NANOMATERIALS-BASED SENSORS FOR PEROXYNITRITE DETECTION AND QUANTIFICATION

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DEDICATION

I dedicate this work to the memory of my respected parents who gave me the life and put me on the track of knowledge. I also extend my dedication to my noble wife Shaimaa Maher for her patience, encouragement, and continuous support; my daughters Mariam, Maram and Mawada; my sons Ahmed and Mohamed; and to my siblings Mohamed and Shaimaa Kalil. I would like also to dedicate this dissertation to Dr. Shehab Sallam, my Egyptian advisor, for his utmost support and motivation during the completion of this work.
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ABSTRACT

Peroxynitrite (ONOO\(^-\), PON) plays a crucial role in several cardiovascular dysfunctions and other diseases triggered by oxidative stress. PON is a strong oxidizing agent produced from the diffusion-controlled reaction between nitric oxide radical (\(\cdot\)NO) and superoxide anion-radical (\(O_2\cdot^-\)). It is also a member of the reactive oxygen nitrogen species family, which attacks vital components inside the body and initiates deleterious effects via direct and indirect interactions. PON reacts directly with lipids, DNA, and proteins, whereas indirectly, it acts as an initiator of radical-chain reactions.

In this work, we have explored various interfaces of manganese-oxide-decorated graphene/hemin and selenium containing compound for PON detection and quantification. The combination of manganese oxide nanoparticles with the graphene/hemin matrix has allowed for more hemin molecules to be adsorbed on the final composite matrix. As a result, the adsorbed hemin has enhanced the catalytic activity of the final composite and improved the sensitivity towards PON detection. In the same context, a selenium-containing compound (aniline-selenide) has been synthesized and grafted on the electrode surface using the chemistry of diazonium salt. The aniline-selenide-modified electrode showed an increase of approximately 40 times the catalytic current as the aniline-modified electrode.

Throughout this project, the preparation methods of the electroactive nanomaterials were described in detail. Moreover, the characterizations of the prepared-materials have
been investigated by various physicochemical methods using scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), ultraviolet/visible measurements (UV/Vis), and X-ray photoelectron spectroscopy (XPS).

This study showed the importance of using selenium and manganese interfaces as sensitive platforms for PON detection. It also provides the initial stage to extend the use of these interfaces to ultramicroelectrode sensors for use at the level of single cells.
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CHAPTER I
INTRODUCTION AND OVERVIEW OF PEROXYNITRITE SYNTHESIS AND QUANTIFICATION

1.1. Introduction

Over the past two decades, several thousand peroxynitrite-related articles based on the data available in PubMed, were published particularly after the discovery of its crucial role in pathophysiology. Historically, peroxynitrite was first identified in 1904, and chemists including Halfpenny and Robinson in 1952, Hughes and Nicklin in 1968, Keith and Powell in 1969, and Mahoney in 1970 had studied the chemistry of peroxynitrite. However, the work done by Beckman et al. in 1990 is considered a key milestone in bringing attention back to this molecule through its biological role. The nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) system suggests oxoperoxonitrate for peroxynitrite anion (ONOO⁻) and hydrogen oxoperoxonitrate for its conjugate peroxynitrous acid (ONOOH).

Peroxynitrite (PON) is a strong oxidizing agent, a potent nitrating anion, and one of the most noxious reactive oxygen-nitrogen species (RO-NS), which has shown a significant impact in cell biology. They are characterized by the high reactivity and a short
lifetime. Although PON is following the same route of free radicals in reactivity and brief lifespan, it does not have any unpaired outer shell electron. Thus, it is an exceptional form of non-radical reactive species. In addition, clinical studies have revealed the reactivity of PON by its direct and indirect incorporation in a several deleterious effects that mark this analyte as a suspicious agent in homeostasis disruption. PON reacts directly with lipids, DNA, and proteins, whereas indirectly, it triggers radical chain reactions.

1.2. Chemistry of Peroxynitrite

Peroxynitrite chemistry has emerged and received more attention after the discovery of nitric oxide and its biological functions. The Nobel Prize was awarded to Robert Furchgott, Louis Ignarro, and Ferid Murad in 1998 for exploring the role of nitric oxide as a signaling molecule. This exploration opened the door of research on its activity in health and disease. It turned out that some of the problems that were assigned to nitric oxide’s pathology are in fact related to peroxynitrite, adherent deleterious product of nitric oxide. Thus, the research marks out the peroxynitrite molecule as a very noxious metabolite in the biological system. Human diseases associated with pollution, toxicity and oxidative stress are attributed to higher levels of PON inside the body, which plays a significant role in pathophysiological and cellular milieu.

Before discussing the chemistry of PON, it is necessary to refer to its precursors: superoxide and nitric oxide, and their biological significance. Nitric oxide (NO), the first PON precursor, is generated from the catalytic oxidation of L-arginine to citrulline by nitric oxide synthase (NOS). The second precursor, superoxide (O$_2^-$), is continuously formed from aerobic cells’ metabolism, such as the mitochondrial respiratory chain,
uncoupled nitric oxide synthesis from NADPH oxidase, xanthine oxidase, and respiratory bursts.\textsuperscript{2,22} Both nitric oxide and superoxide are not as deleterious as their product PON, since each of them has a physiological scavenger. The scavenger works to get rid of the excess level of those precursors (\(\text{NO} \) and \(\text{O}_2^-\)).\textsuperscript{2,23}

Superoxide dismutase (SOD) is responsible for the neutralization of the superoxide excess by catalyzing the reaction between superoxide and hydrogen ions (\(\text{H}^+\)) to form hydrogen peroxide and oxygen molecules (\(k = 2.5 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}\)). On the other hand, the high concentration in nitric oxide is modulated by reactivity with a number of physiological targets. Physiologically, nitric oxide radical is deactivated to nitrate by the reaction with oxyhemoglobin to form methemoglobin within less than two seconds (equation 1). Interestingly, neither superoxide dismutase nor oxyhemoglobin can contend against the formation of PON via the very rapid diffusion-controlled reaction between both precursors (rate constant \(k \approx 2 \times 10^{10} \text{ M}^{-1} \cdot \text{s}^{-1}\)).\textsuperscript{2,20,22}

\[
\text{Hb (Fe}^+\text{O}_2\text{)} + \cdot \text{NO} \rightarrow \text{Hb (Fe}^+\text{)} + \text{NO}_3^- \quad (1)
\]

In biological systems, PON is formed through the reaction of nitric oxide (\(\text{NO}\)) radical with superoxide anion-radical (\(\text{O}_2^-\)).\textsuperscript{24} Since the pKa of peroxynitrous acid is in the range of 6.5 – 6.8, peroxynitrite anion is protonated at physiological pH, and it forms a very unstable acid. However, the peroxynitrite ion is more stable in basic environments with the absence of carbonyl compounds, carbon dioxide, and transition metals.\textsuperscript{25-26} The equilibrium protonation of peroxynitrite anion is given in the following equation:
On the other hand, its conjugate acid, peroxynitrous acid, is an inorganic peroxy acid, which is unstable and decays rapidly by one of two mechanisms given below:

\[ \text{O}_2\text{N}^-\text{O}_2\text{OH} + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{NO}_2^- \quad (2) \]

\[ \text{O}_2\text{N}^-\text{O}_2\text{OH} \rightarrow \text{NO}_3^- + \text{H}^+ \quad 70\% \quad (3) \]

\[ \text{O}_2\text{N}^-\text{O}_2\text{OH} \rightarrow \text{\cdotNO}_2 + \text{\cdotOH} \quad 30\% \quad (4) \]

Peroxynitrite is a structural isomer of nitrate (\(\text{NO}_3^-\)) that contains a peroxo bond (\(\text{O}—\text{O}\)). Geometrically, the unpaired electrons of superoxide anion and nitric oxide radical recombine to form a new bond (\(\text{ON}—\text{OO}^-\)) and create one of two conformational isomers as illustrated in Figure 1.1. Interestingly, the \(\text{cis}\) isomer, as shown by the vibrational spectroscopy measurements, is more stable than the \(\text{trans}\) isomer. This stability of the peroxynitrite in \(\text{cis}\)-conformation results from the facilitated delocalization of the negative charge over the entire molecule. In addition, the \(\text{cis}\) isomer is also the highly reactive species that triggers the nitration process of many targets including proteins.\(^2,17,27\)
Figure 1.1 Conformational isomers of peroxynitrite.

The reactivity of the peroxynitrite anion (ONOO\(^-\)) can be demonstrated from a thermodynamic point of view. Since it has about 180 kJ/mol more energy than the nitrate ion, it should be highly unstable. However, it does not decompose through bond rearrangement. In fact, the decomposition of the PON ion in a basic medium takes place via a set of radical reactions trigger by the slow reaction between two PON anions ON-OO\(^-\) and ONO-O\(^-\) to produce nitrite, nitrate and oxygen. On the other side, the PON anion works as a Lewis base, which reacts rapidly with Lewis acid to neutralize by H\(^+\) or form an adduct (ONO-OL). At this level, PON starts its own oxidative/nitrosative chemistry through very fast decomposition pathways. The first pathway is a radical route through homolytic decomposition, while the other takes place via heterolytic cleavage forming positive and negative ionic species. In the terms of electron transfer, the decomposition process has two possible mechanisms: one-electron transfer (homolysis) involves oxygen radicals; two-electron transfer (heterolysis) includes an oxygen atom transfer.\(^2,27\)

Another very important consideration of PON reactivity is the reaction with carbon dioxide CO\(_2\) to produce a highly unstable form of nitrosoperoxycarbonate (ONOOCO\(_2\)^-). This unstable adduct (ONOOCO\(_2\)^-) undergoes immediate degradation to release carbonate
(CO$_3^-$) and nitrite (NO$_2$) radicals. These radicals are very strong oxidizing and nitrating agents. Both radicals might react together in the absence of reducing agents (reductants) and regenerate carbon dioxide and release nitrate ion as final stable product. Here, it is important to know that the concentration of carbon dioxide in biological fluids can exceed 1 mM, which results in highly favorable reaction with peroxynitrite (rate constant k $\approx 5 \times 10^4$ M$^{-1}$·s$^{-1}$)$^2,17$

The short lifespan of the PON anion is the biggest challenge. PON undergoes several reactions per unit time that prevents its accumulation in biological systems. Therefore, the direct determination of PON concentration is not an easy protocol. Alternatively, indirect methods including EPR, immunohistochemistry, tyrosine nitration, and fluorescence are the common methods to estimate its concentration. However, these methods are not suitable to occur in real time. Besides, they are laborious, inconvenient, and prone to systematic errors. In contrast, electrochemical methods come to the forefront in detecting PON efficiently in real time and directly with reliable promise of sensitive and selective measurements.$^{22,28}$

1.3. Common Methods of Peroxynitrite Synthesis

Peroxynitrite can be rapidly generated \textit{in vivo} by the combination of nitric oxide and superoxide radicals or synthetically in laboratory using different chemical methods. In both ways, the two main precursors of peroxynitrite formation are nitric oxide radical and superoxide anion radical.

Several synthetic methods have been listed in the literature and used for peroxynitrite preparations.$^{29-32}$ The method of preparation plays a crucial role in PON
quantifications and significantly influences its method of detection. This influence can be elevated by the interference with any components that mainly come as a contaminant during the preparation process. These contaminations lead to unreliable concentrations in the detection of PON. Hence, to have an accurate calibration for PON, it is important to find a clean and reliable method of PON preparation. In addition, process optimization including pH, reaction temperature, as well as the method of PON collection and storage must be considered during the experimental procedures.

Most PON synthesis methods produce a contaminated product of PON with one or more reagents that derived from the alkaline solutions and/or other impurities, such as nitrite, nitrate, hydroxylamine, azide, hydrogen peroxide, 2-ethoxyethanol, and/or tetramethylammonium hydroxide. Understanding the activity of PON in health and disease depends on the quality of measurements and ways of monitoring its concentrations. This cannot be fully understood without taken into consideration the methods of preparation.

Here, some of the PON preparation methods have been listed and evaluated. Most of these preparation methods imply the presence of unavoidable interferents, mainly hydrogen peroxide and nitrite. Methods that generate relatively high concentrations of PON with low interferents are of course more favorable. In this section, a summary of various published synthesis methods of PON will be discussed.

1.3.1 Reaction of Nitrite with Acidified Hydrogen Peroxide

As shown in Scheme 1.1, the reaction of nitrous acid with acidified hydrogen peroxide produce a short-lived peroxynitrous acid, which is then quenched immediately to
form peroxynitrite anion. The basic medium is necessary to stop the spontaneous degradation of the peroxynitrous acid and reduce its isomerization to nitrate. The time factor in this method is critical due to the half-life time of peroxynitrite anion/peroxynitrous acid in acidic and neutral pHs. However, peroxynitrite is relatively stable in alkaline solution where pH ~ 9–12. Therefore, PON can be preserved in a strongly basic medium at −20 °C for several weeks or at −80 °C for few months.30,34

![Scheme 1.1 PON synthesis from the reaction of nitrite ion and acidified hydrogen peroxide.](image)

The general yield of peroxynitrite from this method is approximately 85 % at room temperature with nitrite residual. This method has been improved by Saha et al. by using an efficient double mixer with a quenched-flow reactor to minimize the interferents.35

1.3.2 Two-Phase Displacement Reaction

The most reliable method for peroxynitrite synthesis has been reported by Uppu.29 As shown in Scheme 1.2, this method is based on a displacement reaction between the anion of hydroperoxide (HOO−) in the aqueous phase and isoamyl nitrite (3-methyl-1-nitrosooxybutane) in the organic phase. The advantage of this method is shown by the generation of PON in the aqueous phase, while the unreacted isoamyl nitrite along with the new product of isoamyl alcohol will be remained in the organic phase. The lower chance of contamination makes this method more suitable to have a clean PON and minimize its
interference with any of other reaction components. Moreover, the high concentration of PON that is produced from this method adds another advantage to it. However, the only disadvantage here is the time consuming (~ 5-6 hrs.), which sometimes leads to PON self-decomposition. This limitation has been treated by introducing solvents of intermediate-polarity, like isopropyl alcohol, which works to revoke phase separation of the reactant pair hydroperoxide and isoamyl nitrite. This simple modification makes this method more efficient for peroxynitrite synthesis, faster than before, and less contaminated with nitrite, nitrate, and carbonate. Furthermore, it provides high yield of PON with a concentration up to 1 M.

Scheme 1.2 PON synthesis from the displacement reaction of isoamyl nitrite and hydrogen peroxide in strong basic medium.

The concentration of PON can be easily monitored by UV/Vis spectroscopy at wavelength of 302 nm and molar absorptivity of 1700 M$^{-1}$·cm$^{-1}$ as shown in Figure 1.2. This method is reported to produce peroxynitrite with very low residues of nitrite and/or hydrogen peroxide compared to other methods.
Figure 1.2 Assay of PON synthesis using displacement reaction method, absorbance of PON at 302 nm increases as a function of time.

1.3.3 Generation of PON from SIN-1

3-morpholinosydnimine (SIN-1) has been used to simultaneously generate nitric oxide and superoxide, which instantly combine to form peroxynitrite as a final product. This method suggests that the decomposition of SIN-1 in the presence of oxygen releases superoxide and nitric oxide radicals with 1:1 molar ratio that spontaneously form PON at physiological pH. As shown in Scheme 1.3, Kurz and his group suggest that SIN-1 is oxidized to SIN-1A⁺ in the first step to generate superoxide radical from of molecular oxygen. This step is followed by releasing an equivalent mole of nitric oxide radical, which combines rapidly with superoxide anion to generate peroxynitrite.
Scheme 1.3 PON generation mechanism from SIN-1

In this project, we will use SIN-1 as a generator of PON to perform our electrochemical measurements at physiological pH. We will also show the difference of using PON generator versus authentic PON on the sensitivity and limit of detection (LOD) of the bare and modified sensors.

1.4. Peroxynitrite Detection & Quantification

Several methods of PON detection, other than electrochemical methods, are known. However, all these methods depend on a byproduct or a marker of PON instead of the analyte itself. These methods are considered as indirect techniques and may not be suitable for real-time determination of PON. They also may be laborious and costly. In fact, the high reactivity and short lifetime make the accurate detection of this analyte in biological systems a challenging task. In the last decade, tremendous work has been done to develop methods to measure the generation of PON in tissue and cellular levels. These methods can be classified into three major detection categories: 1) nitrotyrosine formation, 2) fluorescent probes, and 3) electrochemical sensors.43
1.4.1. Nitrotyrosine Formation

Detection of nitrotyrosine as a biomarker of PON formation was commonly used as a tool to monitor the levels of PON in biological samples. In fact, tyrosine is easily nitrated by ONOO\textsuperscript{−} to form 3-nitrotyrosine, the latter can then be used as a footprint of peroxynitrite in biological systems. Several methods have been developed to quantify 3-nitrotyrosine, such as immunohistochemistry and high-performance liquid chromatography (HPLC) as a stand-alone or in conjunction with mass spectrometry (MS).\textsuperscript{26}

Nitration of tyrosine is used to understand PON cytotoxicity and nitrosative stress that can be associated in several diseases including neurodegenerative diseases, diabetic complications and cardiovascular diseases. More recently, Chen et al. have measured the amount of nitrotyrosine and used it for evaluating potential drug candidates for treating peroxynitrite-mediated brain damage in cerebral ischemia-reperfusion injury.\textsuperscript{44}

Regardless of the accuracy of using nitrotyrosine as a marker of PON, the method suffers several drawbacks, such as complications in handling of samples, and unavoidable internal contaminations. For example, one method using HPLC/MS showed contamination by peroxide, which leads to artificial generation of peroxynitrite, and overestimation of nitrotyrosine. In antibody-based methods, the low quality of nitrotyrosine antibodies, add another disadvantage. Moreover, the nitration of tyrosine can be easily triggered by another reactive nitrogen species, such as \textsuperscript{\textbullet}NO\textsubscript{2} or \textsuperscript{\textbullet}NO, which does not necessary reflect the nitration of tyrosine by PON. Additionally, in many cases, the production levels of nitrotyrosine are too little to be quantified.\textsuperscript{2}
In conclusion, the formation of nitrotyrosine is not a selective way to quantify the level of peroxynitrite. It also cannot be used for real time analysis to detect an accurately measure peroxynitrite concentration in biological systems.\textsuperscript{2, 45}

1.4.2. Fluorescent Probes

Many fluorescent probes have been designed and developed for monitoring peroxynitrite in real time spatial imaging with high sensitivity in living cells.\textsuperscript{36, 46-47} Currently, dihydrorhodamine (analog of 2,7 dichlorodihydrofluorescein) 123 (DHR) is often used in PON detection. Because it is easily oxidized in the presence of PON to generate the fluorophore rhodamine 123, DHR has been used to quantify PON. However, DHR\textsubscript{123} is readily oxidized by any cell-derived oxidant and not necessarily by peroxynitrite. The readily oxidation of DHR\textsubscript{123} is reported as a significant drawback of using this technique as a specific PON detection tool. Most of the reactive oxygen-nitrogen species can also give fluorescence when reacting with dihydrorhodamine, such as 'OH, 'NO\textsubscript{2}, hypochlorous acid and carbonate radicals. The lack of required specificity makes these fluorescent probes unreliable to detect PON. Furthermore, it is an indirect method that relies on secondary species measurements instead of direct detection of PON.\textsuperscript{46-47}

New generation of fluorescent probes have been developed to react with peroxynitrite with enhancement in the fluorescence signal using boronate and boronic acid derivatives. Although they provide better ways in peroxynitrite detection, it is difficult to use them selectively as peroxynitrite probes, while they also give a fluorescent signal with hydrogen peroxide.\textsuperscript{48}
1.4.3. Electrochemical Sensors

Electrochemistry offers the possibility for real-time measurements of peroxynitrite. This advantage puts electrochemical sensors at the forefront of any direct methods. In addition, electrochemical techniques provide minimum reagent cost, high sensitivity, good selectivity, and calibration simplicity.

Several electrochemical sensors with interfaces, such as manganese phthalocyanine, cyanocobalamin and hemin, have been developed to detect peroxynitrite at the level of cells and in vitro. Amatore and co-workers have provided a method based on direct voltammetric determination of peroxynitrite using platinized carbon microelectrodes under physiological conditions. They measured the oxidation signature of one-electron transfer of peroxynitrite under simulated oxidative stress in a living cell. Bedioui’s group tested a chemically modified platinum ultramicroelectrode by manganese tetraaminophthalocyanine films for the detection of peroxynitrite in alkaline solution (pH=10.2). The sensitivity of the modified sensor was reported at 14.6 nA/µM, while the limit of detection was recorded at 5 µM. Later, Wang and Chen have established a novel method of modification the glassy carbon electrode by electropolymerized-cyanocobalamin in phosphate buffer solution at pH of 9.2.

In the same line of work, Bayachou et al. have showed a significant improvement in the detection of peroxynitrite using interfaces with electrodeposited hemin, and graphene-functionalized hemin, as well as poly (3,4-ethylenedioxythiophene)-hemin films on carbon electrodes in CAPS buffer.
1.5. Project Overview and Significance

The project aims to prepare nanocomposite materials for surface functionalization of electrode interfaces in order to catalytically detect PON. Example of catalytic surfaces include graphene nano-sheets, selenium containing compounds, and melanin films. Synthesis and preparation methods will be discussed in detail followed by characterization of these platforms. Characterization methods include scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), ultraviolet visible (UV/Vis) absorption measurements, and X-ray photoelectron spectrometry (XPS). In addition, the electrochemical performance of the modified sensors will be examined using cyclic voltammetry, dose-response amperometry, and electrochemical quartz crystal microbalance (QCM).

First, we test our bare electrodes with different aliquots of authentic peroxynitrite at pH = 9.5, and then compare this data with the modified electrodes by immobilizing the nanocomposite materials on the electrode surfaces using various techniques, such as drop-casting method, electrodeposition technique, electro-polymerization, and electro-grafting process. After verifying the oxidation profile of peroxynitrite on each interface using cyclic voltammetry, we move to amperometric techniques to monitor the current responses of the modified sensors in the presence of nanomolar concentrations of peroxynitrite. Finally, we compare the sensitivity and the detection limit of our modified sensors to the published data. Furthermore, the reproducibility will be examined for each catalytic interface.

Manganese is a stable metal that has several biological importance, manganese (II) ion serves as a cofactor in some enzymes with many functions. Enzymes containing manganese ions are essential in detoxification of reactive oxygen-nitrogen species, such as
superoxide and peroxynitrite. It also plays a significant role in the oxygen-evolving complex of photosynthetic plants. For example, superoxide dismutase (SOD) is a manganese containing enzyme that acts as antioxidant metalloprotein. As an antioxidant, it works to catalyze the dismutation reaction, which detoxify the reactive oxygen species like $O_2^-$. Here, we take advantage from using manganese oxide nanoparticles in combination with graphene/hemin to enhance the sensitivity of PON sensors.

Selenium is an important member of the chalcogen elements. The electrochemistry of Se finds wide applications in solar and fuel cells as well as in batteries and catalysis. Se-based compounds have been investigated as catalysts for oxygen reduction in fuel cells. Selenium is a catalytic cofactor of endogenous antioxidative systems. It is found in several human proteins (selenoproteins), which are involved in anti-oxidant defense mechanisms.\textsuperscript{58-62} Se plays a key role in redox regulation as a modulator of reactive oxygen species.\textsuperscript{60, 62} Recently, some synthetic selenorganic compounds have been prepared and used as PON scavengers.\textsuperscript{61-62} To the best of our knowledge, no attempt has been reported to quantify peroxynitrite on interfaces modified with selenium compounds.
16. References


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2.1. Introduction

Recent clinical research indicates that the cytotoxicity of peroxynitrite (ONOO\(^-\)) plays a crucial role in several cardiovascular dysfunctions and other diseases triggered by oxidative stress.\(^1\)-\(^8\) Peroxynitrite is a potent oxidative and/or nitrosative agent produced from the diffusion-controlled reaction between nitric oxide radical (\(^\cdot\)NO) and superoxide anion-radical (O\(_2^\cdot\)\(^-\)). It is a well-known member of the reactive oxygen-nitrogen species family.\(^9\)-\(^10\) This non-radical metabolite attacks vital components in cells and initiates deleterious effects via direct and indirect interactions. Peroxynitrite reacts directly with lipids, DNA, and proteins and indirectly triggers several radical chain reactions.\(^10\)-\(^11\) The elevation of peroxynitrite levels is associated with chronic inflammatory disorders including neurological and vascular diseases, as well as many other pathophysiological conditions.\(^3\)-\(^4, 10, 12-15\) Hence, the precise quantification of this analyte in biological systems is of paramount importance not only to understand the genesis and development of diseases at the tissue/cellular level, but also to assess potential therapies and understand its
associated reaction mechanisms. Although progress has been achieved in terms of peroxynitrite detection and quantification, the techniques of detection have not been applied efficiently. This deficiency is due to complicated pretreatment, low selectivity, as well as other technical problems.\textsuperscript{10, 16} Fluorescence, chemiluminescence, electron spin resonance, and immunohistochemistry methods are indirect since they rely on the measurement of secondary species derived from oxidation or nitration reactions mediated by peroxynitrite.\textsuperscript{17-19} Undoubtedly, these indirect methods are costly, laborious, and generally suffer from poor sensitivity and low selectivity. Thus, developing a sensitive, selective, fast-responding, and direct detection method is highly desirable. In this work, we explore an electrochemical method based on manganese-functionalized graphene/hemin modified glassy carbon electrodes as a platform for peroxynitrite detection and quantification.

Electrochemistry offers real-time measurements that put PON electrochemical sensors at the forefront. Several electrochemical sensors based on manganese phthalocyanine, cyanocobalamin, and hemin have been developed to detect peroxynitrite.\textsuperscript{17, 20-21} Amatore and co-workers used a platinized carbon microelectrode under physiological conditions.\textsuperscript{22} They were able to detect the oxidation signature of one-electron transfer of peroxynitrite under oxidative stress at the level of a living cell.\textsuperscript{22} Bedioui’s group reported on a platinum ultramicroelectrode electrochemically modified by manganese tetraamino-phthalocyanine films for the detection of peroxynitrite in alkaline solutions (pH=10.2).\textsuperscript{21, 23} The sensitivity of the modified sensor has shown 14.6 nA/\textmu M and the limit of detection recorded at 5 \textmu M. Later, Wang and Chen have prepared a new platform-modified glassy carbon electrode via electro-polymerized cyanocobalamin layers
to catalyze the oxidation of peroxynitrite using a voltammetric method in solution at pH of 9.2.\textsuperscript{17} Bayachou et al. used electro-polymerization of poly-3,4-ethylenedioxythiophene (PEDOT)-hemin film on carbon fiber electrodes as an interface to detect and measure PON in CAPS buffer with pH of 10.5.\textsuperscript{24} More recently, Peteu and his co-workers used hemin functionalized graphene for peroxynitrite detection. They reported on the environmentally friendly method of reducing graphene oxide by hemin molecules and made a sensitive hybrid interface for peroxynitrite quantification.

Graphene nanosheets have become a very attractive material due to its fascinating physical and electrochemical properties. Graphene is a single atomic layer of graphite arranged in a two-dimensional structure. It is one of the carbon allotropes that is built up of very tightly bonded carbon atoms organized into a hexagonal lattice structure.\textsuperscript{25-27} Furthermore, graphene is considered as the parent of all graphitic structures ranging from graphite to carbon nanotubes and fullerenes. Its sp\textsuperscript{2} hybridization coupled with its ultra-thin atomic thickness (0.345 nm) accounts for graphene’s unique properties and wide applications.\textsuperscript{28-40} Graphene has been proposed as a supporting material for numerous applications, such as drug delivery, catalysis, and sensor development. Metal-decorated graphene could improve the process of electron transport by minimizing the background current and enhancing electron flux for potential electrochemical interfaces. The graphene decoration, whether by metal doping or nonmetal functionality, makes graphene a good candidate for catalysis and sensor applications. Moreover, graphene-based materials generally provide features that lead to an enhancement of the catalytic activity and an improvement in the catalyst performance. For example, graphene provides a two-dimensional support structure with a large open and accessible surface area. Also, graphene
combines with substrates by either $\pi$-$\pi$ stacking or cation-$\pi$ interaction through catalytic metallic centers.\textsuperscript{38, 41}

Metalloporphyrins-modified glassy carbon electrodes (GCE) have shown significant electro-catalytic properties towards peroxynitrite detection, particularly hemin-modified GCE. Manganese phthalocyanine electrodeposited on platinum microelectrodes, also showed a significant increase in electrode sensitivity towards peroxynitrite. Both metalloporphyrins and phthalocyanine compounds have been used as electrochemical interfaces for peroxynitrite detection, and were used as such or in a combination with graphene nanostructures.\textsuperscript{18, 20, 42-46} The advantages of graphene metal nanostructures include a high surface area and tunable redox properties. Therefore, we explored if the combination of metal oxide-nanoparticles (MnO$_2$) and the two-dimensional graphene sheets will result in composite material that will allow for better catalytic detection of PON. The simplicity and low cost of the preparation of graphene-manganese nanostructured/hemin composite is an advantage.

In this work, graphene is used as a scaffold for peroxynitrite sensors modification. We decorated graphene sheets with needle-like MnO$_2$ nanostructures followed by hemin functionalization. These graphene-manganese nanostructures are prepared by a simple synthetic reaction, followed by hemin conjugation. Hemin is incorporated noncovalently into the synthesized matrix using $\pi$-$\pi$ interaction between the graphene-MnO$_2$ nanosheets and the porphyrin rings of hemin.

Manganese-functionalized graphene/hemin nanostructures were formed and cast on carbon electrodes. The morphology and composition of the composite materials were characterized using Scanning Electron Microscopy (SEM), Energy Dispersive X-ray
analysis (EDX), and X‐ray Photoelectron Spectroscopy (XPS). The electrochemical catalytic activity of the prepared interfaces was investigated by cyclic voltammetry and amperometry.

2.2. Experimental

2.2.1. Chemicals and reagents

Ferric protoporphyrin IX chloride (hemin) (~90%), synthetic graphite powder (<20 micron), sulfuric acid (95-98%), hydrogen peroxide (30%), dichloromethane, hexane, manganese (II) chloride tetrahydrate (99%), manganese (IV) oxide (99%), sodium nitrate (99%), sodium chloride (99%), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium permanganate, isopropanol, methanol, diethylene triamine pentaacetic acid (DTPA), ethanol, 3-morpholinosydnonimine hydrochloride (SIN-1) and isoamyl nitrite were purchased from Sigma‐Aldrich (St. Louis, MO) and used as such. Nano‐pure deionized water (specific resistance >18.2 MΩ cm) was supplied by a Barnstead water purification system model D8961. Indium Tin Oxide (ITO) coated glass slides were from Delta Technologies, Ltd. (resistivity Rs = 8 – 12 ohms). All other chemicals were reagent grade and used as received.

2.2.2. Instruments

The electrochemical analyses were performed using CHI‐1030B multi‐mode potentiostat system. Amperometry and cyclic voltammetry measurements were carried out using the above mentioned electrochemical workstation. Ag/AgCl is used as the reference electrode and all potentials reported here are measured versus this reference. A platinum
wire was used as the auxiliary electrode, and modified glassy carbon electrodes (GCEs) were used as working electrodes. UV-Visible absorbance spectra were recorded on Agilent 8453 spectrophotometer using 1-cm path length quartz cuvette. An Accumet AB15 pH-meter was used for pH measurements. SEM and EDX analyses were carried out using Hitachi S-4500 Field Emission Gun Scanning Electron Microscope (FESEM). X-Ray Photoelectron Spectroscopy (XPS) was performed using PHI Versaprobe 5000 Scanning spectrometer at a pressure ~10^{-9} millibar in the vacuum chamber. Monochromatic Al Kα X-ray beam was applied in the survey scans as well as in high-resolution analyses, with an estimated area of 0.35 mm$^2$. A pass energy of 93.9 eV was selected for sample surveys (0-800 eV), and 11.75 eV was chosen for high-resolution C1s (278-298 eV), whereas 23.5 eV was used for Fe 2p and Mn 2p (630-740 eV).

2.2.3. Synthesis and Preparation

2.2.3.1. Graphene Oxide Preparation

In this study, a modified method of Hummers and Offerman has been used to prepare graphite oxide.\textsuperscript{47} This method is based on oxidation of graphite flakes in strong acid. The concentrated sulfuric acid and potassium permanganate followed by the addition of hydrogen peroxide are the main reagents used to oxidize graphite. Briefly, 30 mL of concentrated sulfuric acid was added drop-wise to the solid mixture of 0.5 g of synthetic graphite powder and 0.3 g of sodium nitrate in a volume of 250 mL round flask at 0 °C. The second step consists of slowly adding 2 g of solid potassium permanganate to the reaction vessel, and the reaction mixture was continuously stirred for 1 hr. at 60 °C. Then, deionized water (130 mL) was gradually added to the mixture with continuous stirring for
0.5 hr. Finally, a solution of hydrogen peroxide (30%) was added to the reaction flask until the gas effervescence stops. The final product was centrifuged for 1 hr. to remove water and the excess of oxidizing reagents. The residue was then washed repeatedly with hydrochloric acid solution (5%) followed by washing several times using a deionized water and tested for sulfate ions using a reagent of BaCl₂ by seeing if BaSO₄ precipitates. After the washing solution gave a negative result for the presence of sulfate ions, the final product was dried overnight in an oven at 60°C to obtain the yellowish-brown solid of graphene oxide (GO).

2.2.3.2. Graphene Oxide-MnO₂/hemin Composite

The prepared graphene oxide (GO) was then functionalized with MnO₂ nanoparticles by adding manganese ions and performing further oxidation as reported by Wang.³⁰ Briefly, GO (0.066 g) and MnCl₂·4H₂O (0.27 g) were dispersed in isopropyl alcohol (50 mL), with ultra-sonication for 1 hr. The reaction mixture was heated to ~ 80°C in a water-cooled condenser with vigorous stirring. KMnO₄ (0.15 g) dissolved in 5 mL of deionized water was added rapidly to the above solution. After refluxing for 1 hr., the mixture was cooled to room temperature. Finally, the prepared composite material was centrifuged, washed, and then dried in air at 60 °C overnight. After preparing the functionalized graphene-MnO₂, hemin was incorporated by adding 5 mM solution of iron protoporphyrin IX in 5 mL methanol to 5 mg of graphene-MnO₂ dispersed in 5 mL of deionized water. The sonication was continued for another 5 hrs. followed by centrifugation, washing, and finally drying at 60 °C to obtain the final matrix of GO-
MnO$_2$/hemin. In terms of comparison, we also prepared rGO/hemin by the same method as reported in details in our previous work.$^{18}$

### 2.2.3.3. Peroxynitrite Synthesis and Generation

Peroxynitrite was synthesized as previously reported procedures of Uppu and Pryor with little modifications.$^{48-51}$ A two-phase displacement reaction occurred between isoamyl nitrite (organic phase) and hydroperoxide anion (aqueous phase). Hydrogen peroxide solution 30% (23 mL) in a round flask was chilled in an ice bath and a solution of sodium hydroxide (5 N, 40 mL) was added to the flask. Then, a solution of DTPA (0.04 M) in NaOH (0.05 N, 5 mL) was gently mixed with the reaction solution. The total volume of the buffered hydrogen peroxide was increased using deionized water to reach 100 mL to a 0.2 M final concentration. An equimolar concentration of isoamyl nitrite (0.2 M, 27 mL) was added to the reaction vessel and stirred vigorously for 6 hrs. in an ice bath. Peroxynitrite was produced in the aqueous layer, while the formation of isoamyl alcohol along with unreacted isoamyl nitrite remained in the organic layer. Peroxynitrite was easily separated and purified from the reaction vessel by washing thoroughly with dichloromethane, and then by passing through a column of manganese oxide to remove excess hydrogen peroxide. The concentration of peroxynitrite was determined using a UV/Vis at 302 nm and 1700 $\text{M}^{-1}\text{cm}^{-1}$ as the molar absorptivity. Finally, aliquots of peroxynitrite in 1 mL Eppendorf tubes were kept at −80 °C and used within 6 months from the time of preparation. Stability and concentration are always checked prior to each experiment.
3-Morpholinosydnonimine hydrochloride (SIN-1) was used as a peroxynitrite generator at physiological pH as reported in details in our earlier paper. A molar ratio of 1:100 for [PON]:[SIN-1] has been used in all SIN-1 experiments.

2.2.4. Electrode Modification

Glassy carbon electrodes (GCEs) were polished on alumina pastes (0.3 and 0.05 µm respectively) using a Buehler microcloth polishing disk, followed by sonication in a mixture of ethanol and acetone for 1 minute to remove any polishing residue, then flushed repeatedly with a deionized water. The electrodes are then modified by the drop-casting method. A suspension of GO-MnO₂/hemin or rGO/hemin composite was sonicated in methanol for 1hr and then cast on the surface of GCE. The electrode is left for about 30 minutes to dry. This process was repeated three times using a 10 µL aliquots each time. Finally, all modified electrodes were washed several times with a deionized water and phosphate buffer solution (PBS) pH=7.4 before use in the electrochemical experiments.

2.3. Results and Discussion

2.3.1. Preparation and Characterization

A simple and direct method has been developed to prepare the composite GO-MnO₂/hemin matrix. The formation process started by an acid oxidation of graphite flakes using Hummers’ method to form the GO sheets. The resultant GO was mixed with manganese chloride salt and dispersed in isopropyl alcohol. Ultrasonication of the whole mixture in isopropanol resulted in a homogenous dispersion cocktail. Wang and Zhu have discussed the formation mechanism of GO-MnO₂ nanocomposite and explained the role of
isopropanol-water system in the orientation of 1D growth. However, it has been found that using the minimum amount of water in the reaction vessel leads to good nucleation and nanocrystal growth. MnO$_2$ which is working as an exfoliating agent to exfoliate the GO sheets is more likely to do so in isopropyl alcohol than in isopropanol-water system. Further oxidation was allowed by adding KMnO$_4$ under reflux with stirring to obtain the composite GO-MnO$_2$. Hemin molecules were then introduced to allow π-π stacking interaction under ultrasonication for few hrs. to form the final matrix of GO-MnO$_2$/hemin. Scheme 2.1 illustrates the various preparation steps from graphite to the first composite (GO-MnO$_2$) followed by hemin addition to form the rGO/hemin and GO-MnO$_2$/hemin composite structures.

**Scheme 2.1** Preparation steps of GO, GO-MnO$_2$, rGO/hemin and GO-MnO$_2$/hemin nanostructured materials.
SEM analysis was used to study the change in morphology and surface features of the functionalized-graphene nanostructures. The SEM images of GO, rGO/hemin, GO-MnO₂, and GO-MnO₂/hemin are shown in Figure 2.1. The characteristic micrograph images illustrate the reaction steps of the final product GO-MnO₂/hemin nanocomposite. The solid powder of sample collected at different preparation steps were mounted on carbon tape for the SEM analyses. The SEM image (Figure 2.1 A) shows a random orientation of GO-curved nanolayers. The rGO/hemin image depicts a homogenous feature of the prepared composite confirming the intimate incorporation of graphene-hemin nanosheets (Figure 2.1 B). The SEM image (Figure 2.1 C) illustrates the needle-like structures of GO-MnO₂ nanomaterial with 100-200 nm length and 40-50 nm width, which was consistent with early reported data.²⁹⁻³⁰ The hetero-structure of GO-MnO₂/hemin nanocomposite enables the existence of both morphological characteristics of the GO-MnO₂ needle structures and rGO/hemin sheets (Figure 2.1 D).
Figure 2.1 SEM images of sample collected at different preparation steps of graphene nanostructures: (A) GO, (B) rGO/hemin, (C) GO-MnO$_2$, (D) GO-MnO$_2$/hemin.

We also performed EDX elemental analyses of GO, rGO/hemin, and GO-MnO$_2$/hemin, and the atomic weight ratios are listed in Table I. The carbon/oxygen (C/O) atomic ratio is crucial to determine the degree of graphene oxidation, and assess the nature of oxygen functionalities. It is known that the efficacy of the prepared graphene oxide can be estimated from C/O ratio, which lies between 2.1 to 2.9 atomic ratio for the high-quality GO.$^{47}$ Moreover, the color of the graphitic product when dispersed in deionized water can be used as an indicator of the degree of oxidation. A yellow-brown color means an effective graphene oxide product (sufficient oxygen moieties), whereas a black-brown color reflects
poor oxidation quality (insufficient oxygen moieties). In this work, a high quality of graphene oxide was successfully prepared, which has yellow-brown color and a 2.15 C/O atomic ratio. On the other side, the atomic weight ratio C/O of GO was increased from 2.15 to 2.8 after mixing and sonicating with hemin. This result suggests some degree of reduction on the graphene oxide sheets that reflects the role of hemin molecules as a reducing agent. This suggestion has been reported recently by our research group using the deconvolution of C1s high-resolution XPS.\textsuperscript{18, 53} Here, we report to reconfirm this result using the EDX data as a simple tool instead of XPS analysis. As shown in table I, the C/O atomic ratio increased to 2.8 suggesting the reduction of GO by hemin.

In contrast, the Mn-based matrix, GO-MnO\textsubscript{2}/hemin, has a lower value of C/O atomic ratio (less than 1%), indicating more oxygen in the final composite product. The functionality with MnO\textsubscript{2} nanoparticles obviously increases the number of oxygen atoms, which obscures the reduction role of hemin molecules compared to the GO-hemin case.

Figure 2.2 shows the difference between the EDX spectra of rGO/hemin and GO-MnO\textsubscript{2}/hemin. The peak of Fe increased from 1.75 to 2.87 atomic % weight. Also, a new peak of Mn appeared in the case of GO-MnO\textsubscript{2}/hemin. The data confirm the formation of GO-MnO\textsubscript{2}/hemin nanocomposite. The incorporation of manganese oxide nanoparticles apparently allows for more hemin combination as shown by the increase of the Fe peak in the EDX spectrum.\textsuperscript{30}
Figure 2.2 EDX analysis of rGO/hemin (A) and GO-MnO$_2$/hemin (B).

Table I. EDX Elemental Analysis.

<table>
<thead>
<tr>
<th>Atom Weight Percentage (%)</th>
<th>GO</th>
<th>rGO/hemin</th>
<th>GO-MnO$_2$/hemin</th>
</tr>
</thead>
<tbody>
<tr>
<td>C %</td>
<td>68.13</td>
<td>70.78</td>
<td>30.40</td>
</tr>
<tr>
<td>O %</td>
<td>31.70</td>
<td>25.32</td>
<td>41.73</td>
</tr>
<tr>
<td>Cl %</td>
<td>---</td>
<td>2.15</td>
<td>0.59</td>
</tr>
<tr>
<td>Fe %</td>
<td>---</td>
<td>1.75</td>
<td>2.87</td>
</tr>
<tr>
<td>Mn %</td>
<td>---</td>
<td>---</td>
<td>24.41</td>
</tr>
</tbody>
</table>

We carried out XPS analysis to characterize the modified surfaces, and to identify the chemical changes in the various composites. As shown in Figure 2.3, high-resolution spectra have been obtained for C1s, N1s, O1s, Fe2p, and Mn2p of the prepared composites rGO/hemin and GO-MnO$_2$/hemin. Initially, survey spectra were performed for the decorated graphene nanostructures on the ITO glass substrates after treatment using the
drop-casting method. In addition, a blank ITO slide was scanned using the XPS technique under the same conditions and subtracted from the analyzed samples to eliminate the ITO background contribution.

The deconvolution of the C1s spectrum has been used as an indicator to measure the degree of reduction that has occurred on the GO sheets by hemin molecules. Specifically, the three deconvoluted peaks of C1s at binding energies of 284.8 eV (C-C/C-H), 286.7 eV (C-OH/C-N), and 289.1 eV (C=O) are used to determine the chemical environment (state) of the decorated graphene-based structures. The reduction of GO by hemin conjugation results in significant decrease of the oxygen-bound carbons (C-OH/C=O), with expected increase in sp² carbon hybridization on the sp³ of the oxygen-carbon functionalities. This result is consistent with previous work, and confirms that the graphene oxide/hemin conjugation is in the reduced form as reduced-graphene oxide/hemin functionality (rGO/hemin).

The high-resolution XPS spectra of C1s and N1s have almost the same binding energies for rGO/hemin and GO-MnO₂/hemin at 283.8 and 397.6 eV, respectively, indicating similar chemical states for the both composites with respect to carbon and nitrogen contributions. The intensities of both C1s and N1s peaks of the rGO/hemin are higher than the GO-MnO₂/hemin nanocomposite due to the predominant graphene and protoporphyrin moieties compared to the manganese-oxide nanoparticles in the final matrix. Interestingly, the O1s peak of the GO-MnO₂/hemin matrix shows higher intensity and a slight chemical shift of −1.8 eV in binding energy from the corresponding rGO/hemin at 531.8 eV in binding energy. This negative shift suggests the presence of oxygen as C=O or HO-C=O moieties instead of C-OH chemical state. This conclusion is also supported
by the careful analysis of the C1 spectra of GO-MnO$_2$/hemin, which reveals small peaks at 288, 291, and 293.5 eV, assigned to C=O/HO-C=O groups and $\pi$-$\pi$ conjugation, respectively. The incorporation of MnO$_2$ nanoparticles seems to prevent hemin molecules from efficient reduction of GO sheets. This lower reduction results in more carboxylic moieties in the final composite matrix of GO-MnO$_2$/hemin. In addition, a significant decrease in the C/O ratio of GO-MnO$_2$/hemin has been observed from the EDX analysis, which is consistent with the XPS findings.

The incorporation of hemin in the prepared composites is of course confirmed by the presence of the iron peaks. Interestingly, the rGO/hemin and GO-MnO$_2$/hemin composites both have similar iron peak strengths and the same binding energy, which confirm the equal chemical state of iron in both composites. The spin-orbital coupling of Fe 2p$_{3/2}$ and Fe 2p$_{1/2}$ for both composites appears at binding energies of 712 and 725 eV that is consistent with the published data elsewhere.\textsuperscript{56} However, the EDX analysis recorded an increase in the iron peak of the final matrix, suggesting more hemin is adsorbed on the exfoliated graphene sheets by MnO$_2$ nanoparticles. The EDX info depth ($z$) is > 500 nm compared to only 1-5 nm surface penetration of XPS. The bulk sampling of EDX may explain why this method better reflects the expected higher Fe % in the GO-MnO$_2$/hemin composite.

The Mn2p spectrum in the final matrix of GO-MnO$_2$/hemin exhibits two peaks for 2p$_{3/2}$ and 2p$_{1/2}$ chemical states at 642 and 653 eV respectively, with binding energy difference ($\Delta$) of 11 eV as reported in the literature.\textsuperscript{57} Both Fe2p and Mn2p high-resolution spectra have shown a positive shift in their observed binding energies suggesting the formation of the target GO-MnO$_2$/hemin composite.
Figure 2.3 Survey and High-Resolution XPS analyses of rGO/hemin and GO-MnO$_2$/hemin.
We also used UV/Vis analysis to investigate the formation of GO-MnO$_2$ and GO-MnO$_2$/hemin nanocomposites as shown in Figure 2.4. In addition, UV/Vis absorption analysis of the initial components (starting materials) GO and hemin was performed and studied for comparison. A dispersed sample of GO and GO-MnO$_2$ nanomaterials in water were analyzed to monitor the composite formation and the observed change in GO.

As observed in Figure 2.4A, the UV/Vis spectrum of GO shows a maximum absorption at 228 nm, which is ascribed to the $\pi$-$\pi^*$ transitions of the carbon in GO layers. In addition, a small hump is observed at 298 nm that is attributed to n-$\pi^*$ transitions of carboxylic functionalities. Interestingly, the GO-MnO$_2$ nanocomposite exhibits also two predominant absorption peaks at 219 nm and 430 nm corresponding to GO ($\pi$-$\pi^*$ transitions) and MnO$_2$ (d-d transitions), respectively. The blue-shift of the graphene peak could be attributed to the exfoliation of graphene sheets by MnO$_2$ nanoparticles, whereas the peak corresponding to Mn appears red-shifted due to the growth of MnO$_2$ particles. In this regard, Wang and coworkers have reported on the correlation between the absorbance peak’s shift with the reaction time. They showed that increasing the time of reaction causes the peak of MnO$_2$ to gradually shift from 413 to 436 nm, while the GO peak consistently shifts from 230 to 216 nm.$^{30}$ It has been found that the GO sheets can be easily exfoliated during the nucleation growth of manganese oxide nanoparticles. Our UV/Vis spectra are also consistent with this finding and suggest a high degree of GO exfoliation after one hour of mixing with manganese salt and potassium permanganate in isopropanol.$^{30}$ It also confirms the successful preparation of the GO-MnO$_2$ composite.

Along the same line, we conducted UV/Vis analysis of GO, GO-MnO$_2$, hemin, and GO-MnO$_2$/hemin in methanol as shown in Figure 2.4B. The combination of the GO-MnO$_2$
with hemin molecules in GO-MnO$_2$/hemin reflects the features of all sub-components. The incorporation of hemin in the graphene-manganese composite is indicated by the appearance of Soret band around 400 nm, followed by a couple of weak peaks in between 420 to 640 nm related to the hemin Q-bands.\textsuperscript{18,41} The absorption characteristics of the Soret band of the GO-MnO$_2$/hemin nanocomposite and the hemin alone show approximately the same wavelength (~ 400 nm), suggesting the presence of the monomeric hemin structure in the final nanostructured matrix. The data reveal that the hemin rings were adsorbed as monomeric entities on the GO-MnO$_2$ substrate.

![Figure 2.4 UV/Vis spectra of (A) GO and GO-MnO$_2$ dispersed in water; (B) collection spectra of GO, hemin, GO-MnO$_2$ and GO-MnO$_2$/hemin composite in methanol.](image)

The Q-bands signature of hemin increased in the GO-MnO$_2$/hemin indicating more adsorbed hemin in the final composite. The d-d transition peak of MnO$_2$ was obscured by the intense hemin Soret-band in the final matrix nanocomposite; however, a careful observation still shows the broad shoulder at 430 nm. In conclusion, the overall matrix shows the general features of all matrix components confirming the formation of our target GO-MnO$_2$/hemin nanocomposite.
2.3.2. Electrochemical Measurements and Sensor Performance

The electrochemical performance of the rGO/hemin and GO-MnO$_2$/hemin-modified GCEs was examined side-by-side using cyclic voltammetry analysis at 0.1 V/sec scan rate in phosphate buffer (50 mM, pH=7.4). The brown-red of rGO/hemin and the brown-black of GO-MnO$_2$/hemin were drop-cast on the GCEs. The voltammetric responses in the presence of peroxynitrite (PON) are shown in Figure 2.5. In this case, we used SIN-1 as a peroxynitrite generator in-situ at physiological pH. The rGO/hemin voltammogram shows an oxidative peak response at ~1.1 V attributed to the catalytic oxidation of PON mediated by iron centers of adsorbed hemin. The mechanism of the catalytic activity of hemin-mediated peroxynitrite oxidation has been reported previously$^{18,45}$, and is shown in Scheme 2.2.

**Figure 2.5** Cyclic voltammetry responses of modified GCEs to 1.5 µM of PON released from 150µM of SIN-1 in PBS pH=7.4 at scan rate 0.1 v/sec: (A) Voltammogram of rGO/hemin modified GCE in the absence and presence of PON, (B) Voltammogram GO-MnO$_2$/hemin modified GCE in the absence and presence of PON.
The incorporation of MnO_2 in the graphene hemin composite shifts the peroxynitrite oxidation peak to lower positive potentials by about 200 mV. As a result, a well-defined peak for the catalytic oxidation appears at approximately 0.9 V as shown in Figure 2.5B. The shift of the catalytic peak to positive potentials seem to indicate that the incorporation of MnO_2 particles on GO/hemin nanocomposite helps lower the oxidation barrier of PON at the modified interface. Also, the added exfoliation of GO enabled by the needle-like MnO_2 nanoparticles allows for incorporating more hemin catalyst. Both effects result in higher current response at less positive potential.

Scheme 2.2 Proposed catalytic oxidation of peroxynitite on GO-MnO_2/hemin composite interface on GCE.

Amperometric measurements have been used to compare and contrast the electrocatalytic activity and the performance of the various composite materials. We have used aliquots of SIN-1 from a 1µM stock solution, which generates a10 nM PON for each addition. The amperometric response to added PON aliquots was measured after each injection. The applied potential of 1V was selected based on the cyclic voltammetry.
**Figure 2.6** (A) Typical amperometric responses of rGO/hemin and GO-MnO$_2$/hemin-modified GCEs to PON aliquots released from SIN-1 in PBS pH=7.4 at applied potential of 1V vs. Ag/AgCl; (B) The corresponding calibration curves of current versus PON concentration for rGO/hemin and GO-MnO$_2$/hemin.

Figure 2.6 shows the typical current responses associated with the addition of PON aliquots to the electrochemical cell at physiological pH. We observe a significant increase in the catalytic current of the GO-MnO$_2$/hemin compared to rGO/hemin-modified electrodes. A linear correlation between the measured currents and PON concentration was observed in the range of 2 to 10 nM for both systems. Linear regression equation shows a sensitivity of 23.7 nA/nM for GO-MnO$_2$/hemin as opposed to only ~ 10nA/nM for rGO/hemin. The detection limit of the GO-MnO$_2$/hemin-modified sensors was 2 nM with overall good sensor stability.

### 2.4. Conclusion

A simple preparation method has been applied to prepare hemin functionalized GO-MnO$_2$ nanocomposite. The newly prepared nanostructured showed higher catalytic activity for peroxynitrite detection. This work reported for the first time the effect of MnO$_2$
incorporation in GO/hemin interface on the electrocatalytic detection of PON. The incorporation of MnO$_2$ nanoparticles not only allows for more adsorbed hemin entities into the final composite matrix, but also seems to lower the oxidation barrier of PON. As a result, amperometric calibration curves of the GO-MnO$_2$/hemin-modified sensors demonstrated a significant increase in the sensitivity of peroxynitrite detection (23.7 nA/nM compared to 10.7 nA/nM of the rGO/hemin).
2.5. References


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CHAPTER III
ANILINE-SELENIDE GRAFTED ELECTRODE FOR SENSITIVE PEROXYNITRITE DETECTION

3.1. Introduction

Peroxynitrite (ONOO$^-$, PON) is a reactive oxygen-nitrogen species that has been linked with both oxidation and nitration reactions in biological systems. PON emerged as a major cytotoxic agent that is implicated in a host of pathophysiological conditions.$^{1-2}$ Reports emphasized the deleterious physiological reactivity of PON with different cellular targets including DNA, proteins, and lipids in cell membranes.$^{3-4}$ On the other side, some reports identified counterintuitive cytoprotective roles of PON under certain conditions.$^{4-5}$ The deleterious versus potential cytoprotective roles of PON depend on its dynamic concentration.$^{4-6}$ Accurate determination of PON concentration is inherently difficult due to its high reactivity and the low concentration levels in the relevant biological systems.$^4$ Various methods for PON determination have been reported including indirect spectroscopic assays and, recently, direct electrochemical methods.$^7-12$ Currently, this field is still expanding to find the viable electrochemical probes and catalytic interfaces that give
the highest sensitivity along with lowest limit of detection, and selectivity for PON-sensing.

Over the past two decades, several thousand peroxynitrite-related articles based on the data available in PubMed were published, particularly after the discovery of its crucial role in pathophysiology. Historically, peroxynitrite was first identified in 1904; however, the work done by Beckman et al. in 1990 is considered a milestone in bringing the attention back to this molecule by shedding the light on its various biological roles. The nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) system recommends the names of oxoperoxonitrate for peroxynitrite anion (ONOO\(^-\)) and hydrogen oxoperoxonitrate for peroxynitrous acid (ONOOH). In this study, we use the word of peroxynitrite for representing both the peroxynitrite ion and the peroxynitrous acid. Peroxynitrite is a strong oxidant, a potent nitrating agent, and one of the most noxious reactive oxygen-nitrogen species that has shown a significant impact in cell biology.

Recent clinical research has marked PON as a reactive metabolite associated with several homeostasis disruptions, such as arthritis, age-related dysfunctions, inflammation, apoptosis, carcinogenesis, stroke, Alzheimer’s, Huntington’s and Parkinson’s diseases, AIDS, acute ischemia-reperfusion injury, and heart failure. Biologically, PON is produced from the diffusion-controlled reaction between nitric oxide (NO) and superoxide (O\(_2^-\)) radicals. Despite the expanding body of work on PON, its true impact on biological systems in health and disease is still a controversial topic. PON attacks vital components inside the body and initiates deleterious effects via direct and indirect interactions. It reacts mainly with lipids and DNA through direct reaction, while it triggers radical chain reactions by indirect pathways through its known homolytic cleavage. In addition,
PON is involved in a side reaction with CO\textsubscript{2}, which inflicts further chemical damage.\textsuperscript{4} Elevation in peroxynitrite levels is associated with chronic inflammatory disorders including neurological and vascular diseases, as well as other pathophysiologic dysfunctions. Hence, the accurate detection of this analyte’s levels under physiological conditions and settings is of paramount importance, not only to determine and understand the genesis of the diseases, but also to develop effective therapy, and understand the pharmacological reaction mechanisms.

Selenium (Se) is considered as an essential micronutrient element due to its role in medicine and biology.\textsuperscript{23-24} Its properties are similar to sulfur (S) with some small differences in redox potentials and oxidation state stabilities.\textsuperscript{23, 25-26} These differences are in part due to the change of electronegativity of selenium vs. sulfur, which affect their oxidation potentials. Also, the selenium atom is more polarizable than the sulfur atom. These differences between Se and S are among some of the reasons why selenium compounds have distinct catalytic activity and other biological functionalities compared to their sulfur analogs at very low concentrations. In fact, selenium is the catalytic cofactor of important endogenous anti-oxidative systems in the human body, which attracted considerable attention in research.\textsuperscript{14, 23-26} It exists in several human proteins (selenoproteins), many of them involved in anti-oxidant defense systems.\textsuperscript{14, 26} Overall, it plays some roles in redox regulation and as a modulator of reactive oxygen species (ROS). Recently, several synthetic organoselenium compounds have been prepared as antioxidants in medicinal chemistry.\textsuperscript{26-31} Previous electrochemical data in our hands showed that some organoselenium compounds have specific redox activity with peroxynitrite in solution. We hypothesized that such selenium compounds may act as efficient catalytic entities for
mediated PON oxidation. One of these compounds is 4,4' diaminodiphenyl selenide, which offers the possibility to be electrochemically grafted on electrode surfaces. We have prepared selenium interfaces, characterized them, and evaluated their performance in catalytic oxidation of PON and, thus, as PON electrochemical sensors.

Grafting chemistry has been applied to immobilize organic layers on conductive surfaces such as carbon surfaces (graphite, graphene, carbon nanotubes, carbon fiber) and metals (Cu, Ni and Fe).\textsuperscript{32-33} Aryl diazonium salts are commonly used for the modification of such electrode surfaces. The diazotization of primary aromatic amines is the result of nitrosation by nitrous acid formed \textit{in situ} by the reaction of nitrite with added hydrochloric acid. As such, aromatic amines, such as aniline or \textit{p}-nitroaniline, can be quantitatively converted to the corresponding diazonium salts under the proper conditions. The electrochemical reduction of such salts displaces the stable byproduct N\textsubscript{2} and generates a radical that immediately attaches covalently to the conductive surfaces.\textsuperscript{34} Several methods have been established for surface grafting using diazonium salts, such as electrochemical reduction as well as other chemical and photochemical methods.\textsuperscript{35-37}

In this work, we used the chemistry of phenyl diazonium derivatives to immobilize 4,4' diaminodiphenyl selenide on the surfaces of graphite electrodes in order to use these modified surfaces as catalytic platforms for peroxynitrite detection and quantification. Cyclic voltammetry was used for electrochemical-grafting of the diaminodiphenyl selenium compound. 4,4' diaminodiphenyl selenide was grafted by two consecutive potential scans from + 0.8 to − 0.8 V. Aniline was also grafted on the electrode surface to use as a “blank” interface for performance comparison purposes. In this regard, the 4-nitrophenyl diazonium salt was used as the source of the electrografted aniline interface.
Two steps are required in this case: the first is the regular electrografting the 4-nitrophenyl after electrochemical reduction of the initial diazonium salt; and the second step is to reduce the terminal nitro group to the desirable amine functionality by scanning the modified electrode in 1 M sulfuric acid down to − 0.8 V.

We first report on the characterization of the prepared interfaces using various physicochemical methods including scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), and X-ray photoelectron spectroscopy (XPS). Then, we characterized the performance of the electro-grafted aniline selenide-modified electrodes as peroxynitrite sensing interfaces using voltammetry and dose-response amperometry. We also compared and contrasted the performance of closely related compounds in effort to highlight the crucial importance of the selenium atom in the grafted catalytic selenium compound. To the best of our knowledge, this is the first time a selenium-based compound electrochemically grafted at an electrode surface has been used for catalytic detection and quantification of peroxynitrite.

3.2. Experimental

3.2.1. Chemicals and Reagents

4-nitrobenzendiazonium tetrafluoroborate, acetonitrile, sulfuric acid, hydrogen peroxide (30%), dichloromethane, hexane, potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, manganese (IV) oxide, sodium nitrate, sodium nitrite, sodium hydroxide, aniline, ethanol, diethylene triamine pentaacetic acid (DTPA), tetrabutylammonium tetrafluoroborate (TBABF₄), 4,4' diaminodiphenyl sulfide, 3-morpholinosydnonimine hydrochloride (SIN-1), selenium powder, 4-iodoaniline, ethylene
diamine, Cu$_2$O, 4,4' diaminodiphenyl oxide, 4,4' diaminodiphenyl methane and isoamyl nitrite were purchased from Sigma-Aldrich (St. Louis, MO) and used as such. Nano-pure deionized water (specific resistance >18.2 MΩ cm) used throughout this work was supplied by a Barnstead water purification system model D8961. All other chemicals not listed above are reagent grade.

3.2.2. Instruments

The electrochemical analyses have been performed using CHI-1030B multi-mode potentiostat system. Amperometric measurements and cyclic voltammetry were carried out using the above mentioned electrochemical workstation. All potentials reported here are versus silver/silver chloride (Ag/AgCl) reference electrode. A platinum wire was used as the auxiliary electrode. The pyrolytic graphite or ITO electrodes (bare or modified) were used as the working electrode. UV-Visible absorbance spectra were recorded on an Agilent 8453 spectrophotometer using a 1-cm path length quartz cuvette. An Accumet AB15 pH-meter was used for pH measurements. Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray analysis (EDX) characterizations were carried out using a Hitachi S-4500 Field Emission Gun Scanning Electron Microscope (FESEM). X-Ray Photoelectron Spectroscopy (XPS) was performed using PHI Versaprobe 5000 Scanning Spectrometer at a pressure of $\sim 10^{-9}$ millibar in the vacuum chamber. Monochromatic Al Kα X-ray beam was used for survey and high-resolution analyses with estimated spot size area of 0.35 mm$^2$. A pass energy of 93.9 eV was selected for sample’s survey (0-800 eV) and 23.50 eV was chosen for high-resolution Se3d (50-70 eV) with 0.2 eV step size and 50ms/step for 30 times.
3.2.3. Synthesis and Preparation

3.2.3.1. Preparation of Peroxynitrite

Peroxynitrite was prepared according to previously reported procedures of Uppu and Pryor with small adjustments using a two-phase displacement reaction between isoamyl nitrite (organic phase) and hydroperoxide anion (aqueous phase).\textsuperscript{38-41} Briefly, hydrogen peroxide solution 30\% (23 mL) in a round flask was chilled in an ice bath. A solution of sodium hydroxide (5 N, 40 mL) was added to the flask. Then, a solution of DTPA (0.04 M) in NaOH (0.05 N, 5 mL) was gently mixed with the reaction solution. The total volume of the buffered hydrogen peroxide was increased to 100 mL using deionized water to a 0.2 M final concentration. Finally, an equimolar solution of isoamyl nitrite (0.2 M, 27 mL) was added to the reaction vessel and stirred vigorously for 5 hours in an ice bath. Peroxynitrite was produced in the aqueous layer, while the formation of isoamyl alcohol along with the unreacted isoamyl nitrite remained in the organic layer. Peroxynitrite was separated and purified from the reaction vessel by washing the aqueous phase repeatedly with dichloromethane. The resulting aqueous solution was then passed through a column of manganese oxide to remove excess hydrogen peroxide. The concentration of peroxynitrite was determined by UV/Vis spectrometry using the characteristic absorbance of PON at 302 nm with 1700 M\textsuperscript{−1}·cm\textsuperscript{−1} molar absorptivity. The purified solution of PON was separated into small aliquots that were kept at −80 °C in the freezer for later use. As needed, PON aliquots are thawed and kept on ice prior to use in experiments. It is worth noting that the concentration of peroxyntirite is checked by UV/Vis prior to each experiment.
3.2.3.2. Generation of Peroxynitrite

3-Morpholinosydnonimine hydrochloride (SIN-1) can also be used as a peroxynitrite donor. SIN-1 derives from the deacetylation of the known prodrug molsidomine. SIN-1 spontaneously decomposes to release 1:1 molar ratio of superoxide and nitric oxide. Under physiological pH, SIN-1 breaks down by hydrolysis and opens its oxadiazolium ring. As a result, the molecular oxygen is reduced to generate superoxide, while the oxidized form of oxadiazolium ring further releases one equivalent of nitric oxide. Subsequently, the rapid combination of superoxide and nitric oxide generates peroxynitrite in situ (Scheme 3.1). Practically, a stock solution of 5 mM of SIN-1 was prepared by dissolving 10 mg of SIN-1 in 10 mL of deoxygenated phosphate buffer solution pH=7.4 in air-tight sealed flask. Using an airtight syringe, the 10 mL SIN-1 stock solution was divided to 1 mL aliquots in sealed vials and stored at –80 °C for one-time use per experiment. The peroxynitrite concentration was obtained using to the known concentration ratio (1/100) for PON/SIN-1. Peroxynitrite was generated in situ in the electrochemical cell by adding a calculated aliquot from the SIN-1 (5 mM) stock solution to the oxygenated phosphate buffer solution pH= 7.4.

![Scheme 3.1 PON generation mechanism from SIN-1](image-url)
3.2.3.3. Synthesis of 4,4' Diaminodiphenyl Selenide

A 50 mL round bottom flask was charged with 4-iodoaniline (1.10 g, 5.00 mmol, 1.00 eq), Se (0.316 g, 4.00 mmol, 0.800 eq), Cu$_2$O (0.143 g, 1.00 mmol, 0.200 eq), ethylene diamine (0.060 g, 1.00 mmol, 0.200 eq), KOH (0.560 g, 10 mmol, 2.00 eq) and DMSO (10 mL) under nitrogen and magnetic stirring. The reaction mixture was heated at 120 °C in an oil bath for 20 hours. The mixture was allowed to cool to room temperature and then poured over dichloromethane (50 mL) and deionized water (50 mL). The organic layer was washed with deionized water (4 x 30 mL) and a brine solution (1 x 30 mL), dried with MgSO$_4$, and concentrated under vacuum. The crude product was purified by column chromatography on silica gel eluted with 1% triethanolamine (TEA) in 1:1 hexanes/ethyl acetate mixture. The yield in purified 4,4' diaminodiphenyl selenide was, 0.653 g, 62%; m.p. 115 °C (lit. 117 °C); $^1$H NMR (400 MHz, CDCl$_3$), δ (ppm): 7.31 (d, $J = 8.8$ Hz, 4H), 6.60 (d, $J = 8.8$ Hz, 4H). 3.69 (bs, 4H).

3.2.4. Electrode Modification

Pyrolytic graphite electrodes are first grinded on CarbiMet paper (grit 400/p800) and then polished on two different sizes of alumina pastes (0.3 and 0.05 µm, respectively) using a Buehler micro-cloth disk, followed by sonication in a mixture of ethanol and acetone for 2 minutes. The electrodes are then washed with deionized water. The polished electrodes were modified by electrografting using cyclic voltammetry. Briefly, a solution of 10 mM of 4,4' diaminodiphenyl selenide (or an equivalent substrate) was dissolved in a 5 mL of 0.5 M hydrochloric acid with stirring under a blanket of nitrogen gas; an equimolar solution of sodium nitrite (10 mM) was then added to the reaction compartment while the
temperature was kept at 0 °C for 5 minutes to generate the diazonium salt derivative of the selenium compound.

The aniline selenide compound was electrochemically grafted on the surface of pyrolytic graphite electrode by reducing the corresponding diazonium salt in situ. Two consecutives cyclic voltammograms are scanned in the potential window from +0.8 V to −0.8 V at 0.1 V/sec scan rate. For the comparison interface (without the Se atom), 10 mM solution of 4-nitrobenzendiazonium tetrafluoroborate was dissolved in degassed acetonitrile and further purged with nitrogen gas for 15 minutes. The diazonium salt is then electrochemically reduced and the nitrobenzene is grafted on corresponding electrode surface. The nitro group is then electrochemically reduced in 1M sulfuric acid by cycling the potential from +1 to −0.8 to form the “blank” aniline-grafted interface. Finally, all modified electrodes were washed several times with deionized water and phosphate buffer before use in electrochemical experiments to avoid artifacts from any non-covalently adsorbed contaminants.

3.3. Results and Discussion

The chemistry of selenium has grown dramatically in terms of its chemistry and biology. Selenium-containing compounds, compared to sulfur-containing analogs, have higher reaction rates with oxidants such as peroxynitrite. The behavior can be rationalized by the overall higher polarizability of Se and the resulting relative ease of oxidation. This can also explain the antioxidant effect of the selenium-containing compounds. Based on preliminary data of redox interaction of some aromatic selenium compounds with peroxynitrite, we prepared electrode interfaces grafted with a selenium-
based organic compound as a potential catalytic entity for mediated catalytic oxidation. In this work, we focused on 4,4’ dianimodiphenyl selenide.

The synthesized selenium compound was characterized using NMR, chromatography, and mass spectrometry to verify the structure and purity. The selenium containing-compound was then electro-grafted in situ by electrochemically reducing the corresponding diazonium salt formed in situ. Scheme 3.2 illustrates the grafting process. Stoichiometric amounts of sodium nitrite are first added to the solution of the synthesized selenium compound in 0.5 M hydrochloric acid. The formation of the diazonium salt in situ is directly followed by an electrochemical reduction using cyclic voltammetry. The potential window from +0.8 to −0.8 V is scanned over two consecutive cycles. The electrochemical reduction of the diazonium salt displaces the dinitrogen molecule and generates a reactive radical that covalently attaches to the graphite electrode surface.

Scheme 3.2 Electro-grafting of 4,4’ dianimodiphenyl selenide on graphite electrode via the electrochemical reduction of the corresponding diazonium salt generated in situ.

Similarly, 4-nitrobenzenediazonium tetrafluoroborate was electrografted on the electrode surface but in acetonitrile in the presence of 100 mM tetrabutylammonium tetrafluoroborate (TBABF₄) as a supporting electrolyte. The grafted p-nitrophenyl was then reduced in a second step by scanning the electrode potential to −0.8 V in 1 M H₂SO₄ (scheme 3.3).
Scheme 3.3 Electro-grafting of 4-nitrobenzendiazonium tetra fluoroborate followed by electrochemical reduction to form aniline using cyclic voltammetry.

3.3.1. Surface Characterization

The ITO electrodes were modified using the same procedures of electrochemical grafting for aniline and aniline selenide compounds on pyrolytic graphite electrodes that were mentioned in the experimental section. Figure 3.1 illustrates the electrochemical grafting of aniline selenide compound and of aniline using their respective diazonium salts on the ITO electrodes. The major reduction peak in the first cycle of each case corresponds to the reduction of the diazonium salt and subsequent electrografting of the organic moiety on the electrode surface.\textsuperscript{32,45}
Figure 3.1 Cyclic voltammograms (two consecutive cycles: black is first cycle and red trace is the second cycle) on ITO electrodes in the presence of the diazonium salt of (A) 10 mM of 4,4’ diaminodiphenyl selenide generated in situ in aqueous medium as described in the experimental section, and in (B) the presence of 10 mM of 4-nitrobenzendiazonium tetrafluoroborate in acetonitrile.

Figure 3.2 shows the SEM morphology of the grafted selenium compound on an ITO electrode. The SEM micrographs show the difference between bare ITO vs. the ITO modified with the grafted selenium compound. Figure 3.2 also represents the elemental analysis of the bare and modified ITO slides using EDX analysis. The black trace shows the signature peaks of selenium, which confirms the grafting of the electrode interface with the aniline-Se compound. The underlying ITO surface of the electrode itself gives rise to peaks of various elements (O, Na, Mg, Al, Sn, and In) (see Red EDX trace in Figure 3.2). The chlorine peak observed in the modified ITO slide is probably due to the use of hydrochloric acid. When these background peaks are subtracted from the EDX trace of the ITO electrode modified with the aniline selenide compound, we notice net increases in the peaks of oxygen and nitrogen along with the appearance of a new peak of carbon. Together,
the appearance of the selenium signature peaks along with the increase in oxygen and nitrogen peaks and the appearance of carbon peak indicate the covalent attachment of the target organoselenide compound on electrode surface.

![EDX analysis and SEM images of the bare ITO slide (red) and the grafted aniline-selenide (black) on ITO electrodes.](image)

**Figure 3.2** EDX analysis and SEM images of the bare ITO slide (red) and the grafted aniline-selenide (black) on ITO electrodes.

We also conducted XPS surface characterizations. The survey of the bare ITO substrate was performed first, and was subtracted from the survey trace of the ITO electrode modified with aniline selenide compound. Figure 3.3 shows the resulting XPS survey trace of the modified electrode and the high-resolution analysis around the characteristic 3d$_{5/2}$ Se signature peak. From XPS peak analysis, we found that the carbon-to-nitrogen atomic ratio is about 12:1, consistent with the expected ratio of carbon-to-nitrogen atomic ratio in the molecular structure of the grafted-aniline-Se. The XPS analysis also shows the peaks of selenium in the region from 200 to 50 eV binding energy. The
main characteristic peak of selenium is known as 3d$_{5/2}$ that appears at a binding energy of 56 eV. It is worth noting that there is no evidence for the selenium oxide $^{46-49}$ peak as shown in Figure 3.3B, which gives the possibility to monitor if a selenium oxide is involved in the interaction of the grafted organoselenide compound with the oxidant PON.

**Figure 3.3** XPS analysis of aniline-Se grafted on ITO slide; (A) XPS survey and (B) high-resolution XPS of 3d$_{5/2}$ Se signature peak.

3.3.2. Electrochemical Analysis of Se-modified Electrode and Performance as PON Sensor

3.3.2.1. Interface Characterization

Figure 3.4A shows the cyclic voltammograms of 10 mM 4-nitrophenyl diazonium tetrafluoroborate in acetonitrile on pyrolytic graphite electrode. The voltammogram features two major reduction peaks on the first cycle that correspond to the reductive grafting of 4-nitrophenyl diazonium. Evidence for grafting is indicated by the absence of cathodic current in the second and subsequent cycles. This is consistent with previous reports on the same system.$^{32}$
Figure 3.4 Cyclic voltammetry of (A) 10 mM 4,4’ diaminodiphenyl selenide after reaction with equimolar amount of sodium nitrite in 0.5 M HCl and (B) 10 mM 4-nitrophenyl diazonium tetrafluoroborate in acetonitrile and 100 mM TBABF$_4$ as a supporting electrolyte. Both cyclic voltammograms are performed on pyrolytic graphite electrodes at a scan rate of 100 mV/s.

Figure 3.4B shows the voltammograms of the electrochemical reduction of the diazonium salt of 4,4’ diaminodiphenyl selenide compound. The diazonium derivative is formed in situ using a stoichiometric molar ratio of 4,4’ diaminodiphenyl selenide and NaNO$_2$ in 5 mL of 0.5 M HCl. This step results in the replacement of one of the two terminal amine functionalities of the organoselenium compound to diazonium salt (ph-N≡N$^+$Cl$^-$). Electrochemical reduction of the diazonium salt at the electrode surface in cyclic voltammetry in the potential window between +0.8 to −0.8 V vs. Ag/AgCl ultimately results in grafting of the selenium compound on the electrode surface. The mechanism of reductive grafting of organic diazonium salts on electrode surfaces is well established in the literature.$^{35-36, 50-53}$ However, the observed cyclic voltammetry behavior of this electrochemical grafting exhibits a slightly unusual first cycle compared to known
reductions of aryldiazonium salts. In fact, starting from the from initial potential at +0.8 V vs. Ag/AgCl, the first cycle scan shows an early reductive peak around +0.5 V, followed by another irreversible cathodic peak around −0.4 V. The reverse scan towards positive potentials during the first cycle shows only one anodic wave around +0.58 V, which seems to be coupled to the first reduction process at +0.5V. In fact, in the second cycle and subsequent cycles, the combined anodic and cathodic peaks show the signature of a quasi-reversible redox couple with a mid-potential around +0.54 V. The irreversible cathodic peak at −0.4 V in the first cycle disappears in the following cycles. This peak is typical of the one-electron reduction of the aryl diazonium salts that results in irreversible grafting of the aryl moiety. Upon reduction of the aniline-selenide diazonium derivative at the electrode surface along with the concerted departure of dinitrogen, a reactive radical covalently attaches to the graphite surface. In the second cycle, a significant decrease in current is observed, which is expected since the grafted aniline-selenide moieties block further electron transfers at the electrode interface. The modified electrode surface does not prevent the persistence of the redox couple around +0.54 V. However, the grafted layer probably contributes to the resistance to electron transfer through it, which may explain the observed quasi-reversible redox couple at +0.54 V ($\Delta E_p=80$ mV). The assignment of the quasi-reversible couple at positive potentials is not clear, particularly since its reductive component at +0.5V is observed even before the aniline-selenide diazonium compound is irreversibly grafted on the electrode surface. The presence of reduction peak at +0.5 V in the first forward cycle may be the signature of an existing layer that results from a spontaneous grafting process in open circuit potential prior to potential scanning. Such process is well established and has been reported in related systems. While the
spontaneous grafting can be triggered by a number of processes, available sources of reducing equivalents in solution seem to encourage this route.\textsuperscript{55} Also, the nature of the diazonium salt and the substituents it bears may be a factor. In this regard, the known electron-rich diaryl selenide, particularly with the amino substituents in this case, is itself likely to act as a source of reducing equivalents in open circuit potential.\textsuperscript{57} The early oxidation peak around +0.58 V is unlikely due to the terminal amino groups oxidation of the grafted aniline-selenide. Rather, the quasi-reversible couple may be the signature of redox activity of possibly more complex adducts such as the azo-products that result from the condensation of the original diazonium ion on previously –and spontaneously– grafted aryl-selenides moieties.\textsuperscript{51} Such non-radical process, despite the low electrophilicity of diazonium ions, may be enabled by the amino substituents as well as the electron-rich selenium atom of the grafted aniline-selenide.

![Cyclic voltammery of aniline-modified pyrolytic graphite electrodes](image)

**Figure 3.5** Cyclic voltammetry of aniline-modified pyrolytic graphite electrodes prepared by the reduction of surface grafted 4-nitrophenyl groups on the electrodes in 1 M H\textsubscript{2}SO\textsubscript{4} with a scan rate of 100 mV/s (A) at the potential window from +1.0 to −0.7 V, and (B) at the potential window from +1.0 to −0.2 V.
Figure 3.5 shows the behavior of the pyrolytic graphite electrode modified with grafted 4-nitrophenyl diazonium compound as described in the previous section. It is worth noting that the surface-grafted 4-nitrophenyl moieties, in acidic medium, can be electrochemically reduced to hydroxylamine and to amine, Scheme 3.4.\textsuperscript{53, 58}

\begin{equation}
\text{Ph-NO}_2 + 2H^+ + 2e^- \rightarrow \text{Ph-NO} + H_2O \quad (1)
\end{equation}

\begin{equation}
\text{Ph-NO} + 2H^+ + 2e^- \rightarrow \text{Ph-NHOH} \quad (2)
\end{equation}

\begin{equation}
\text{Ph-NHOH} + 2H^+ + 2e^- \rightarrow \text{Ph-NH}_2 + H_2O \quad (3)
\end{equation}

**Scheme 3.4** The electrochemical reduction steps of surface grafted 4-nitrophenyl molecules to amine functionality using cyclic voltammetry.

Figure 3.5A, in the first forward scan, shows only one irreversible reduction process around −0.6 V. The irreversible peak corresponds to the reduction of the 4-nitrophenyl group to the hydroxylamine and amine forms. The reverse scan, on the other hand, shows an anodic peak around 0.37, which appears to be part of a reversible redox couple assigned to the nitroso/hydroxylamine form of the grafted moieties as reported in previous reports (see Scheme 3.4, Equation 2).\textsuperscript{53, 58} The fact that we observe a small but measurable current for the reversible redox around +0.35 V that corresponds to the reoxidation of hydroxylamine groups shows that the original nitrophenyl functionalities are not completely converted to amines.\textsuperscript{33, 54}
Figure 3.5B shows that the reversible wave with the mid-potential around +0.35V corresponding to the redox couple 4-nitroso/4-hydroxylamine moieties is very stable when the potential window is restricted to this process after the initial reduction of the grafted nitro-groups, consistent with literature reports.\textsuperscript{54}

3.3.2.2. Sensor Performance

Now that the two sets of modified electrodes are prepared and characterized, we address in this section the performance of the grafted amino-selenide compound as a sensor for PON detection and quantitation. To this end, we use cyclic voltammetry and amperometry to study the electrochemical response of the interface upon addition of PON aliquots. We selected the potential window of 0 to +1 V to study the catalytic oxidation of PON on the modified electrodes. Figure 3.6A shows the electrode response in cyclic voltammetry of the grafted aniline-selenide in response of added PON aliquots in the 0-100 µM concentration range. This figure shows that the observed oxidation current increases with the increased PON concentration. In contrast, a similar electrode modified with the aniline electrode shows very weak current responses in the same PON concentrations range. The relatively mute response of the interface modified with aniline compared to the response of the interface with grafted aniline-selenide highlights the critical role of the selenium sites in the mediated oxidation of PON. The significant current response of aniline-selenide electrodes upon PON addition can be rationalized with an electrocatalytic process involving the PON analyte and the oxidized form of the aniline-selenide moieties as outlined in Scheme 3.5. It is conceivable to have a more favorable oxidation of the electron-rich selenium atoms of the grafted molecules.\textsuperscript{57} The direct
chemical interaction of PON with the oxidized chalcogen atom is expected to result in a favorable inner-sphere interaction that would ultimately result in the oxidation of PON and regeneration of the selenium catalytic site.

Scheme 3.5 Postulated mechanism for the electrocatalytic oxidation of peroxynitrite by the aniline-selenide grafted interface.
Figure 3.6 Typical voltammograms of modified pyrolytic graphite electrodes at a scan rate of 100 mV/s with added aliquots of peroxynitrite in pH 9.2 to (A) Aniline-selenide grafted electrode and (B) Aniline-modified electrode.

In effort to highlight the critical importance of the Se atom in the grafted interface in supporting the mediated catalytic oxidation of PON, we substituted the aniline-selenide moieties with equivalent molecules in which the selenium atom is swapped for other centers for including oxygen, sulfur, and methylene, Scheme 3.6.

Scheme 3.6 Alternate compounds used to test the role of Se in the grafted sensing interface
We used the same diazonium grafting method described for the aniline selenide compound. Figure 3.7 shows the cyclic voltammograms observed for the grafting of the 3 alternate compounds.

**Figure 3.7** Voltammograms recorded in electro-grafting using (A) 10 mM 4,4’ diaminodiphenyl methane, (B) 4,4’ diaminodiphenyl oxide, and (C) 4,4’ diaminodiphenyl sulfide with equimolar amount of sodium nitrite in 0.5 M HCl on pyrolytic graphite electrodes at a scan rate of 100 mV/s.

The relative dose responses of the sensing interfaces with the three alternate compounds and with the aniline-selenide is shown in Figure 3.8 for amperometry. Of all
compounds, the grafted aniline-selenide shows by far the highest catalytic current. This result highlights the critical importance of the Se center in the proposed mediated catalytic sensing of PON.

![Amperometric responses of aniline-selenide-modified pyrolytic graphite electrode and of electrodes with other alternate grafting compounds upon addition 1 µM PON aliquots (from SIN-1) at an applied potential of +1.0 V.](image)

**Figure 3.8** Amperometric responses of aniline-selenide-modified pyrolytic graphite electrode and of electrodes with other alternate grafting compounds upon addition 1 µM PON aliquots (from SIN-1) at an applied potential of +1.0 V.

We then examined the performance of the aniline-selenide grafted electrodes in sensing PON in dose-response amperometry. We measured current response of the selenide-based sensing interface at an applied potential of +1.0 V. Figure 3.9A shows the expected increasing current responses upon gradual increase of PON analyte concentration.
In contrast, the aniline-grafted control interface shows very minimal current response. The corresponding calibration curves for the selenide-based sensing interface and the control are shown in Figure 3.9B. We show that the aniline-selenide electrode provides a sensitivity of 200 nA/µM, which is at least 40 times that of the selenide-devoid control sensor. Also, the observed limit of detection with this selenide interface is calculated to be ~ 250 nM.

**Figure 3.9** (A) Typical amperometric responses of the selenide-based sensor and the control electrode upon stepwise increase of PON concentration at an applied potential of 1.0V in phosphate solution pH 9.2 (B) The corresponding calibration curves.

### 3.3.3. Interferents

We tested the selectivity of the aniline-selenide sensing electrode for PON over related biological interferents. We compared the relative responses of the sensor for PON and related analytes including nitrate (NO$_3^-$), nitrite (NO$_2^-$), and nitric oxide (˙NO). Because we wanted to compare the relative responses at physiological pH we also used
here the PON generator SIN-1, which, as we explained in the background section, is known to release set amounts of PON.\textsuperscript{42-43}

Figure 3.10 is a bar graph comparing sensor responses for PON and other interferents. The bar graph shows that although the concentration of the interfering analytes is 100-fold higher than PON (100 µM interferent versus 1 µM PON), the response of the selenide-based sensor for PON is still significantly higher than responses for all other analytes. Together, these results show that the grafted aniline-selenide sensor is more significantly more sensitive for PON compared to the other biologically related species.

\textbf{Figure 3.10} Bar graph comparing sensor responses of 1µM PON to 100-fold of its most interfering species.
3.4. Conclusion

We have prepared pyrolytic graphite electrodes decorated with a diaryl selenide derivative (4-aminophenyl-selenide) using diazonium chemistry. We have also characterized the modified electrode electrochemically as well as spectroscopically. We show that grafting the selenide derivative on graphite transforms the electrode surface to a very sensitive sensor for peroxynitrite. The electrochemical detection of PON on the selenide-grafted interface takes place through an electrocatalytic process where the selenium center plays a crucial role in PON mediated oxidation. In fact, we show that swapping the Se moiety with other molecules containing other related heteroatoms (S, O) or simply a methylene unit does not reproduce the very sensitive response observe on the aniline-selenide derivative. Finally, we show that the electrocatalytic detection of PON on the selenium-modified graphite electrodes seems to respond better and selectively toward PON as a target analyte. In fact, we show that the electrochemical response for PON is significantly higher compared to responses recorded for other related interferents such as nitric oxide, nitrite, and nitrate.
3.5. References


54. Ortiz, B.; Saby, C.; Champagne, G.; Bélanger, D., Electrochemical modification of a carbon electrode using aromatic diazonium salts. 2. Electrochemistry of 4-nitrophenyl


CHAPTER IV
CONCLUSION & FUTURE DIRECTIONS

We introduced a method to prepare graphene oxide that is decorated with manganese oxide particles (GO-MnO₂) and functionalized with hemin as a catalyst platform for peroxynitrite detection and quantification. We have characterized the morphology as well as the composition of this nanostructured matrix. We showed that this prepared nanostructured matrix exhibits catalytic properties towards peroxynitrite’s mediated oxidation. This study shows for the first time the rational design of a new electrochemical sensing composite platform based on catalytic properties of individual components that work in synergy to enhance the overall sensor performance. The combination of MnO₂ nano needles into graphene sheets functionalized with hemin resulted in enhanced catalytic oxidation of PON and therefore better sensor performance. Not only the oxidation potential of peroxynitrite was decreased by about 200 mV, but the related catalytic current also approximately doubled compared to the previous rGO/hemin interface. The resulting sensor sensitivity of the combined matrix of GO-MnO₂/hemin reached 23.7 nA/nM with detection limit down to 2 nM.
Another bottom-up rational design of a catalytic interface for PON detection centered around known properties of selenium compounds and their reactivity with PON. In this regard, we synthesized an aniline-selenide compound, 4,4’-diaminophenyl selenide, which we grafted onto graphite electrodes through the electrochemical reduction of its diazonium salt. The selenide modified electrochemical sensor interface was then tested for the detection and quantification of peroxynitrite in solutions. Interestingly, the graphite electrodes grafted with the aniline-selenide catalyst showed an excellent response towards peroxynitrite detection through mediated oxidation. The selenium-modified electrodes also showed great selectivity for PON compared to related interferents such as nitric oxide, nitrite, PON’s isomer, nitrate. Swapping the selenium with other elements in essentially similar structures that were also grafted on electrodes showed the critical importance of the selenium atom in the mediated oxidation catalytic process. Overall, we showed that the grafting of aniline-selenide compound on a graphite surface significantly increased the electrode sensitivity and improved its selectivity towards peroxynitrite quantification both at alkaline as well as physiological pHs.

One of the major challenges in the analytical quantification of peroxynitrite under physiologic conditions is its short half-life due to the fast reactivity with many cellular targets. Spectroscopic and other detection methods for peroxynitrite have been proposed, but a major challenge is the problem of real-time detection, particularly when localized measurements are needed. As shown in Table II, the electrochemical catalytic detection of peroxynitrite is growing as a feasible and more convenient for real-time quantification of this analyte. In the past, we described the use of polymerized hemin and other porphyrins on graphite electrodes as platforms for amperometric measurement of peroxynitrite both in
standing solutions and under flow conditions. This work has been extended to reduced graphene oxide functionalized with hemin as a catalytic platform for PON oxidative detection. In addition, we have shown that strategies using grafted selenide compounds or graphene decorated with manganese nanoparticles both enhance the sensitivity of the resulting peroxynitrite sensors. In the future, and as an extension of the selenium-based grafted PON sensing interfaces, we will develop and test catalytic interfaces modified with 3,4-ethylenedioxyxselenophene (EDOS).\textsuperscript{1-2} It is the analogue of 3,4-ethylenedioxythiophene (EDOT) that can also be electropolymerized on electrode surfaces. The polymerized EDOS has higher conductivity and with the unique interaction of selenium with PON is expected to result in a very sensitive interface for PON detection.

BDD electrodes have the advantage to provide a relatively wide electrochemical potential window as well as low capacitive currents. Moreover, diamond has some unique properties such as high thermal conductivity, high mechanical stability, and high electrochemical activity, which results in improve the signal to noise ratio and subsequently lower the limit of detection. Recently we have presented some work on the potential of using modified boron-doped diamond electrodes as very sensitive and robust interfaces for PON detection and quantification.\textsuperscript{3} In the future work, we will apply our catalytic nanomaterials on BDD to compare and contrast the performance of the new sensors particularly in terms of lowering the limit of detection. Boron-doped diamond based microarray electrodes have been proposed as potential devices for neuronal stimulation. Recent studies suggest that the generated PON at the array-tissue interface is a major cytotoxic intermediate leading to ultimate tissue death. Developing an electrode based on the catalytic detection of PON on boron-doped diamond electrodes is a step towards using...
individually addressable electrodes in a BDD electrode array for both electrical stimulation and monitoring of PON levels as a predictor of tissue deterioration and, ultimately, tissue death. Understanding the fundamental processes involved during the catalytic oxidation of PON on modified BDD offers the possibility to fine-tune the surface to optimize PON detection.

Table II. Electrochemical modification of PON sensors vs. our work.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Modifier</th>
<th>Source of PON</th>
<th>Buffer</th>
<th>pH</th>
<th>PON Oxidation Potential (V)</th>
<th>Sensitivity</th>
<th>LOD</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>Carbon fiber</td>
<td>Platinum</td>
<td>Single cell</td>
<td>PBS</td>
<td>10.5</td>
<td>0.29 vs. SCE 0.304 Ag/AgCl</td>
<td>-----</td>
<td>-----</td>
<td>Amatore⁴ 2001</td>
</tr>
<tr>
<td>Glassy carbon</td>
<td>Cyano-cobalamin</td>
<td>PON</td>
<td>PBS</td>
<td>9.2</td>
<td>0.836 vs. SCE 0.85 Ag/AgCl</td>
<td>-----</td>
<td>-----</td>
<td>Wang⁵ 2010</td>
</tr>
<tr>
<td>Carbon fiber</td>
<td>Hemin-PEDOT</td>
<td>PON</td>
<td>CAPS</td>
<td>10.5</td>
<td>0.75 vs. Ag/AgCl</td>
<td>13 nA/µM</td>
<td>0.2 µM</td>
<td>Bayachou⁶ 2010</td>
</tr>
<tr>
<td>Glassy carbon</td>
<td>Hemin</td>
<td>PON</td>
<td>CAPS</td>
<td>10.5</td>
<td>1.1 vs. Ag/AgCl</td>
<td>70 nA/µM</td>
<td>5 µM</td>
<td>Bayachou⁶ 2013</td>
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<tr>
<td>Glassy carbon</td>
<td>rGO/hemin</td>
<td>SIN-1</td>
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<td>1.1 vs. Ag/AgCl</td>
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<td>5 nM</td>
<td>Peteu⁷ 2013</td>
</tr>
<tr>
<td>Glassy carbon</td>
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<td>SIN-1</td>
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<td>2 nM</td>
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<tr>
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<td>PON</td>
<td>PBS</td>
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<td>1 vs. Ag/AgCl</td>
<td>200 nA/µM</td>
<td>0.2 µM</td>
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4.1. References


