops of KOH helped compound 1 dissolve into water. The test solution was prepared by adding 0.25 mL of the stock solution to 1.75 mL citrate phosphate buffer (pH=4.48). The test solution was deoxygenated for 15 mins using argon gas before running cyclic voltammetry.
3.3.2. Electropolymerization of Pyrrole with 1

Compound 1 was entrapped by electropolymerization of pyrrole through bulk electrolysis. The test solution was prepared by adding 7 mg of KCl into 1 mL of the 2.8 x 10^{-4} M stock solution. The test solution was purged with argon gas for 15 mins, and 30 μL of 0.1 M pyrrole was added. The pyrrole solution was distilled before using. A cleaned and pre-treated glassy carbon electrode was immersed into the solution. Three drops of HCl was added into the solution to adjust pH to acidic. A fixed potential of 630 mV was applied. Deposition of polypyrrole was continued until the desired thickness was achieved. According to Inoue,29 a charge of 100 mC cm^{-2} can produce a polypyrrole film containing Cl\(^-\) counterions about 125 nm in thickness. The modified electrode was washed with DI water, and dried in air. The entrapment and stability of the modified electrode was measured in 0.1 M citrate phosphate buffer (pH=3.12).

3.3.3. Preparation of Compound 1/ Nafion/SWNTs-COOH/GCE

A solution of single wall carbon nanotubes was prepared. 0.6 mg of SWCNTs-COOH was dispersed in 1 mL of DMF to give a 0.6 mg/mL black suspension, which also contains 5 μL of Nafion (0.5%, v/v).

Glassy carbon electrodes were modified, according to Tachikawa’s procedure.68 5 μL of the SWNTs-COOH solution were placed on the clean glassy carbon electrode surface and dried at room temperature. 3 μL of the 2.8 mM solution of compound 1 was added to the surface of the GCE electrode and allowed to dry.
3.4 Results and Discussion

3.4.1. Synthesis

This simplest substituted benzimidazole quinone 1 of minimum molecular size was synthesized according to the method of Gupta. The carbonyls of the ortho-quinone moiety were placed immediately adjacent to the imidazole ring, analogous to the structure of PQQ. Methyl substituents were incorporated into the unsubstituted positions of the quinone ring to inhibit the Michael-type addition of reactive nucleophiles. The synthetic scheme and all the NMR spectra are attached in the appendix.

Compound 7 is commercially available. Electrophilic bromination of compound 7 with N-bromosuccinimide gave compound 8 with excellent yield (Scheme 3-1). Compound 8 was treated with n-butyllithium followed by methyl iodide to obtain intermediate compound 9 in good yield. Dinitration of compound 9 with nitric acid at 0 °C gave compound 6 in low yield. Compound 6 was refluxed in formic acid with Pd/C overnight to give compound 10. Demethylation with 33% HBr in acetic acid then gave 5, which was oxidized with manganese dioxide to quinone 1.
3.4.2. Electrochemical Properties of 1

Previous research has found that pyrroloquinoline quinone exhibits a reversible, two electron, two proton electron-transfer reaction. The synthetic benzimidazole quinone 1 is expected to have similar behavior (Scheme 3-2).

Scheme 3-2. Proposed mechanism for the reversibility of 1.
There are three criteria that to determine the reversibility of an electrochemical reaction. First, the separation of peak potentials $\Delta E_p = E_{pa} - E_{pc} \approx 0.059/n \, V$ for all scan rates, where $n$ is the number of electrons transferred in the electrode reaction. Second, for a reversible redox couple, $i_p$ increases with $v^{1/2}$ and is directly proportional to concentration according to the Randles-Sevcik equation for a diffusion controlled reaction.

$$i_p = 2.69 \times 10^5 n^{3/2} A D^{1/2} C v^{1/2} \text{(3-1)}$$

*i*$_p$ = peak current, A  

*n* = electron stoichiometry  

*A* = electrode area, cm$^2$  

*D* = diffusion coefficient, cm$^2$/s  

*C* = concentration, mol/cm$^3$  

*v* = scan rate, V/s

Third, the ratio of $i_{pc} / i_{pa}$ should be close to one; however, chemical reactions coupled to the electrode process can significantly alter the ratio of peak currents.

Figure 3-1 shows the cyclic voltammogram of compound 1 at pH 4.48 and a scan rate 100 mV/s. The ratio between $i_{pc}/i_{pa}$ is approximately equal to 0.92, suggesting reversibility of the reaction. The peak separation for 1 is $\Delta E_p = E_{pa} - E_{pc} = 109 - 72 = 37$ mV. The $\Delta E_p$ is a little higher than the theoretical value for a two electron involved reversible redox reaction, where $\Delta E_p$ is expected to be around $\Delta E_p = 59 \, mV/2 = 29.5$ mV. From the forward scan, the quinone moiety is reduced to hydroquinone, and then during the reverse scan the hydroquinone is oxidized back to the quinone. As the scan rate increases, the peak current also increases (Figure 3-2).
linear relationship was shown in Figure 3-6 between the square root of the scan rate (mV/s) and cathodic current ($i_{pc}$).

Figure 3-1. Cyclic voltammogram of 35 μM 1 in solution, pH 4.48 citrate phosphate buffer, scan rate 100 mV/s.
Figure 3-2. (a) Cyclic voltammogram of 35 μM 1 in solution, pH 4.48 citrate phosphate buffer, scan rate 100 mV/s (A), 10 mV/s (B); (b) plot of Cathodic peak current vs square root of scan rate.

3.4.3. Electropolymerization of Pyrrole in the presence of 1

Figure 3-3 shows the cyclic voltammogram of the 1/PPy modified electrode. No quinone peaks were found from Figure 3-3.

Figure 3-3. Cyclic voltammogram of 1/PPy/GCE, pH 3.12 citrate phosphate buffer, scan rate 100 mV/s.
3.4.4. Deposition 1 and Nafion with SWNTs-COOH

Figure 3-4 shows the cyclic voltammogram of electrode modified by 1/Nafion/SWNTs-COOH/GCE. The quinone peaks were found from Figure 3-4, but decrease after first run and disappear after several runs.

![Cyclic voltammogram of 1/Nafion/SWNTs-COOH/GCE](image)

Figure 3-4. Cyclic voltammogram of 1/Nafion/SWNTs-COOH/GCE, pH 3.12 citrate phosphate buffer, scan rate 100 mV/s.

3.5. Conclusion

The simplest, minimum molecular size substituted benzimidazole quinone was synthesized and its electrochemical properties evaluated. The \( \text{o-quinone moiety} \) was shown to have a diffusion controlled, reversible, pH dependent two-electron, two-proton redox reaction.

Unfortunately, the electrochemistry of the modified electrode did not provide a stable reproducible response for 1. Thus, the use of 1 as an electrocatalyst for quantitative thiol detection is not possible.
Chapter 4

Future Work

It would be interesting and useful to synthesize other quinone compounds that can be used in modified electrode using same method as PQQ in this study, especially Au-PPy nanocomposites combination of SWNTs, which were shown to be stable enough to detect thiols. It would be very interesting and useful to expand the Au-PPy project to study the activity of the enzyme, such as, acetylcholine esterase or glucose oxidase by the amperometric monitoring of thiocholine or H$_2$O$_2$. Furthermore, this modified electrode can be used to detect pesticides and AChE inhibitors.

Also it would be interesting and useful to explore other matrixes that can incorporate the quinone compounds which would prevent of leaching for the detection of thiols, and different nanoparticles.

Different electrodes can be used, such as gold electrode, since PQQ and its derivatives can be directly attached to gold electrode surface through forming molecular wires.
References


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Appendix A

1H NMR Spectra of Synthesis

A1. $^1$H NMR spectra of compound 8.
A2. $^1$HNMR spectra of compound 9.
A3. $^1$H NMR spectra of compound 6.

A4. $^1$H NMR spectra of compound 10.
A5. $^1$H NMR spectroscopy of compound 5.

A6. $^1$H NMR spectroscopy of compound 1.