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NEW GENERATION OF ELECTROCHEMICAL SENSORS FOR NITRIC OXIDE: RUTHENIUM/CARBON-BASED NANOSTRUCTURES AND COLLOIDS AS ELECTROCATALYTIC PLATFORMS

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DEDICATION

This dissertation is dedicated to

My son Haridu, wife, Reshani and for my Parents
NEW GENERATION OF ELECTROCHEMICAL SENSORS FOR NITRIC OXIDE: RUTHENIUM/CARBON-BASED NANOSTRUCTURES AND COLLOIDS AS ELECTROCATALYTIC PLATFORMS

W. PUBUDU M. PEIRIS

ABSTRACT

Nitric oxide (NO) is an important intercellular messenger that acts in many tissues to regulate a diverse range of physiological and pathological processes. The physiologically implications of NO function are far from being completely understood. The multifaceted reactivity of NO prompted the need for accurate determination of the concentration of this molecule. However, it is difficult to detect nitric oxide, particularly in biological media and near live cells due to its short half-life, a result of its reactivity and the low levels of NO produced in vivo. As a result, the accurate and reliable detection of NO under varying experimental conditions has always posed a challenging task. The main goal was to develop ultra-sensitive electrocatalytic sensors for accurate quantification of NO. We report the fabrication and characterization of improved NO sensors based on electrocatalytic platforms such as ruthenium (colloids, nanoparticles, and nanotubes) and carbon (pastes and nanotubes), acting as catalytic sites for NO oxidation. These sensors are characterized using various surface analytical tools. The electrocatalytic oxidation of NO is assessed by cyclic voltammetry and amperometry both in solution phase and gas phase. Excellent sensitivity and linearity are observed for
our modified electrodes towards NO quantification. Our new NO detection sensors also show superior limit of detection and selectivity against common interference species. Our NO sensors are tested for various applications including in the measurement of NO released from human umbilical vein endothelial cells (HUVECs).
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CHAPTER I

STATE OF THE ART: NITRIC OXIDE SENSORS FOR ULTRA-SENSITIVE AND SELECTIVE NO DETECTION

1.1 Introduction

Nitric oxide (NO) is one of the simplest and smallest, yet one of the most biologically important molecules in nature. The importance of nitric oxide research was realized when NO was announced as the molecule of the year in 1992 by Science Magazine [1]. The abundance of research into this area further grew with the creation of a professional society and a scientific journal devoted entirely to nitric oxide. The attention that this small molecule drew culminated in 1998 when the Nobel Prize in Medicine was awarded to Furchgott, Ignarro, and Murad for their discovery of NO as a signaling molecule in the cardiovascular system [2]. NO is an intra- and extracellular messenger that mediates diverse signaling pathways in target cells and is known to play an important role in many physiological processes [3-9].
Currently, over 3,000 scientific articles about the biological roles of nitric oxide are published yearly. Now NO is widely accepted as a multifunctional signaling molecule, and it is a major player in controlling a myriad of cellular functions in the body. Its catalytic biosynthesis is complex and it is mediated by a class of enzymes called nitric oxide synthases (NOSs) [2, 4].

Due to its various physiological roles in regulating diverse cellular functions, imbalance of NO production and use is known to the development of many different pathophysiological states. A deficiency in NO production has been associated with several vascular diseases including hypertension, arterosclerosis, ischemia/reperfusion, stroke, and myocardial infarction [4, 10-12]. Overproduction of NO is a contributor to hypotension, septic shock, arthritis, reperfusion injury and cancer [13, 14]. More importantly, a small deviation from the normal physiological NO concentration may have a significant effect on the function of the cardiovascular system [10, 13-15]. Thus, from a biochemical and medical perspective, tools that offer the possibility to quantify the details of NO production in real-time under normal and pathological conditions are of paramount analytical importance.

Measurement of NO in biological media is very difficult due to its low concentration and short half-life. Since it is a free radical, it reacts extremely fast with superoxide and other reactive radicals, moderately fast with transition metals ions, and is also reactive with molecular oxygen [16, 17]. Therefore, a very sensitive and selective method has to be used to accurately detect NO in biological systems. Of the many
methods that have been proposed for the analysis of NO [18-29], electrochemical techniques are the most promising methods because they can be applied for real-time *in vivo* and *in vitro* measurements with appropriate spatial and temporal resolution [18, 28, 29].

However, direct electro-oxidation of NO does not produce the necessary degree of sensitivity, and their selectivity is poor at bare, nondiscriminatory electrode surfaces. A large body of literature has demonstrated that NO electro-oxidation is surface–dependent and can be catalyzed using a supported catalyst and/or defects on the electrode surface [30-33]. Also, currently available modified methods to measure NO in real time are relatively complex and their sensitivity and selectivity have not yet been fully addressed. Thus, improvements in NO sensors are still needed.

Our main focus in this study is to develop ultra-sensitive electrocatalytic sensors for accurate quantification of NO. After fabricating different types of highly sensitive and selective NO sensors, we will examine their performance in determining real time NO concentration *in vitro* and *in vivo*. 
1.2 Physical and Chemical Properties of NO

Nitric oxide is a heteronuclear diatomic molecule formed from the simple combination of nitrogen and oxygen. It is one of the smallest molecules present in nature, first discovered by Joseph Priestley in 1772 [34]. NO is a colorless gas at standard temperature and pressure with a boiling point of -151.7 °C and a melting point at -163.6 °C. Gaseous NO is the most thermally stable oxide of nitrogen and is also the simplest known paramagnetic molecule, as depicted in the following Lewis structure.

\[
\cdot\text{N}==\text{O}:
\]

NO contains eleven valence electrons and it has an electronic configuration of \((\sigma_{2s})^2 (\sigma_{2s}^*)^2 (\pi_{2p})^4 (\pi_{2p}^*)^1\) as represented in Figure 1.1. The paramagnetic property of NO is due to its unpaired electron in the \(\pi_{2p}^*\) orbital, which essentially represents the highest occupied molecular orbital (HOMO). This unpaired electron weakens the overall bonding of the nitrogen and oxygen resulting in a net bond order of 2.5, which is responsible for the unique properties of NO. The bond distance reflects this, as the bond length for NO is 1.10 Å, which is intermediate between the average double bond length of 1.15 Å and the triple bond length of 1.05 Å [35]. Bond energy for NO is 149.9 kcal/mol.
Figure 1.1. Molecular orbital diagrams for NO in its ground state.
Due to its small dipole moment (0.7 D), NO is essentially hydrophobic and freely diffuses through lipophilic biological membranes. The diffusion coefficients of NO in aqueous solution and under physiological conditions at 37 °C are $4.8 \times 10^{-5}$ cm$^2$s$^{-1}$ and $3.3 \times 10^{-5}$ cm$^2$s$^{-1}$, respectively [36]. Due to its hydrophobic nature, NO exhibits a low level of solubility in water (~1.9 mmol/l at 22 °C), and is approximately nine times more soluble in hydrophobic solvents such as hexane [4, 36]. NO can rapidly and directly react with the unpaired electron on other free radicals to yield a variety of highly reactive intermediates because of its thermodynamic instability [4, 37]. However, despite this thermodynamic instability, its decomposition is kinetically delayed; thus NO gas can be stored at 1 atm pressure at ordinary temperatures without detectable decomposition. In addition, NO can function as a ligand in a variety of metal complexes (vid
er infra).

NO may be oxidized by extracting one electron to yield the nitrosonium ion (NO$^+$) (no unpaired electron in its $\pi^*$ orbital and isoelectronic with carbon monoxide) or reduced by one electron to form the nitroxyl anion (NO$^-$) (contains 2 electrons in the $\pi^*$ orbital). Both nitrosonium and nitroxyl ions are important intermediates in the chemistry of NO (equations 1.1 and 1.2) [38].

\[
\begin{align*}
\text{NO} & \rightarrow \text{NO}^+ + e^- & E^o = +1.2 \text{ V (vs NHE)} \quad (1.1) \\
\text{NO} + e^- & \rightarrow \text{NO}^- & E^o = -0.33 \text{ V (vs NHE)} \quad (1.2)
\end{align*}
\]
1.3 Nitric Oxide in Biological Systems

Nitric oxide is released by many cells in mammalian systems [2-5, 9, 11, 40-42]. NO is an important signaling molecule that acts in many organs to regulate a range of physiological processes. Since NO is such a small and hydrophobic molecule, it can diffuse rapidly through the cytosol and across cell membranes without the need of receptors or channels and, depending on the conditions, is able to diffuse distances of more than several hundred microns. NO is a highly reactive free radical, and in biological systems it is considerably less stable with a half-life of less than 10 seconds [43]; this means that NO is quickly consumed close to the point where it is synthesized.

A short half-life and high affinity, especially for transition metals, are also important aspects that make NO an ideal signaling molecule. The reaction of NO with some transition metal complexes results in the formation of metal-nitrosyl adducts. The vast majority of these reactions \textit{in vivo} are with iron-containing metalloproteins. The reaction between NO and the ferrous iron in heme cofactors within these proteins are important in the regulation of guanylate cyclase activity [2]. NO has been shown to bind to the heme moiety of this protein, thereby stimulating the conversion of guanosine triphosphate GTP) to cyclic guanosine monophosphate (cGMP), which leads to many of its biological effects [4].
NO reacts with oxy-hemoglobin (HbO₂) or oxy-myoglobin to form nitrate and met-hemoglobin (MetHb) or met-myoglobin (equation 1.3) [4]. This concentration-dependent reaction has been proposed as a key mechanism to control NO concentrations in vivo. Therefore, the specificity of NO towards transition metals allows target tissue to receive the information based on NO concentration.

\[
\text{Hb(Fe-O₂)} + \text{NO} \rightarrow \text{MetHb(Fe(III))} + \text{NO}_3^- \quad (1.3)
\]

### 1.3.1 Biosynthesis of Nitric Oxide

Endogenous NO is generated from L-arginine by a specific enzyme family, nitric oxide synthases (NOSs) [40]. The regulation of NOS enzymes is complex and requires cofactors such as heme, tetrahydrobiopterin, calmodulin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) as well as L-arginine, O₂, and NADPH. As described in Scheme 1.1, in this two-step process, one of the guanidino nitrogen atoms of L-arginine undergo a five-electron oxidation to yield NO via an enzyme bound intermediate N⁰-Hydroxy-L-arginine. The overall reaction requires 1.5 equivalents of NADPH and 2 equivalents O₂ [44].
There are three types of NOS enzymes: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) [40]. Two isoforms, nNOS and eNOS, are Ca$^{2+}$-dependent. Chemical agonists such as bradykinin and ATP stimulate synthesis of small quantities of NO from these two types of NOS by stimulating Ca$^{2+}$ flux through the cell membrane. In contrast, iNOS is not calcium dependent and is not expressed under normal conditions, it is rather stimulated by endotoxins or cytokines. However, its activity, like all of the NOS isoforms, is still dependent on the binding of calmodulin [4].

Increases in cellular calcium levels increase the binding of calmodulin to eNOS and nNOS. This leads to a transient increase in NO production by these isoforms. By
contrast, iNOS is able to bind very tightly to calmodulin even at very low cellular concentrations of calcium [42, 45], and is therefore not depend on calcium concentration. Consequently, iNOS activity is not very much affected by changes in calcium levels in the cell. As a result, iNOS can produce NO at high nanomolar concentration levels for long periods of time. However, production of NO by nNOS and eNOS occurs at low levels (pico to nanomolar) for only short periods of time, and only after the appropriate stimulus [40, 46].

1.4 Health Relevance

NO is an important regulatory molecule, which affects a number of important physiological processes; it has thus been implicated in a variety of diseases (pathology of NO, Table 1.1) [47]. Now it is widely accepted as a major mediator in the cardiovascular, nervous, and immune systems. NO is used as a signaling molecule in the cardiovascular system and in the nervous system (brain, spinal cord) [9, 10]. NO can be produced in large amounts by white blood cells, helping to fight infections by killing bacteria and parasites[8]. NO controls our blood pressure, giving more blood when needed, and reducing blood flow when the body is at rest [39]. NO is known to affect memory processes and related diseases [9].
Table 1.1. Pathologic effects of NO

<table>
<thead>
<tr>
<th>NO concentration</th>
<th>Too Low</th>
<th>Too High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseases</td>
<td>Acute hypertension</td>
<td>Hypotension</td>
</tr>
<tr>
<td></td>
<td>Artherosclerosis</td>
<td>Septic shock</td>
</tr>
<tr>
<td></td>
<td>Diabetes,</td>
<td>Vascular leakage</td>
</tr>
<tr>
<td></td>
<td>Ischemia (stroke, heart attack)</td>
<td>Meningitis</td>
</tr>
<tr>
<td></td>
<td>Parkinson’s disease</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td>Alzheimer’s dementia</td>
<td>Post-ischemic brain and reperfusion injury</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>Cancer</td>
</tr>
<tr>
<td></td>
<td>Myocardial infarction</td>
<td></td>
</tr>
</tbody>
</table>

NO has been assigned both protective and deleterious properties in biological systems. The correct amount of NO production is essential for optimal function; too much or too little can be harmful and even deadly. In most life-threatening diseases like hypertension, atherosclerosis, ischemia and diabetes, the net concentration of NO is lower than under normal conditions [4, 10-12]. The low production of NO in an atherosclerotic cardiovascular system leads to vasoconstriction, increased platelets aggregation and thrombus formation, which are prime causes of heart attacks [12, 47].

High concentrations of NO can be problematic, as well. Consistently high concentrations of NO in the heart combined with stress can lead to heart attack [10, 13].
It can also lead to septic shock, where the immune system, attempting to fight infection, releases so much NO that the system goes out of control [8, 48]. High concentrations of NO can also dramatically decrease blood pressure, which may be followed by failure of vital organs, especially the liver, kidney and heart. A massive release of NO is observed during heart attacks, brain strokes, and any kind of condition that limits the supply of blood and O$_2$ (ischemia) to an organ [4].

Moreover, the simultaneous release of both NO and superoxide anion (O$_2^-$) results in reactive nitrogen oxide species (RNS) such as peroxynitrite, which may lead to serious, sometime irreversible chemical damage, especially in the brain and the heart [4]. Both acute and chronic neurological diseases, ranging from stroke, epilepsy, head trauma, Parkinson’s disease, and Alzheimer’s dementia are implicated due to subsequent generation of RNS and are involved in clinical conditions such as hypercholesterolemia, diabetes, pulmonary and coronary artery disease [4, 15, 48-54]. Elevated concentrations of NO, at least 100-fold higher than regular NO generated from vascular endothelial cells, can affect the function of iron-sulfur containing proteins. NO is vital to the nonspecific immune defense system, showing significant cytostatic and cytotoxic effects on parasitic microorganism and tumor cells [55]. NO plays both protective and destructive roles in a given cell milieu during cancer development [56, 57].

The nanomolar concentrations of NO generated by endothelial NOS, protect against the insurgence of pathologies and higher concentrations of NO (micromolar) generated from inducible NOS promote the previously described pathologies either directly or via the formation of reactive nitrogen species. Thus, from a biochemical as
well as medical perspective, it is important to quantify the details of NO production in real-time under normal and pathological conditions.

1.5 Current Nitric Oxide Measurement Techniques, Critical Evaluations of Existing Knowledge and Importance of the Research

To aid understanding of the important physiological and pathological roles of nitric oxide, sensitive and selective methods for its in vivo and in vitro detection are vital. The detection of NO in living tissues is challenging due to the low nanomolar concentrations found in tissues [4, 58], and because NO is highly reactive and unstable under normal physiological conditions. For these reasons it has been necessary to develop tools for the detection of NO that are very sensitive and specific to this reactive species. Several techniques have been developed for the measurement of NO, as briefly reviewed below.

1.5.1 Non-Electrochemical Methods for Nitric Oxide Detection

Various non-electrochemical methods have been reported, but most are indirect strategies based on detection of a secondary species such as nitrite, nitrate (both NO oxidation products) [22, 23] or L-citrulline (a coproduct of NO synthesis) [21]. The hemoglobin-dioxy assay [24] in another indirect method takes advantage of the Soret band shift that occurs when the hemoglobin-dioxy species reacts with NO to form methemoglobin and nitrate.
The most common procedure to detect NO is using the Griess reagent [59]. It is based on indirect measurements using nitrite and nitrate ions. Nitrate is first reduced to nitrite [60] before carrying out the Griess assay. The assay is a two-step diazotization reaction in which $\text{NO}_2^-$ in the sample reacts with sulfanilamide to produce a diazonium intermediate, which is then coupled to N-(naphthyl) ethylenediamine to form the final chromophoric azo-derivative [61]. The Griess reaction is simple with good inter-assay reproducibility, but it has a poor detection limit for $\text{NO}_2^-$, between 0.1-1 μM [62]. The lack of sensitivity restricts the application of this colorimetric method for quantifying nanomolar levels of $\text{NO}_2^-$ and $\text{NO}_3^-$ in biological samples [63]. Also, this type of assay is not applicable for real-time monitoring of NO-concentration in biological systems.

Scheme 1.2. Chemical reactions involved in the measurement of $\text{NO}_2^-$ using the Griess Reagent System.
Non-electrochemical methods that are more direct include chemiluminescence, mass spectroscopy, Raman spectroscopy, electron paramagnetic resonance (EPR) spectroscopy, and gas chromatography [19, 20, 25, 26].

The chemiluminescence technique [25, 64] is based on the reaction of NO with ozone (O₃) to produce nitrogen dioxide in the excited state, reaction 1.4 and 1.5. The reaction with O₃ is known for its chemiluminescent property that produces a deep red glow. This emission spans the range of 600-3000 nm [64].

\[
\begin{align*}
\text{NO} + \text{O}_3 & \rightarrow \text{NO}_2^* + \text{O}_2 \quad (1.4) \\
\text{NO}_2^* & \rightarrow \text{NO}_2 + \text{hv} \quad (1.5)
\end{align*}
\]

Detection of the luminescence reaction in a dark environment can therefore provide a sensitive way to resolve trace amounts of NO as they react with an external source of ozone. The chemiluminescent detection has been applied to measuring NO in exhaled breath [65-67]. A limiting factor for the use of this technique is the necessity for real-time measurements of NO. Gas phase chemiluminescence detection offers very good sensitivity and selectivity but it has some drawbacks such as bulky instrumentation, amount of time required, cost, and the need for multiple reagents.
Fluorometric techniques are very useful in bio-imaging of NO [61, 68]. The batch fluorometric assay is based on the reaction of NO with 2,3-diaminonaphthalene as an indicator of NO formation; the assay is conducted under acidic conditions to yield the fluorescent product 2,3-naphthotriazole [68]. The major drawbacks are reagent preparation, time consumption, and lack of selectivity at low pH due to the interference of nitrite.

Electron paramagnetic resonance (EPR) spectroscopy provides a specific tool to monitor molecules with unpaired electrons. Relaxation from field-induced excited states produces characteristic spectra. Even though NO is a paramagnetic molecule with the unpaired electron in the $\pi_{2p}^*$ orbital, NO and NO$_2$ cannot be studied by simple EPR, as the relaxation time of the stimulated electron to ground state is too rapid to be detected [69]. The unpaired electron of NO can only be detected by EPR spectroscopy after reaction with a spin-trap compound that reacts with the radical to form a more stable paramagnetic species. For example, heme-containing proteins, particularly hemoglobin, can be used as a spin trap for NO; the resulting stable nitrosyl adduct can then be detected [70, 71].

EPR offers the advantage of continuous real-time monitoring of NO with a detection limit of 0.5 $\mu$M, but it has a few drawbacks including the need for specialized operators, cost, size, and interference from many redox species.
1.5.2 Electrochemical Methods for NO Detection

Electrochemical methods offer a number of advantages that are not available with non-electrochemical techniques, especially the capability of measuring NO concentrations in vivo. Using small electrodes, we create the possibility of performing direct, continuous, accurate detection of NO. In addition, electrochemistry is the most suitable method for measuring NO in biological samples due to minimal reagent requirements, long-term stability, good selectivity towards NO, simplicity of calibration, and low cost.

Amperometric and voltammetric methods are the most commonly used techniques for detection of NO. In principle, these systems consist of a sensing/working electrode, counter electrode, and a reference electrode immersed in a liquid electrolyte. As a result of applying a suitable potential between the working and reference electrode, the current flowing through the sensor is proportional to the concentration of NO in the medium [20, 72]. The redox chemistry of NO allows its detection by either oxidative or reductive processes. There are two approaches for electrochemical measurements of NO, using direct oxidation/reduction, and using catalytic oxidation/reduction.

1.5.2.1 Direct Electrooxidative Detection of NO

The first electrochemical approach based on the direct oxidation of NO on a platinum electrode was reported by Shibuki [73, 74], using a miniature Clark-type electrode (oxygen electrode). The probe uses platinum as a working electrode and a silver
wire serves as both the counter and reference electrode. The electrodes are inserted in a capillary tube filled with a sodium chloride/hydrochloric acid solution separated from the analyte by a chloroprene gas-permeable membrane, Figure 1.2. A current generated from the electrochemical oxidation of NO on the platinum surface is measured after application of a constant potential of 0.9 V vs. Ag wire.

The sensitivity of this type of sensor shows a wide variation between probes that is largely attributed to inconsistencies in rubber membrane thickness, permeability, elasticity, and other physical variations that are difficult to control. Due to the time needed for NO to penetrate the rubber membrane, the response time of these sensors is slow and the fluctuations of the background signals make this Clark-type NO sensors useless for most *in vivo* applications.
Figure 1.2. Schematic drawing of the Clark type NO microsensor developed by Shibuki (adapted from[74]).

Shibuki’s prototype allowed World Precision Instruments, Inc. Sarasota, FL, USA (WPI) [29] to develop a commercially available NO sensor, the ISO-NOP. The ISO-NOP sensor consists of a platinum disk working electrode and an Ag/AgCl reference electrode. Both electrodes are encased within a protective shielded stainless steel sleeve that contains an electrolyte solution. The tip of the sleeve is further covered with an NO-selective membrane. The basic design of this type of NO sensor is illustrated in Figure 1.3. Because of the delicate nature of their design, WPI’s ISO-NOP sensors require extreme precautions when using in order to ensure their proper use. Besides the fragility
of the NO-selective coating membranes, the major limitation of these sensors is related to difficulties involving their calibration.

**Figure 1.3.** Schematic drawing of the WPI ISO-NOP-sensors NO developed by WPI, Inc. (Adapted from [29]).

Several NO-electrochemical micro-sensors have been developed on the basis of this pseudo-type Clark’s electrode by using carbon, glassy carbon, platinum or gold electrode covered by selected types of membranes [75-80]. Pt/Ir alloy electrodes modified by nitrocellulose and silicon have also been used for the detection of NO generated from aortic rings [81]. Pt electrodes modified with different selective membranes such as polystyrene [82], carbon fiber electrodes modified with cellulose [76], o-phenylenediamine [77], nafion® and cellulose acetate [78] have also been used as NO sensors.
Of all these sensors, electrodes coated with polymeric ion exchangers are the most useful for this purpose. In particular, the use of nafion® (perfluorinated sulfonic acid ionomer), due to its impermeability to anions, has been successfully developed to provide a sufficient barrier for common anionic interfering analytes, especially nitrite or ascorbate. However, the transport properties of neutral and cationic substrates across the nafion® coating, which are affected by the film morphology, permeability, and thermal treatment, have to be well understood to better evaluate the performances of such recast ionomer films [18]. In addition, membrane modified electrodes are limited by low sensitivity, slow response time, and high operating potentials. Moreover, there are some variations between sensors of the same kind due to differences in membrane thickness, which is difficult to control. In addition, the current response of these electrodes are temperature dependent [20].

1.5.2.2 Catalytic Electrooxidative Detection of NO

Sensitivity and selectivity of NO electrochemical determination can be achieved through electrode surface modification using electrocatalytic platforms with high affinity for NO. Indeed, various publications have demonstrated that NO electro-oxidation is surface-dependent and can be catalyzed using a surface defects or supported catalysts [30-33]. This approach to NO detection is performed by coating an electrode surface with specific, catalytically active, compounds, mostly based on metal complexes. These metal catalysts list also includes metalloporphyrins such as nickel(II)meso-tetrakis(3-methoxy-
4-hydroxyphenyl) porphyrin (Ni-THMPP),[30, 83] manganese porphyrins,[84] and iron porphyrins[85, 86].

Malinski and Taha reported in 1992 on the very first application of an electropolymerized nickel porphyrin film electrode for *in situ* amperometric detection of nitric oxide in biological systems [30]. The authors coated carbon fibers (0.5-1mm length; 0.8-30 µm diameter) with thin polymeric nickel porphyrin layers and Nafion® to minimize anionic interference. Figure 1.4 shows a schematic drawing of the sensor, which can be operated in either the amperometric or voltammetric mode. Although these electrodes were used successfully in several applications [87-89], subsequent studies have revealed that carbon fibers modified using metal porphyrin without the coordinated metal can also detect NO with the same sensitivity [77, 80, 90].

![Figure 1.4. Schematic drawing of the nickel porphyrin / Nation modified NO sensor (Adapted from [30]).](image)
Other types of NO sensors have been reported using metal phthalocyanins [18], metal shiff bases [91], and/or inorganic modifiers (organometallic compounds with Ni, Fe, Mn, Co and Cu centers [18, 28, 29, 92, 93]). Most of the modification procedures are carried out by electrochemical polymerization of related monomers. The sensitivity and selectivity of these NO sensors vary significantly from electrode to electrode and depend not only on the potential at which NO oxidizes, but also on the surface effects, axial ligation to the central metal, the modification/treatment procedure, and other experimental variations.

Other reported coatings include multiple membranes [94], heat-denatured cytochrome C [95], Nafion®-CoII-1,10-phenanthroline [96], and ferrioxamine [97]. Meyerhoff’s group described an improved planar amperometric NO sensor based on a platinized anode [32, 98] and its application for measurement of NO release from NO-donors. Scheler, et al. explored using myoglobin-clay modified electrodes for NO detection [99].

Unfortunately, despite the various approaches used in the previous years, none of the sensors have stood the test of time, due mostly to various practical difficulties and/or poor sensitivity/selectivity [28, 29]. Furthermore, the usual lack of data of these sensors potentially in biological systems limits conclusions on the potential of their widespread use. In addition, a major drawback of some of these methods is the incorporation of inaccessible mediators that require synthesis as well as complex modification procedures.
Therefore, investigation of suitable electrocatalysts that improve sensitivity and selectivity towards NO is still much needed.

1.6 Electrocatalytic Properties of Transition Metals Towards NO as an Analyte

Nitrosonium ion (NO⁺) is isoelectronic with carbon monoxide (CO), therefore many transition metals that form carbonyl complexes are also capable of forming isostructural or isoelectronic nitrosyl complexes [100]. However, differing from carbonyl complexes, there are two principal binding modes in nitrosyl complexes, linear M-N-O groups, and bent M-N-O groups [4]. Figure 1.5 represents the two different geometries of M-NO complexes.

Linear geometry is the most common and, in this situation, the net bonding interaction between the metal and NO consists of both σ donation from NO to the metal and π backbonding from occupied d-orbitals of the metal to the π* antibonding orbital on NO [101]. In the bent geometry, the metal can be envisioned to donate an electron to NO, which then binds the metal in a σ interaction. This leaves an electron pair localized in an sp² orbital on the nitrogen atom of the ligand. As a result, short M-N bond length and high NO vibration frequencies (> 1700 cm⁻¹) are best represented by a nitrosyl complex (M (n-1)+ NO⁺) with a linear geometry (M=N=O). A longer M-N bond and lower frequencies (1525-1590 cm⁻¹) usually characterize a metal to NO electron transfer (M (n+1)+ NO⁻) and a bent geometry [101, 102].
Due to this important interaction between transition metal and NO, a variety of metal complexes as electrocatalysts and their coordination chemistry have been examined extensively [30, 83-85]. In fact, several organometallic compounds with transition metal Ni, Fe, Mn, Co and Cu centers have been employed as electrocatalytic platforms for NO quantification [18, 28, 29, 92, 93]. However, the mode of interaction of NO with the transition metal complexes varies, involving different orbitals, which could affect the catalytic activity of a particular catalyst. NO reacts with the metal center of the complex via the nitrogen atom, forming a bond that strongly depends on the nature of the metal and its oxidation state.

**Figure 1.5.** Geometry and hybridization of N in M-NO complexes.
1.6.1 Transition Metal Ruthenium as Electrocatalyst for NO Quantification

Ruthenium (Ru), a rare transition metal of the platinum group, first discovered by the Russian scientist Karl Klaus in 1844 [103], and its oxides are extensively used as catalysts in various applications, especially in fuel cells. The oxidation states of ruthenium range from -2 to +8, and oxidation states of +2, +3, and +4 are the most common ones. Due to the unique combination of chemical and physical characteristics, such as metallic conductivity, high chemical and thermal stability, high specific capacitance, and electrochemical redox properties, ruthenium oxides demonstrate great promise as electrocatalysts [104, 105].

The association reactions of Mn, Fe, and Ru with NO have been investigated and they exhibit little or no activation barriers [106]. The relative reactivity of these transition metal atoms towards NO falls in the order Ru > Fe > Mn, indicating that Ru has higher affinity for NO compared to other metals [106]. The interaction of Ru and NO is well known in organometallic chemistry. Ruthenium forms more nitrosyl complexes than any other metal [100]. Ru(III) complexes react rapidly with NO to form six coordinate Ru(II) mononitrosyl containing a linear Ru-NO bond, an example is given in Figure 1.6. The Ru-NO bond is very stable, and able to resist a variety of redox and substitution reactions, and consequently the nitrosyl moiety is not easily displaced [104, 107].
Nitrosyl derivatives of ruthenium are well known intermediates in redox catalysis [107, 108]. The Ru-NO bond is extremely stable, persisting through a variety of both redox and substitution reactions. For example, polyamino-carboxylate-ruthenium complexes such as potassium chloro[hydrogen (ethylenedinitrilo)tetraacetato] ruthenate (complex I) with NO in aqueous solution involves formation of the aqua derivative, aqua[hydrogen (ethylenedinitrilo)tetraacetato]ruthenium (complex II), followed by rapid substitution of H₂O for NO (Figure 1.7) [108]. The binding of NO by these complexes is extremely rapid (k = 2.6 x 10⁷ M⁻¹ s⁻¹ at pH 7.4 and 7.38 °C [107].

Figure 1.6. Six coordinate Ru(II) mononitrosyl complex. Adapted from [107].
**Figure 1.7.** Reaction between complex I and NO in aqueous solution. The resulting adduct contains a stable, linear Ru(II)-NO bond (See Figure 1.6). Figure reprinted from Ref. [110]

Complexes of ruthenium were employed as a potential NO scavenger in order to regulate NO levels for therapeutic gain [109]. In fact, in the early exploration of possible electrode materials for the detection of nitric oxide, the response of ruthenium metal was rationalized in terms of formation of surface nitrosyls [110].

This rich coordination chemistry of ruthenium makes it possible to develop ruthenium-based high-affinity electrocatalytic surfaces for NO detection and quantification. Our current quest for suitable electrocatalysts to improve sensitivity and selectivity towards NO targets applications in biological systems with medical interest, where real-time measurement and monitoring of NO is critical.
In the subsequent chapters we describe the development of a nitric oxide sensor based on electrocatalysis on ruthenium as a platform. We will describe how this system can be used to improve selectivity, sensitivity, and other analytical problems associated with current NO sensors.

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CHAPTER II

THE ELECTROCATALYTIC EFFECT OF RUTHENIUM ON NO USING RUTHENIUM OXIDE COLLOID-MODIFIED CARBON PASTE ELECTRODES

2.1. Introduction

The need for accurate, reliable, and real-time methods to monitor nitric oxide grew fast in parallel with the explosion of research on this physiological messenger. Very early in this development, the power of electrochemical methods and their inherent sensitivity and selectivity were recognized. As explained in section 1.5.2, a variety of electrochemical approaches for the quantification of NO have been in steady development [1-7]. Proposed methods ranged from simple oxidation of NO analyte on solid materials such as platinum, carbon, iridium, or ruthenium, to modified electrodes with mediators such as metal porphyrins [8-10]. A common goal of all methods targeted lowering the potential at which the oxidation of NO can be monitored and the sensitivity in terms of the current generated per amount of analyte added. While some proposed
methods were able to generate mediated oxidation current the sensitivity was still relatively low [11]. In addition, the major drawback of some of these methods suffered from the use of inaccessible mediators that required synthesis as well as complex modification procedures.

When appropriate, carbon paste electrodes (CPEs) are among the most popular platforms for electrochemical detection because they have a wide working potential range and are inexpensive and can easily be renewed. Also, the electrode material is easy to prepare, and can be conveniently formulated with mediators and/or other modifiers.

CPEs consist of a mixture of graphite powder and a pasting liquid such as mineral oil, and, at times, a modifier is added. Various oils and formulations have been used to suit particular applications [12, 13]. Particularly, polychlorotrifluoroethylene oil (PCTFE or Kel-F®, a registered 3M trademark) has been used as a pasting liquid for the graphite powder [14]. In addition to providing compact and smooth surfaces, Kel-F® seems to also provide a good hydrophobic microenvironment suitable for NO partitioning and detection.

Chemically modified electrodes containing redox mediators have been employed because of their increased sensitivity and selectivity that can be geared towards the detection of targeted analytes. In the case of nitric oxide, carbon paste electrodes modified with 6,17-diferrocenyl-dibenzo[b,i]5,9,14,18-tetraaza[14]annulen-nickel(II) was used in NO detection [11]. However, the response of this modified CPE to NO was
found to be not reproducible, probably as a result of electrode fouling by the cumulative oxidation products resulting from repetitive NO determinations.

Our group explored the use of easily accessible modifiers that can be embedded in carbon pastes without prior or exhaustive modification, and that are able to reliably mediate the oxidation of nitric oxide. As modifier, ruthenium oxide colloids (RuO₂) have recently attracted great interest because of their unique physical and chemical properties, and potential applications in high-charge capacitors [15]. As described in section 1.6.1, the formation of nitrosyl complexes is a marked feature of ruthenium chemistry [16, 17]. In fact, in the early exploration of potential electrode materials for nitric oxide, the response of ruthenium metal was rationalized in terms of formation of surface nitrosyls [18]. Recently, nitrosyl complexes of ruthenium porphyrins were used as fluorescent probes for NO sensing [19]. Also, complexes of ruthenium, were employed as potential NO scavengers in order to regulate therapeutic NO levels [20]. Because of the rich redox and coordination chemistry of ruthenium and the unique properties of the Ru-NO chemical bond, the ruthenium oxide colloids may provide the right platform for the catalytic detection and quantification of NO.

In our current study, we report the fabrication and characterization of improved NO sensors based on the electrocatalytic activity of small amounts of RuO₂ colloids embedded in carbon paste and acting as catalytic sites for NO oxidation as illustrated in Scheme 2.1. Cyclic voltammetry is used in the electrochemical characterization of our RuO₂-modified carbon paste electrodes, while amperometry is used to assess their
performance in NO determination both in standing solution and under hydrodynamic regime using rotating disc electrodes (RDE).

Scheme 2.1. RuO$_2$ colloids serve as electrocatalytic sites for enhanced sensing of nitric oxide on carbon paste.

2.2 Experimental Design

2.2.1 Materials and Apparatus

Graphite powder (1-2 µm, synthetic), RuO$_2$.xH$_2$O, NaH$_2$PO$_4$, Na$_2$HPO$_4$, KOH, NaNO$_2$, NaNO$_3$, diethyl ether, pyrogallol, hemoglobin, ascorbic acid, L-arginine, bradykinin and sodium hydrosulfite are purchased from Sigma-Aldrich Corp (St. Louis, MO). Kel-F® oil (no.10) is purchased from Ohio Valley Specialty Chemicals (Marietta, OH). Compressed nitric oxide (99.5%) and nitrogen gas is purchased from Praxair (Burr Ridge, IL). Nanopure deionized water (specific resistance >18.2 MΩ.cm) used
throughout the experiment is from a Barnstead water purification system model D8961. All other chemicals are reagent grade and are used as received.

Cyclic voltammetry and amperometric measurements are performed using CHI-440 electrochemical workstations. Ag/AgCl is used as the reference electrode. All potentials reported here are versus Ag/AgCl. A platinum wire is utilized as the auxiliary electrode and RuO₂-modified or unmodified CPEs are used as working electrodes. UV-Visible absorbance spectra are recorded on Agilent 8453 spectrophotometer using 1-cm pathlength UV-visible cells. An Accumet AB15 pH-meter is used for pH measurements. Rotating disc electrodes measurements are recorded using an Autolab RDE setup (Brinkmann Instruments Inc, Westbury, NY).

2.2.2. Preparation of Modified Electrodes

Carbon pastes are prepared by hand-mixing graphite powder and Kel-F® oil (ratio 6:4 w/w). RuO₂ modified carbon paste is optimized from an exhaustive series of trials. A ratio of 6:3:1 (w/w) graphite/oil/RuO₂ is selected as the optimum proportion, and is prepared by adding RuO₂ and graphite powder to 10 ml of diethyl ether. This mixture is sonicated until all the ethyl ether is evaporated, and then the desired amount of Kel-F® oil is added. The resulting paste is packed tightly into the cavity of BAS electrodes (3mm diameter/2mm depth or 1mm diameter/1mm depth; Model MF-2010 and MF-2015h, respectively). Finally, the electrode surface is polished smooth on transparent weighing
paper. Rotating disc carbon paste electrodes are constructed using empty RDE tips (4 mm diameter, Brinkmann Inc.). The empty RDE electrode housing is filled with the prepared carbon paste as described above.

2.2.3 Preparation of NO Stock Solution

NO stock-solutions are made by bubbling pure NO gas through degassed water using the following procedure. First, 200 ml alkaline pyrogallol (5% w/v) is degassed for 30 minutes with N₂. Deionized water is also degassed in a small 10-ml-vial capped with a rubber septum. NO gas is purified by sparging through the 5%-pyrogallol solution in saturated KOH to remove trace oxygen, and then through a 10% (w/v) KOH solution to remove other nitrogen oxides (NOₓ). NO gas downstream of the pyrogallol/KOH system is then bubbled through the degassed water for 30 min. The concentration of NO in the saturated aqueous solution is 2.0 mM, which is independently confirmed via a UV-vis spectrophotometric method based on the stoichiometric conversion of oxyhemoglobin to methemoglobin by NO [21]. Both NO stock and standard solutions are freshly prepared through serial dilution of the NO-saturated solution prior to each experiment.

2.2.4 Electrochemical Experiments

All electrochemical experiments are performed with a three-electrode setup at room temperature in 10 mM pH 7.0 phosphate buffer unless otherwise indicated. The buffer is purged with purified nitrogen gas for at least 20 minutes prior to experiments.
naturally blanketed is then kept over the test solution throughout the experiment. Time-based amperometric experiments are performed by applying the desired potential to a solution stirred at *ca.* 250 rpm, allowing the transient background current to decay to a steady-state value in the absence of NO. The NO analyte in the standard solution is introduced with an airtight Hamilton syringe. Amperometric rotating disc CPE measurements are carried out in a similar manner keeping the rotation rate of the electrode at 2000 rpm.

### 2.3 Results and Discussion

#### 2.3.1 Electrocatalytic Activity of RuO2 Colloids in CPE Towards Nitric Oxide.

The RuO2 modified carbon paste electrode is first characterized and its electrocatalytic activity towards NO is examined. Cyclic voltammetry is used as a monitoring tool in this regard. Figure 2.1 shows the typical cyclic voltammograms obtained at 100 mV/s for CPE modified with RuO2 colloids as compared to non-modified CPEs. The response of unmodified CPEs in the absence (Figure 2.1a) and presence (Figure 2.1b) of 4.0 μM NO is minimal, with very little differentiation at this concentration. In contrast to this muted response, Figure 2.1c and Figure 2.1d illustrate how the RuO2-modified CPE increases the current response in the presence of 4 μM NO. An additional point of differentiation via a change in potential is also noted for RuO2-modified electrode. The current response in Figure 2.1d is due solely to the oxidation of the added nitric oxide. The oxidation potential of NO on unmodified carbon paste
electrode at about +1.15 V is similar to the potential measured on solid graphite electrodes [22], whereas in the presence of the RuO$_2$ colloids, the oxidation of NO occurs at much less positive potentials, with the onset of oxidation current starting at about +0.5V. These early oxidations potential, together with the larger currents observed in the presence of NO, are both indicative of electrocatalytic oxidation of nitric oxide by the RuO$_2$ colloids.

Figure 2.1. Cyclic voltammograms of unmodified CPE in the absence of NO (a) and in the presence of 4.0 µM NO (b), as compared to modified CPE in the absence of NO (c) and in the presence of 4.0 µM NO (d). Scan rate, 100 mVs$^{-1}$
The broad nature of the voltammetric response of the RuO$_2$-modified electrode suggests that changes in oxidation states for the oxide particles occur over wide potential ranges. The accurate assignment of a standard redox potential is thus difficult on modified CPE because of the ill-defined voltammetric peaks, particularly at intermediate pH [23].

To gain more insight on the behavior of the RuO$_2$-modified CPE in the absence and presence of NO, we carefully examine cyclic voltammograms over various potential windows. Figure 2.2 represents cyclic voltammograms obtained with RuO$_2$-modified CPEs with various potential ranges, namely from 0.0 V to +0.85 V and then from 0.0 V to +1.0 V. The observed background current at the RuO$_2$-modified electrodes in pH 7 buffer is as expected for the embedded ruthenium oxide material [23, 24]. The subtle anodic and cathodic peaks around +0.5 V are very close to the redox couple assigned previously to the Ru$^{3+}$/Ru$^{4+}$ [23, 25]. Another redox couple is easily identified at more positive potentials, ca. +0.8 V, and is assigned to the Ru$^{4+}$/Ru$^{6+}$, Equation 2.1, based on previous reports on the behavior of RuO$_2$ electrodes.

\[
\text{RuO}_2 + 4 \text{OH}^- \rightarrow \text{RuO}_4^{2-} + 2 \text{H}_2\text{O} + 2e^- \quad (2.1)
\]
Figure 2.2. Cyclic voltammograms of RuO$_2$-modified CPEs in the absence of NO (a) and presence of 4.0 µM NO (b) in pH 7.0 phosphate buffer solution with different potential windows. Potential range is indicated on graphs A and B.

The addition of NO causes the anodic current of the Ru$^{4+/6+}$ couple to increase with concomitant loss of reversibility. This behavior is a typical signature of an electrocatalytic process triggered by the oxidation of NO on RuO$_2$-modified CPE at the level of the Ru$^{4+/6+}$ redox couple, Equation 2.2.

\[
\begin{align*}
\text{RuO}_2 & + 4 \text{OH}^- \rightarrow \text{RuO}_4^{2-} + 2\text{H}_2\text{O} + 2e^- \quad (2.1) \\
\text{RuO}_4^{2-} & + \text{NO} \xrightarrow{k} \text{RuO}_2 + \text{Products} \quad (2.2)
\end{align*}
\]
Using a classical electrocatalytic scheme whereby the oxidation of the nitric oxide is mediated by RuO$_4^{2-}$ species on the colloid sites with the concomitant regeneration of RuO$_2$ (Equation 2.1 and Equation 2.2), one can estimate the rate constant $k$ for the elemental step limiting the catalytic oxidation of nitric oxide. This can be done for instance using the Nicholson and Shain treatment and their plots of the ratio of kinetic peak current for the electrocatalytic scheme in the presence of the substrate to the current in the absence of substrate, i.e. NO in this case [26, 27]. Due to the nature of the embedded redox couple catalyst, the peak currents are, in our case, ill-defined and only a lower limit of the current ratio is determined from Figure 2.2B (scan rate = 0.1 V/s) in the presence of 4.0 $\mu$M NO, Equation 2.3:

$$\frac{i_{NO}}{i_0} = \sim 1.75$$  \hspace{1cm} (2.3)

This catalytic situation yields a general second order rate constant $k=1.1 \times 10^6$ M$^{-1}$ s$^{-1}$ for the Ru$^{4+}$-driven catalytic oxidation of NO on RuO$_2$ colloids. This is in fact just a lower limit of this rate constant since the catalytic peak current is not reached and the wave is not well defined at the level of Ru$^{4+/6+}$ redox couple. This large rate of catalytic oxidation of NO at the RuO$_2$ colloid sites explains the great performance of RuO$_2$-modified paste electrode for the detection and quantification of NO in solution as shown in Figure 2.1. This will be further reflected in the use of these RuO$_2$-modified CPEs as NO sensors in the next section addressing the amperometric detection of NO.
It should be noted that the current of the electrocatalytic oxidation of NO on RuO$_2$-modified paste is significantly higher than the current observed on unmodified CPE, even at potentials as low as +0.5 V, Figure 2.2B. This property, as we will see, provides the advantage of using less positive applied potentials for the detection of NO, which, by itself, can enable discrimination against potentially interfering species that oxidize at higher (more positive) potentials.

### 2.3.2 Amperometric Detection of NO

#### 2.3.2.1 Sensitivity and Limits of Detection

Constant potential amperometry was used to further investigate the improved response of RuO$_2$-modified CPEs to NO as an analyte. Figure 2.3A represents typical amperometric responses for low $\mu$M aliquots of NO on unmodified (a) and RuO$_2$-modified (b) CPEs. As clearly shown, the modified CPE exhibits a significantly enhanced electrochemical response compared to the unmodified CPE. This is likely a direct result of the electrocatalytic response of NO on embedded RuO$_2$ particles. In general, the observed sensitivity of RuO$_2$-modified CPEs for the micromolar range of NO is around 100 pA/1nM at +0.8 V potential, and is at least 10 times more sensitive than unmodified CPEs, Figure 2.3B. As will be shown and explained later, much higher sensitivities are observed for lower ranges of NO concentrations.
Figure 2.3. (A) Amperometric responses of unmodified (a) and RuO$_2$ modified (b) CPEs with successive additions of NO to pH 7.0 phosphate buffer solution. Applied potential is +0.8V vs. Ag/AgCl. (B) Resulting calibration plots based on amperometric responses with respect to NO concentration recorded in (A).
Figure 2.4A shows typical responses for RuO$_2$-modified CPEs in the nanomolar (nM) range using +0.8V applied potential. As shown in Figure 2.4B, a linear response is also observed in this range, with a lower detection limit of 100 pM, as shown in Figure 2.4C.

Figure 2.4. (A) Amperometric responses of RuO$_2$-modified CPE to NO in the nanomolar range at 100, 200, 400, and 800 nM NO. (B) Calibration plot resulting from the dose-response amperometric curve. Applied potential is +0.8V vs. Ag/AgCl. (C) Typical detection limit of RuO$_2$-modified CPEs at 100 pM NO at +0.8V vs. Ag/AgCl.
Unmodified CPEs have a very low and nonlinear response at this concentration range at the +0.8V potential, thus we applied a +0.9 V potential in order to properly assess the gain in sensitivity from unmodified CPEs to RuO$_2$-modified CPEs. Even with this potential bias, the modified electrode still gives at least a 30x enhancement in response, as shown in Figure 2.5.

**Figure 2.5.** Typical calibration plot resulting from amperometric responses of RuO$_2$-modified CPE (b) to NO in the nanomolar range with +0.8V applied potential. Response of unmodified CPE (b) in the same nanomolar range is very low and not reproducible at +0.8 V and thus a +0.9 V was applied to assess the gain in sensitivity brought by RuO$_2$ catalytic sites.
It should be noted at this point that an observed trend is that higher sensitivities are obtained at lower concentration ranges, such that the slope of the NO-dose/current response plot is higher at the lower concentration range. Figure 2.6 clearly shows this, as the slope in the nanomolar concentration range is \( \sim 500 \) pA/nM NO, five times higher than the \( \sim 100 \) pA/nM NO slope in the micromolar range.

**Figure 2.6.** Slope increase of the response of RuO\(_2\)-modified CPE as one moves from micromolar to nanomolar NO concentration range.
A possible rationale for this behavior can be drawn from the electrocatalytic nature of the NO determination and the finite ruthenium oxide sites within the carbon paste. In the low NO concentration range, the ruthenium catalytic sites are used efficiently and the limiting factor is the NO analyte itself. On the other hand, in the higher NO concentration range, a phenomenon of pre-saturation of the catalytic sites by the flux of NO analyte is conceivably taking place, which would limit the extent of the catalytic process, and thus lower the sensitivity observed.

Although sensitivity plays a major role in analytical determination, the actual achievable lower detection limit is also vital. The electrocatalyst is obviously critical in the catalytic detection of NO, though larger amounts of this catalyst do not necessarily translate into a better signal-to-noise ratio, especially as we approach the lower detection limit. While the response to NO aliquots increases with larger amounts of embedded RuO$_2$ colloids, the background current also increases dramatically, making it difficult to detect low concentrations of NO. This was our rationale for selecting the optimized ratio of 6:3:1 for the graphite/Kel-F/RuO$_2$ composition for the modification of our CPEs as NO sensing platforms, which was used throughout this work. Using this optimized electrode formulation at +0.8V, we found that we can reliably detect as low as 100 pM NO concentration, based on the analytical criterion of signal to noise ratio (S/N) greater than 3, as was shown earlier in Figure 2.4C. This remarkable detection limit is a direct result of the fact that the response of our RuO$_2$-CPE catalytic system is better and particularly enhanced at low NO concentration.
While Figures 2.3 and 2.4 show that the RuO₂-modification significantly enhances the response to nitric oxide compared to unmodified carbon paste, this performance is maintained even when compared with other systems reported for nitric oxide determination. Table 2.1 shows a comparison of performance in terms of sensitivity and detection limits for systems of the same geometry (disk) and where the electrode size is in the same range (i.e. radii 1.5-5.0 mm). For comparative purposes, we report sensitivities in terms of current densities (current/mm²) per nanomole of NO. Our RuO₂-modified carbon paste electrode provides significantly higher sensitivity in terms of current density (pA/mm²) per nanomole of NO, and provides one of the lowest detection limits as compared to similar NO-sensing platforms.

Table 2.1. Comparison of normalized sensitivities and detection limits of similar systems used as platforms for NO detection and quantification. For RuO₂-modified carbon paste (this work) we report two values for two ranges of NO concentrations (see text).

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Modifier(s) (inner/outer layer)</th>
<th>Potential at which NO is determined</th>
<th>Normalized sensitivity (pA/nM mm²)**</th>
<th>Detection limit</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glassy carbon (disk; diameter = 3 mm)</td>
<td>CuPtBr₆</td>
<td>0.83 V/Ag-AgCl*</td>
<td>4.1</td>
<td>1.2 nM</td>
<td>[28]</td>
</tr>
<tr>
<td>Pt (disk; diameter = 1.6 mm)</td>
<td>CytC-PITO / (Nafion)</td>
<td>0.75 V/Ag-AgCl</td>
<td>2.5</td>
<td>20 nM</td>
<td>[29]</td>
</tr>
<tr>
<td>Pt (disk; diameter = 2 mm)</td>
<td>None (WPI membrane)</td>
<td>0.86 V/Ag-AgCl</td>
<td>0.32</td>
<td>1 nM</td>
<td>[3]</td>
</tr>
<tr>
<td>Carbon paste (disk; diameter = 3 mm)</td>
<td>RuO₂</td>
<td>0.80 V/Ag-AgCl</td>
<td>14.1 –for µM range 71.0 –for nM range</td>
<td>100 pM</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Potential value converted from the SCE reference used in that work.

** Values calculated from reported sensitivities and electrode size used.
2.3.2.2 Selectivity of the RuO$_2$ CPEs towards NO

In addition to sensitivity, selectivity is also important for electrochemical NO determination in biological systems. In this regard, at high potentials many other electroactive biological species may be oxidized and may interfere with the electrochemical detection and quantification of NO as the target analyte. Therefore, it is advantageous to use potential-based selectivity to make electrochemical determinations at lower potentials in order to avoid these possible interferences.

In previous reports of existing electrochemical sensors, the applied potential used to detect NO is normally between +0.85 and +1.1V vs. Ag/AgCl [15-17]. While we have shown that the RuO$_2$-modified CPEs can be used to detect nM levels of NO at +0.8V vs. Ag/AgCl with great sensitivity, potentials as low as +0.6 V can also be used to determine NO with our system.

In fact, Figure 2.7 shows that RuO$_2$-modified electrodes maintain the 10-fold increase in sensitivity for NO quantification over the unmodified CPEs with good linear response at an applied potential as low as +0.6 V vs. Ag/AgCl.
Figure 2.7. (A) Amperometric responses of unmodified (a) and RuO$_2$-modified (b) CPEs with successive additions of NO, at a lower potential of +0.6 V vs Ag/AgCl. (B) The resulting calibration plots based on responses observed in (A). (C) Amperometric responses of RuO$_2$-modified CPEs with successive additions of 1 mM L-Arginine (L-Arg), 1 mM nitrate (NO$_3^-$), 100 µM nitrite (NO$_2^-$), 10 µM AA and 1 to 5 µM aliquots of NO at +0.6 V.
Nitrite and nitrate, the stable by-products of NO metabolism, are the main interfering substances in the determination of NO, and their concentration in biological fluids can fluctuate during NO production. Other NO interferents that are in high biological concentrations in and around NO-producing systems are L-Arginine and ascorbic acid (AA).

In order to test the selectivity of our modified CPE at this +0.6V potential, we add these potential interferents and measure current response. Figure 2.7C demonstrates the current responses of RuO₂-modified electrodes with successive additions of 1 mM L-Arginine (l-Arg), 1 mM nitrate (NO₃⁻), 100 µM nitrite (NO₂⁻), 10 µM AA, and 1 to 5 µM aliquots of NO at +0.6 V vs. Ag/AgCl. By using observed sensitivity values, the % interference is calculated according to Equation 2.4 and values are summarized in Table 2.2. RuO₂-modified CPE did not show any significance interference to nitrate, nitrite and L-arginine (the substrate for nitric oxide synthase). However, ascorbic acid shows ~35 % interference at + 0.6 V vs. Ag/AgCl.

\[
\text{Interference species } X[\%] = \left( \frac{\text{Sensitivity of } X}{\text{Sensitivity of NO}} \right) \times 100 \tag{2.4}
\]
Table 2.2. Interference studies of selected compounds with the RuO₂-modified CPE.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tested Concentration (µM)</th>
<th>Sensitivity (pA/nM)</th>
<th>Interference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>1.00 x 10⁻³</td>
<td>4.59 x 10⁻⁴</td>
<td>0.01</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>1.00 x 10⁻³</td>
<td>7.00 x 10⁻⁴</td>
<td>0.001</td>
</tr>
<tr>
<td>NO₂⁻</td>
<td>100.00</td>
<td>1.00 x 10⁻⁴</td>
<td>0.3</td>
</tr>
<tr>
<td>Ascorbic acid (AA)</td>
<td>10.00</td>
<td>1.80 x 10⁻³</td>
<td>35.1</td>
</tr>
</tbody>
</table>

2.3.3 Determination Under Hydrodynamic Conditions on Rotating Disc Electrodes

The RuO₂-modified carbon paste system can be amenable to determinations of nitric oxide under forced convection (i.e. convective mass transport of analytes) by using the modified paste on rotating disc electrode housing. Figure 2.8 illustrates the amperometric response of a typical rotating disc electrode (RDE) prepared the same way as stationary CPE electrodes. The rotation speed of the RDE was kept at 2000 rpm throughout.
Figure 2.8. (A) Amperometry of unmodified (a) and RuO$_2$-modified (b) rotating disc CPEs with successive additions of NO. The rotation rate of the electrode is 2000 rpm and the applied potential is $+0.8\text{V}$ vs. Ag/AgCl. (B) Calibration plots resulting from amperometric responses.
Figure 2.8A represents the response for the high nM to low µM range of NO on unmodified (a) and RuO₂-modified (b) carbon paste on a rotating disc electrode. As expected, the modified rotating CPE exhibits enhanced electrochemical response compared to the unmodified rotating CPE in the presence of NO. As a result, the observed sensitivity with RuO₂ colloids in the rotating CPE is significantly enhanced over the unmodified rotating electrode. Under the standard rotation speed used, the slope of the current-concentration plot is in the range of ~1.0 nA/1nM (normalized sensitivity of ~80 pA/1nM.mm²) of NO at an applied potential of +0.8 V.

Lower concentrations of NO were also explored using this hydrodynamic method. In this regard, the typical staircase amperometric responses for RDEs are displayed in Figure 2.9 along with the corresponding calibration plots. It is important to note that the RuO₂-modified rotating disc CPEs also show higher sensitivity for NO determination in the low nM range, as compared to higher concentrations in the micromolar range.

Like the case of stationary electrodes, the trend of enhanced NO sensitivity observed at the low nanomolar range under hydrodynamic conditions may also be rationalized by the fact that the available RuO₂ catalytic sites in the modified RDE are not all recruited at low analyte concentrations. Also, by using hydrodynamic conditions under forced convective mass transport, it is possible to obtain sharp and enhanced current responses even for the low nanomolar range.
Figure 2.9 represents the typical calibration curves for unmodified (a) and RuO\(_2\) modified (b) rotating disc CPEs under these conditions. In the low nM range, the sensitivity of RuO\(_2\)-modified electrodes for NO detection was found to be 6.1 nA/nM, which is about 100-fold higher than unmodified electrodes.

**Figure 2.9.** The typical calibration curves for unmodified (a) and RuO\(_2\) modified (b) rotating disc CPEs at low nM concentration of NO at 0.8 V vs. Ag/AgCl. **Inset:** Responses of unmodified (a) and RuO\(_2\)-modified (b) rotating disc CPEs with successive additions of low nM aliquots of NO. The rotation rate of the electrode is 2000 rpm and the applied potential is maintained at 0.8V vs. Ag/AgCl.
2.4 Summary for Chapter II

Composite carbon paste with RuO₂ colloids bonded with the hydrophobic Kel-F® oil was developed as an electrode material for the electrocatalytic detection and quantification of nitric oxide in aqueous solutions. The ruthenium redox couples of colloid particles have been characterized on the modified paste. Addition of nitric oxide triggers an electrocatalytic oxidation current mainly at the level of the Ru⁴⁺/Ru⁶⁺ redox couple.

The RuO₂-modified carbon paste electrodes have been characterized as platforms for electrochemical sensing and quantification of NO. We have shown that the added RuO₂ colloids act as efficient electrocatalytic sites for the oxidation of NO. While the electrocatalytic oxidation of the NO analyte is likely mediated by the Ru⁴⁺/Ru⁶⁺ redox couple, the NO-driven current increase is apparent at potentials as low as +0.5 V vs. Ag/AgCl. As a result, the NO response of RuO₂-modified CPEs is greatly enhanced compared to unmodified carbon paste electrodes. With the RuO₂-CPE system, the sensitivity to NO is particularly enhanced in the low nanomolar range, and this results in a measured lower detection limit of 100 pM. Under hydrodynamic conditions, and particularly at the low nM range of NO concentration, the RuO₂-modified rotating disc carbon paste electrodes show ca. 100-fold enhanced current response compared to unmodified electrodes.

The preparation of the carbon paste electrodes modified with RuO₂ colloids is
very simple, straightforward, and quite easy to use. The method described here and which has been extended to other systems allows the determination of NO at relatively lower applied potential (+0.6 to +0.8 V range) and with excellent linear ranges. The lower detection limit (ca. 100 pM) and the high sensitivity of our RuO$_2$-modified carbon paste electrodes show superior performance over other similar systems for NO detection. The method described in chapter I was extended to other systems, which allows real time NO quantification with miniaturized sensor platforms as will be addressed in chapters III and IV.

2.5 References


CHAPTER III

NANO-STRUCTURED RUTHENIUM OXIDES MODIFIED ULTRA-MICROELECTRODES AS NO SENSING PLATFORMS


3.1.1 Introduction

Carbon fiber microelectrodes (CFEs) are widely used for electrochemical detection of many analytes including NO, as they are easy to fabricate and are size-compatible to offer non-invasive, non-destructive close proximity to the site of NO release in biological media. As we demonstrated in chapter two, sensitivity and selectivity of NO by this method can be improved through electrode surface modification using
electrocatalytic platforms with high affinity for NO. Because of the rich coordination chemistry of ruthenium and the unique properties of the Ru-NO chemical bond, as well as high surface area, ruthenium oxide nanoparticles may provide the right platform for the catalytic detection and quantification of NO.

The preparation of modified electrodes generally requires a reproducible procedure and good control over the permeability characteristics of electrode coatings. An elegant method of electrode surface modification is electrodeposition. Progress in this area is strongly connected to the design of new electroactive systems and to the success in forming thin, insoluble, stable, reproducible, and adherent films on electrode surfaces. The advantage of using electrodeposition in this capacity is the ability to coat very small electrode surfaces of various shapes.

Electrochemical deposition of ruthenium oxide particles and anodic oxidation of deposited ruthenium is a well-known method for preparation of ruthenium oxide coating on various substrates to improved response characteristics [1, 2]. In this chapter, we describe the fabrication and characterization of improved NO sensors based on carbon microelectrodes modified with nano-structured ruthenium oxide. The ruthenium oxide nanoparticles are deposited on a single carbon fiber electrode by continuous cycling electrodeposition. We demonstrate how these ruthenium catalytic sites improve NO sensitivity compared to bare CFEs. Furthermore, we illustrate these results in terms of amperometric NO detection, both in standing solution and in flow cells. Finally, CFEs have been successfully used to monitor the production of NO from human umbilical vein endothelial cells (HUVECs).
3.1.2 Experimental Section

3.1.2.1 Materials

Carbon fibers (7µm and 30µm) are obtained from Goodfellow (Devon, PA) and World Precision Instruments (Sarasota, FL) respectively. Compressed nitric oxide (99.5%) and nitrogen gases are purchased from Praxair (Burr Ridge, IL). RuCl₃·xH₂O, NaNO₂, NaNO₃ diethyl ether, pyrogallol, hemoglobin, ascorbic acid, L-arginine, bradykinin and sodium hydrosulfite are products of Sigma-Aldrich Corp (St. Louis, MO). Highly Oriented Pyrolytic Graphite (HOPG) surfaces (SPI-1 Grade) are obtained from SPI supplies (West Chester, PA). Silver conductive epoxy is purchased from Chemtronics (Santa Ana, CA). Nanopure deionized water (specific resistance >18.2 MΩ cm) used throughout the experiment is supplied by a Barnstead water purification system model D8961. All chemicals are reagent grade and used as received.

3.1.2.2 Apparatus

Cyclic voltammetry measurements are performed using BAS-100B electrochemical workstations. Amperometric measurements are carried out using CHI-440 electrochemical workstation and a multi-mode potentiostat system. The electrochemical cell setup is as previously reported in section 2.2.1.
Scanning Electron Microscopy (SEM) characterization is carried out on uncoated or palladium-coated samples using Hitachi S-4500 Field-Emission Gun Scanning Electron Microscope (FESEM) equipped with energy dispersive x-ray (EDX) elemental analysis capabilities. Auger electron spectroscopy (AES) data is collected using a Perkin-Elmer PHI-680 scanning Auger microprobe system. AFM is performed with a Molecular Imaging pico-SPM using the MAC mode interfaced with a PicoScan controller.

A flow-cell is fabricated in-house out of transparent Plexiglas® for use with the carbon fiber microelectrodes, and is fitted with an Ag/AgCl reference electrode as well as a stainless steel auxiliary electrode tube, which is also used as the outlet. The flow-cell setup is completed with a Masterflex peristaltic pump and a Rheodyne 5020 manual injector (Sigma-Aldrich Corp, St. Louis, MO) with a 500 μL injection loop. Cell culture experiments are carried out on the stage of an Olympus IX-71 inverted microscope with the microelectrode positioned 10-15 μm above the cell surface with the use of a micromanipulator.

3.1.2.3 Preparation of Integrated Single Carbon Fiber Ultra-microelectrode

Carbon fiber microelectrodes (7 or 30-μm diameter) are prepared in-house using published procedures [3, 4]. Briefly, single fibers are isolated and consecutively sonicated in acetone, 50% nitric acid, and distilled water. A dried fiber is then mounted at the end of a copper wire and fixed with conductive silver epoxy. The mounted carbon fiber is inserted into a pulled glass capillary and sealed with non-conductive epoxy, leaving ca. 2
mm fiber exposed. A copper wire is then fixed to the stem of the glass tube with the non-conductive epoxy glue. The pulled end of the glass capillary is coated with Ag/AgCl ink. A layer of insulating material is placed over the body of the sensor leaving 3 mm of the Ag/AgCl reference electrode exposed.

3.1.2.4 Electrodeposition of Ruthenium-oxide-type Catalyst on Carbon Fiber Microelectrode

The ruthenium oxide catalyst is typically preformed on the carbon fiber surface by electrochemical deposition using previously published methods [1]. This procedure requires optimization for our application and is described as follows: A freshly prepared carbon fiber electrode is immersed in an electrochemical cell with 10 ml of 10 mM HClO₄ containing 20 µM RuCl₃ precursors while potential is continuously cycled between -0.85 V and +0.65 V at a scan rate of 100 V/s for a period of 20 minutes. Nafion® coating is applied by dip-dry procedure. Briefly, the active surface area of the fiber is carefully dipped into a Nafion® solution at room temperature for 3 s and dried for 4 min at 120° C. This procedure is repeated until three coats have been applied. After each electrode modification, the electrode is gently washed with deionized water, and allowed to dry before use.
3.1.2.5 Cell Culture

Human umbilical vein endothelial cells (HUVEC) are obtained from American Type Culture Collection (ATCC, VA, USA) and grown in F-12K medium (Cleveland Clinic Foundation, Cleveland). To complete the growth medium, 0.1 mg/ml heparin and 0.03-0.05 mg/ml endothelial cell growth supplement (ECGS) (Sigma-Aldrich) are added to the F-12K medium and adjusted to a final concentration of 10% fetal bovine serum (Sigma-Aldrich). The medium is renewed every 2 days until confluence (4-5 days). The cells are then rinsed with a solution of 0.25% (w/v) Trypsin and 0.53 mM EDTA (CCF, Cleveland), and are detached by incubating in trypsin-EDTA for 5-10 min at room temperature. The cell suspensions are then transferred to new cell culture dishes and allowed to grow in 37 °C in the presence of 5% CO₂ in a humidified environment. Cells are washed with phosphate buffer saline (PBS) and experiments are performed in PBS at room temperature.

3.1.2.6 Procedure

Similar procedures are carried out as was outlined in chapter I. All electrochemical experiments are performed as previously described. The surface properties and composition of the ruthenium oxide modified carbon fiber electrodes is characterized by field emission scanning electron microscopy (FESEM), energy dispersive X-ray spectrometry (EDX), Auger electron spectroscopy (AES), and atomic force microscopy (AFM). Amperometry is the technique used to assess the performance
of the modified fibers in terms of NO determination both in standing and dynamic flow-through NO solutions.

Prior to the cell stimulation experiment, the culture medium is removed and adherent cells are incubated with PBS buffer solution. Our ruthenium oxide modified carbon microelectrode is positioned 10-15 µm above the cell surface. A potential of 0.8 V is then applied to the NO sensor. Once a steady background current is obtained, stimulants are injected into the cell medium.

3.1.3 Results and Discussions

3.1.3.1 Electrodeposition of Ruthenium-Oxide-type Catalyst on Carbon Fiber Microelectrode

Electrodeposition is an effective process for producing metallic coatings on a surface. Today, with the impressive progress and deeper understanding of the underlying electrochemical principles of electrodeposition, sophisticated methods have been developed and are being routinely employed [5]. Dip/drop-dry coating, constant potential electrolysis, repetitive cyclic voltammetry, and differential pulse amperometry have all been employed for the deposition of catalytically active materials on electrode surfaces [6]. Generally, dip/drop-dry coating has been reported to be largely irreproducible. With the use of electropolymerization or electrodeposition, the thickness of the film formed on
the electrode surface can be accurately controlled by monitoring either number of cycles, number of pulses, or the quantity of charge passed, depending on the technique employed, for improved reproducibility.

Cyclic voltammetric continuous scanning electrodeposition technique is widely used in numerous applications [6]. Figure 3.1 shows typical cyclic voltammograms obtained during continuous scanning modification of a typical 7-μm carbon fiber electrode in a RuCl₃/HClO₄ solution. For clarity, only the initial portion of the modification is shown, and each trace (a-f) represents 100 cyclic voltammograms.
Figure 3.1. A cyclic voltammogram for 20 μM RuCl₃ in 10 mM HClO₄ solution recorded at the carbon fiber microelectrodes of 7-μm diameter. The potential of the electrode was cycled between -0.85 and 0.65 V at 100 V/s over 20 min. The curves were recorded every 3 s and each trace (a-f) represent 100 cyclic voltammograms.

As previously reported [1], Figure 3.1a reveals that during the early stages of modification the reductive current increases only in the region of the most negative
potentials used, between -0.65 to -0.85 V. This triggers the initial nucleation of ruthenium-based electrodeposited material on the carbon fiber surface. After the initial steps of continuous scanning, the backward reductive current starts to increase rapidly at less negative potentials (Figure 3.1 (b-f)). Also, the forward oxidative current starts to increase at less positive potentials. Presumably, after the formation of slowly growing ruthenium nuclei on the carbon fiber surface, additional electrode processes lead to the electrodeposition of ruthenium oxides.

Initially deposited ruthenium can be easily converted into ruthenium oxides because the growth of surface oxide of Ru begins at potentials as low as 0.0 V [1, 7]. Due to the effects of certain processes, such as proton and oxygen reduction, the electrode/solution interface becomes less acidic than the bulk solution. Such conditions can lead to hydrolysis of RuCl₃ and precipitation of ruthenium oxides. Hydrolysis of RuCl₃ solution is a well-known technique for precipitation of RuO₂.xH₂O [8]. Since the reduction of ruthenium surface oxides is a relatively slow process [7], during the course of continuous scanning they can accumulate on the electrode surface.

The voltammetric current increases to a maximum value in the 20 min period of continuous cycling (not shown). The capacitor-like behavior observed for electrodeposited coatings in ruthenium solutions is also a characteristic feature of ruthenium oxides [9-11].

These voltammograms provide evidence that the modification by continuous scanning voltammetry in a RuCl₃ solution leads to an electrochemical deposition of
ruthenium-oxide-type nanoparticles on a carbon fiber microelectrode. Indeed as we will see, SEM and AFM images provide the morphological evidence of the modified fiber surface.

### 3.1.3.2 Scanning Electron Microscopic Characterization

The scanning electron microscope (SEM) is a type of electron microscope capable of producing high-resolution images of a sample surface. Due to the way in which the image is acquired, SEM images have a characteristic three-dimensional appearance and are useful for judging the surface morphology of a sample.

Field emission scanning electron microscopic (FESEM) images of modified and bare carbon fibers are shown in Figure 3.2. The surface morphology of electrodeposited ruthenium oxides on a modified carbon fiber surface appear as characteristic granular type nano-structures (Figure 3.2c and 3.2d) and present a clear difference as compared to bare fibers (Figure 3.2a and 3.2b).

Highly-magnified FESEM images (Figure 3.2e and 3.2f) reveal the existence of typical nano-structured clusters of ruthenium oxide in the ca. 100 nm ranges. These structures closely resemble the ruthenium oxide nanoparticles grown on graphite surfaces [12, 13] and provide the morphological evidence of the existence of the ruthenium oxide nanoparticles on carbon fiber electrode, which is further confirmed by Atomic Force Microscopy.
Figure 3.2. FESEM images of typical bare 7-µm (a), 30-µm (b), ruthenium oxide modified 7-µm (c), and 30-µm (d) carbon fibers. Also shown are high-magnification SEM images of carbon fiber surfaces with ruthenium oxide granules, 7-µm (e), and 30-µm (f) respectively.
The modification of electrodes with electrodeposited ruthenium oxide was monitored over time. Figure 3.3 shows particle distribution of ruthenium oxide at different deposition times. Images a, b, and c are taken at 10, 15, and 20 min respectively. The images clearly show that, as expected, the particle density increases with deposition time.

Full coverage of the ruthenium oxide on the carbon fiber appears after 20 min of deposition time. Exceeding 20 min causes additional clustering of ruthenium oxide nanoparticles. Considering both voltammetric behavior and FESEM morphology, 20 min deposition time is determined to be the optimized time for the modification process.
Figure 3.3. FESEM images of ruthenium oxide modified carbon fiber surfaces with different deposition times. Images a, b, and c are taken at 10, 15, and 20 min. respectively.
3.1.3.3. Atomic Force Microscopy (AFM) Characterization

Atomic Force Microscopy (AFM) is currently a well-established technique that enables one to view and understand events as they occur at the molecular level. Atomic Force Microscopy is extensively used in the characterization of ruthenium oxide in high storage supercapacitors [14].

Figure 3.4a presents typical two-dimensional AFM images of the freshly cleaved highly oriented pyrolytic graphite (HOPG) substrate utilized during the surface analysis experiments. The bare HOPG is extremely smooth, which enables the identification of the topographical changes when the surface is modified with ruthenium oxide. The same electrodeposition technique used to modify the carbon fibers is employed here to modify the HOPG. Figure 3.4b represents the two-dimensional AFM image of the ruthenium oxide modified HOPG surface. This deposit possesses a rough morphology containing many nano-scale spherical grains aggregated to form larger oxide particles. These ruthenium oxide aggregates very closely resemble the ruthenium oxide nanoparticles found in other modification techniques and characterized by AFM [14].
Figure 3.4. Atomic Force Microscopy images of (a) bare and (b) ruthenium oxide modified HOPG.

3.1.3.4 Energy Dispersive X-ray Spectroscopy (EDX) Characterization

Energy dispersive X-ray spectroscopy (EDX) is most often used for qualitative elemental analysis to determine which elements are present, and to conclude their relative abundance. This technique is utilized as a qualitative analytical tool to characterize the surface composition of the modified carbon fiber. Figure 3.5 shows the EDX spectrum of modified carbon fibers with the expected ruthenium peaks. The two most intense transitions for Ru, Lα₁ and Lβ₁ correlate exactly with reported values [13, 15]. No peaks are observed in that region for the bare carbon fiber. A palladium peak is evident in both spectra, as the palladium sputter coatings are applied prior to the all FESEM imaging/EDX analysis.
The ruthenium layer is too thin to allow for a further quantitative analysis. This is not surprising because EDX probes need to go 4-5 µm deeper into the material to quantify [16], whereas the observed thickness of our ruthenium oxide layer is less than 1 µm, as confirmed by SEM and AFM. Therefore, quantitative analysis of modified layer using EDX is not an accurate approach.

Figure 3.5. Energy dispersive x-ray (EDX) spectra of bare (dash line) and ruthenium modified (solid line) after 15 min. palladium sputter coating.
3.1.3.5 Auger Electron Spectroscopy (AES) characterization

Auger Electron Spectroscopy (AES) is a surface specific technique that uses the emission of low energy electrons in the Auger process, and is one of the most commonly employed surface analytical techniques for determining the composition of the surface layers of a sample. This technique provides the accurate quantification of the modified electrode surface within the 1 µm thickness of our ruthenium oxide coating [17].

Figure 3.6 shows the AES spectra of bare and ruthenium oxide modified carbon fiber electrodes. The Auger electron peaks of ruthenium at 273 eV (Ru₁) and carbon at 272 eV (C₁) closely resemble each other. The ruthenium oxide modified fiber also displays Auger electron peaks at 231(Ru₂), 200, 184, 176, and 150 eV. These peaks are signatures of ruthenium metal and correlate with published data [17].

The Ru₂ peak can be utilized for accurate surface composition determination, which shows the modified fiber surface contains 95.5% ruthenium. As expected, the only other significant response observed is for oxygen (not shown). These results confirm that electrodeposition of ruthenium oxide via RuCl₃ in perchloric acid solution results in small nucleation sites of ruthenium, ultimately grown into nano-structured clusters of ruthenium oxides on carbon fiber.
### 3.1.3.6 Electrocatalytic Activity of Nano-Structured Ruthenium Oxides on the Modified Carbon Fiber Ultra-Micro Electrodes towards NO.

The ruthenium oxide modified carbon fiber microelectrode is first characterized and its electrocatalytic activity towards NO, and is examined by cyclic voltammetry. Key features that we monitor are the current response and the potential at which NO is

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**Figure 3.6.** Auger electron spectra of bare (dash line) and ruthenium modified (solid line) 7-μm carbon fibers.
oxidized. Figure 3.7 shows the electrochemical response of both modified and bare microelectrodes with and without NO present. Figure 3.7B shows the typical cyclic voltammograms obtained for CFE modified with ruthenium oxides in the absence (Figure 3.7B(a)) and the presence (Figure 3.7B(b)) of 80 µM NO in pH 7.0 phosphate buffer. This is compared to the response of unmodified CFEs in the absence (Figure 3.7A(a)) and the presence of 80 µM NO (Figure 3.7A(b)).

The oxidation potential of NO on bare carbon fiber electrode at about +1.05 V is not different from the NO oxidation potential measured on solid graphite electrodes [18]. As we noticed with RuO₂ modified carbon paste electrodes (section 2.3), in the presence of ruthenium oxide nanoparticles, the oxidation of NO occurs at a much less positive potential with the onset of oxidative current starting as early as ~+0.5V. The early oxidation potential together with the larger currents observed in the presence of NO are both indicative of electrocatalytic oxidation of nitric oxide, comparable to RuO₂ colloids.
Figure 3.7. (A) Cyclic voltammograms of bare CFEs in the absence of NO (a) and presence of 80µM NO (b) in pH 7.0 phosphate buffer. (B) RuO$_2$-modified CFEs in the absence of NO (a) and presence of 80µM NO (b) in same conditions.
The observed background current at the ruthenium oxide modified electrodes is as expected [19]. The subtle anodic and cathodic peaks around 0.6 V are very close to the redox couple assigned previously to Ru$^{3+}$/Ru$^{4+}$ [19, 20]. Another redox couple is easily identified at more positive potentials at around +0.8 V, and is assigned to the Ru$^{4+}$/Ru$^{6+}$, according to previous reports (see equation 2.1, Chapter I) [19, 20].

As in the case of RuO$_2$ modified CPE, we can predict that the addition of NO causes the anodic current of the Ru$^{4+/6+}$ couple to increase with concomitant loss of reversibility. This behavior, a typical signature of an electrocatalytic process triggered by the oxidation of NO, is exactly what is observed on our ruthenium-modified CFE. Refer to equation 2.2 for this reaction scheme.

Using the Nicholson and Shain treatment [21, 22], as previously described in section 2.3.1, and their plots of the ratio of kinetic peak current (equation 2.3) the peak currents are, in our case, ill-defined and only a lower limit of the current ratio is determined from Figure 3.7B (scan rate = 0.1 V/s) in the presence of 80 µM NO.

This catalytic situation yields a second order rate constant $k=1.5 \times 10^6$ M$^{-1}$ s$^{-1}$ for the Ru$^{4+}$-driven catalytic oxidation of NO on ruthenium oxide modified microelectrode. This is in fact just a lower limit of this rate constant since the catalytic peak current is not reached and the wave is not well defined at the level of Ru$^{4+/6+}$ redox couple. This large rate of catalytic oxidation of NO at the ruthenium nanoparticle sites explains the great performance of modified electrodes for the detection and quantification of NO in
All of these characterizations reveal that electrodeposited materials on the fiber surface predominantly consist of nano-structured ruthenium oxide particles, which provide a uniform coating of catalyst to bind and electrocatalytically detect NO. This modified platform on carbon fiber is expected to provide an ideal surface for enhanced NO detection, akin to the performance of ruthenium oxide colloids in carbon paste electrodes (Chapter I). Thus, the nano-structured ruthenium oxides on the modified carbon fiber ultra-micro electrodes with compatible spatial and temporal resolution of single cell levels was utilized for the same purpose to push the limits of selectivity and sensitivity of NO detection.

3.1.3.7 Amperometric Detection of NO: Sensitivity and Detection Limit

Constant potential amperometry was used to further investigate the electrocatalytic activity of nano-structured ruthenium oxides on the modified carbon ultramicroelectrodes towards nitric oxide. Figure 3.8A represents typical amperometric responses obtained for high nM aliquots of NO on a 7-µm unmodified (a) and ruthenium oxides modified (b) CFEs. As clearly shown, the modified CFE exhibits a significantly enhanced electrochemical response compared to the unmodified CFE.
As previously explained, the improved sensitivity is due to the electrocatalytic activity of ruthenium oxide nanoparticles on the carbon fiber surface that provide faster kinetics of NO oxidation. As NO decomposes rather quickly, a rapid response time is also very important for quantifying this analyte. Analysis of this data indicates a 95% response was observed in less than 8 seconds (2-7 s) for the electrode modified with ruthenium oxides. The observed response time is fast enough to detect physiological levels of NO [23].

Figure 3.8B shows resulting calibration plots for unmodified (a) and ruthenium oxide modified (b) CFEs in high nanomolar (nM) to low micromolar (µM) concentration range of NO. An excellent linearity is observed for the modified electrodes with correlation coefficient ($R^2$) value of 0.9996.
Figure 3.8. (A) Amperometric responses of 7-µm bare (a) and ruthenium oxides modified (b) CFEs with successive additions of high nM aliquots of NO. The applied potential is 0.8V. (B) The resulting calibration plots with respect to concentration.
In most applications the sensitivity and detection limit are usually the most important features that determine the efficiency of an electrode. Sensitivity refers to the gradient/slope response of the sensor observed when plotting the redox current against NO concentration. The sensitivity of an NO sensor depends largely on the active surface area of the sensor and the electrode materials used in the design. An electrode with a small active surface area will generally have a lower sensitivity compared to one with a larger surface area. The electrocatalytic nature and larger reactive surface of nano-structured ruthenium oxide will naturally increase the sensitivity of our modified CFE.

As indicated from the slopes of calibration plots, the observed sensitivity for the ruthenium oxide modified CFEs, 12.41 pA/ nM is about ~25 fold higher compared to bare CFEs (0.54 pA/ nM). These results are attributed to the efficient electrocatalytic oxidation of NO by the deposited ruthenium oxide catalytic sites present on the carbon fiber surface.

Biologically more relevant, lower concentrations of NO are also explored using this amperometric method. The typical staircase amperometric responses obtained for low nM concentration of NO are displayed in Figure 3.9, along with the resulting calibration curve (inset b). We observed higher sensitivities at lower concentration ranges, as we found earlier with the RuO₂ modified CPEs. Shin and co-workers recently indicated that their microelectrode system for measuring NO also observed the high sensitivities for lower concentration [24].
In our case, the typical slope measured for the NO-dose/current response plot for the range 2 - 16 nM is 18.96 pA/nM. This value is to be compared to only 12.41 pA/nM NO from 0.1 to 3.2 µM ranges. Both concentration ranges are combined in Figure 3.10 for visual comparison of the change in sensitivity.

A rationale similar to the CPE Case can be used to explain this behavior. As before, in the low NO concentration range, the ruthenium catalytic sites are used efficiently and the limiting factor is the NO analyte itself. In the higher NO concentration range, pre-saturation of the catalytic sites by the flux of NO analyte is believed to limit the catalytic process.
Figure 3.9. Amperometric responses of ruthenium oxides modified CFEs with successive additions of ultra low nM aliquots of NO. The applied potential is 0.8V. Insets: (a) Typical detection limit of modified CFE at 200 pM NO. (b) The typical calibration curve for the modified 7 µm CFEs with successive additions of ultra low nM aliquots of NO.
As with the CPEs discussed in section 2.3.2.1, it is necessary to balance between having a sufficient quantity of the RuO$_2$ necessary to drive the electrocatalytic process and the accompanying increase in the background current that adversely affects the lower detection limit. This was our rationale for selecting the optimized electrodeposition time of 20 min for the modification of our CFEs as NO sensing platforms, which was used throughout this work. Using this optimized condition, we found that we can reliably detect as low as 200 pM NO concentration, based on the analytical criterion of signal to

**Figure 3.10.** Slope increase of the response of ruthenium oxide modified CFE as one moves from micromolar to nanomolar NO concentration range.
noise ratio S/N > 3, which is shown in inset (a) of Figure 3.9. This remarkable detection limit is a direct result of the fact that the response of our ruthenium oxide catalytic system is better and particularly enhanced at low NO concentration.

While Figures 3.8 and 3.9 show that the ruthenium oxide nanoparticle modification significantly enhances the response to nitric oxide compared to unmodified carbon fiber, this performance is maintained even when compared with other systems reported for nitric oxide determination. Table 3.1.1 shows a comparison of performance in terms of sensitivity and detection limits for systems of similar geometry (cylindrical or even in some cases conical) and where the electrode size is in the same range (i.e. radii in micrometers). More detailed comparisons of NO detection systems can be found in several review articles published elsewhere [25-27]. For comparative purposes, we report sensitivities in terms of current densities (current/µm²) per nanomole of NO.
**Table 3.1.** Comparison of normalized sensitivities and detection limits of few systems used as platforms for NO detection and quantification. For ruthenium oxide modified carbon fiber (this work) and Pt/ Pt black (Xerogel) we report two values for two ranges of NO concentrations (see text).

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Modifier(s)</th>
<th>Potential at which NO is determined (reference electrode)</th>
<th>Sensitivity (pA/ nM)</th>
<th>Normalized sensitivity (pA/ nM µm²)**</th>
<th>Detection limit</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon (fiber; cylindrical; diameter = 7µm length = 2 mm)</td>
<td>None (Nafion®/WPI membrane)</td>
<td>0.86 V/Ag-AgCl</td>
<td>1.03</td>
<td>2.33 x 10⁻⁵</td>
<td>5 nM</td>
<td>[3]</td>
</tr>
<tr>
<td>Carbon (fiber; cylindrical; diameter = 7µm length = 2 mm)</td>
<td>None (Nafion/cellulose acetate)</td>
<td>0.91 V/Ag-AgCl*</td>
<td>0.44</td>
<td>5.83 x 10⁻⁷</td>
<td>1 µM</td>
<td>[28]</td>
</tr>
<tr>
<td>Carbon (fiber; cylindrical; diameter = 8µm length = 2 mm)</td>
<td>Ni-THMPP (Nafion®)</td>
<td>0.79 V/Ag-AgCl*</td>
<td>6.28</td>
<td>1.2 x 10⁻⁴</td>
<td>1 nM</td>
<td>[29]</td>
</tr>
<tr>
<td>Carbon (fiber; cylindrical; diameter = 7.8µm length = 0.5 mm)</td>
<td>PBPB (Nafion®)</td>
<td>0.80 V/Ag-AgCl</td>
<td>1.04</td>
<td>2.17 x 10⁻⁵</td>
<td>5 nM</td>
<td>[30]</td>
</tr>
<tr>
<td>Tungsten (fiber; conical; diameter = 10µm length = 0.055 mm)</td>
<td>Pt/ Pt black (Xerogel)</td>
<td>0.80 V/Ag-AgCl</td>
<td>7.60 (0.2 – 3.0) µM</td>
<td>4.27 x 10⁻³</td>
<td>83 pM</td>
<td>[24]</td>
</tr>
<tr>
<td>Carbon (fiber; cylindrical; diameter = 7µm length = 2 mm)</td>
<td>Ruthenium oxide ((Nafion®)</td>
<td>0.80 V/Ag-AgCl</td>
<td>12.41 (0.1–3.2) µM</td>
<td>2.82 x 10⁻⁴</td>
<td>200 pM</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Potential value converted from the SCE reference used in that work

** Values calculated from reported sensitivities and electrode size used.
It should be noted that the ruthenium oxide modified carbon fiber provides a significantly higher sensitivity compared to other cylindrical carbon fiber electrodes (both sensitivity and normalized sensitivity in terms of current density (pA/µm²) per nanomole of NO). Recently reported tungsten conical fiber electrode shows better-normalized sensitivity. It is also worth noting that our ruthenium oxide modified CFEs as NO-sensing platforms provide one of the best detection limits seen for similar systems. Shin and co-workers claimed 83 pM limit of detection for their sensor but no actual data is provided.

3.1.3.8 Amperometric Detection of NO: Selectivity of the ruthenium oxide modified CFEs towards NO

As with the CPE electrodes, it is important to establish that NO interferents that are high in biological concentration in and around NO-producing systems do not interfere with NO detection (section 2.3.2.2). Nafion® (perfluorinated sulfonic acid ionomer), due to its impermeability to anions, has been widely used for elimination of interference from anionic molecules such as nitrite and ascorbic acid during NO measurements [4, 25, 27, 31]. After performing dip-dry procedure to coat three successive layers of Nafion®, selectivity tests are carried out for our modified electrodes.

Figure 3.11 demonstrates the current responses of Nafion® and ruthenium oxides modified CFEs with successive additions of 1 mM L-Arginine (l-Arg), 1 mM nitrate (NO₃⁻), 100 µM NO₂⁻, 100 µM ascorbic acid and 0.1 to 0.4 µM aliquots of NO. The % interference is calculated according to equation 2.4 (Chapter II) and values are
summarized in table 3.2. The % Interference values clearly indicate Nafion® significantly improved selectivity of our microelectrode.

**Figure 3.11.** (A) Amperometric responses of 7-µm ruthenium oxides modified CFEs with successive additions of 1 mM L-Arginine (l-Arg), 1 mM nitrate (NO$_3^-$), 100 µM nitrite (NO$_2^-$), 100 µM ascorbic acid and 0.1 to 0.4 µM aliquots of NO, respectively, in a pH 7.0 Phosphate buffer solution. The applied potential was 0.8V vs. Ag/AgCl.
Table 3.2. Interference studies of selected compounds. Concentration is chosen higher than the biological availability.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tested Concentration (mM)</th>
<th>Sensitivity (pA/ nM)</th>
<th>Interference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine</td>
<td>1.0</td>
<td>$3.59 \times 10^{-5}$</td>
<td>0.0003</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>1.0</td>
<td>$2.00 \times 10^{-5}$</td>
<td>0.0002</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>0.1</td>
<td>$1.00 \times 10^{-3}$</td>
<td>0.011</td>
</tr>
<tr>
<td>Ascorbic acid (AA)</td>
<td>0.1</td>
<td>$1.80 \times 10^{-3}$</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Many selective membranes decrease the interference but adversely affect the sensitivity of the sensor, so it is prudent to measure the sensitivity of the Nafion® modified electrode. We compare the calibration plots with and without Nafion® modification, as shown in Figure 3.12. The data shows that 96% of the response is retained for NO after the Nafion® modification. This clearly indicates our microelectrode still maintains its sensitivity towards NO.
Figure 3.12. Comparison of calibration plots without (a) and with Nafion® (b) modification for the ruthenium oxide modified 7-µm diameter CFEs with successive injection of high nM aliquots of NO.

3.1.3.9 Flow Injection Analysis for Dynamic Detection of NO

Flow Injection Analysis (FIA) is an automated, continuous flow approach for performing chemical analysis, based on injecting a small, well-defined volume of sample into a continuously flowing carrier stream, whereby a concentration gradient of the sample is created. At physiological conditions, NO quickly diffuses and creates concentration gradients. To mimic actual flux of NO in biological systems, dynamic NO quantification is carried out using flow injection analysis with the aid of our homemade
flow cell. Figure 3.13 shows the flow injection analysis system equipped with our sensitive CFE microelectrode employed for dynamic NO measurements. The components and schematic of this flow cell were previously described in section 3.1.2.2.

**Figure 3.13.** Photographs and schematic representation of the flow injection analysis (FIA) system with homemade flow cell.

Figure 3.14 shows typical amperometric responses for unmodified (a) and ruthenium oxide modified (b) 30-µm diameter CFEs in our FIA electrochemical cell. As in previous standing experimental trials, modified CFEs give expected enhanced signals for NO as compared to bare electrode. The typical calibration curves are obtained from the normalized area under current responses. The sensitivity of the ruthenium oxide
modified CFE, 0.5 \mu C/nM, which is at least a factor of 50 times greater than unmodified CFE.

As explained earlier, pre-saturation of the catalytic sites can be a limiting factor in sensitivity. The large surface area of 30-\mu m carbon fiber as compared to the 7-\mu m fiber allows for more ruthenium catalytic sites, and the NO analyte does not become a limiting factor to the same extent as with the smaller electrode. Also, the flux of NO analyte in the FIA system limits pre-saturation and allows for further increases in sensitivity. Again this is indicative of the efficient electrocatalysis of the oxidation of NO and correlates with the results obtained from amperometry in the standing solution.
Figure 3.14. (A) Amperometric flow injection analysis responses of unmodified (a) and ruthenium oxide modified (b) 30-µm diameter CFEs with successive injection of high nM aliquots of NO, to a pH 7.0 phosphate buffer flow-through system. The applied potential is 0.8V. (B) Shows a plot of charge vs. concentration NO.
3.1.3.10 Direct Detection of NO Release from Human Umbilical Vein Endothelial Cells (HUVECs)

The endothelium is a dynamic endocrine organ that regulates secretory, contractile, and mitogenic activities in the vessel wall, and the hemostatic process within the vascular lumen [32]. Nitric oxide produced from endothelium cells is involved in the regulation of vascular tone and blood pressure by stimulating smooth muscle relaxation, inhibiting platelet aggregation, and preventing smooth muscle proliferation [32].

Endothelial nitric oxide synthase (eNOS) catalyzes NO production in the endothelium from L-arginine. The generation of NO in endothelial cells can be stimulated by mechanical stimuli (shear stress) and chemical stimuli (acetylcholine, ATP, ADP, bradykinin, serotonin, thrombin and others). As shown in Figure 3.15 bradykinin for instance can be used as a chemical stimulator. Bradykinin (BK) acutely increases endothelial NO production by activating eNOS [32].
As specifically mentioned in the background section, a deficiency in NO production has been associated with dysfunction of the endothelium [32-34]. Therefore, the direct detection of NO released from the endothelium can offer important insights into the modulatory role of NO in cardiovascular system.

In this study, the detection of NO release is successfully performed on HUVECs, where the generation of NO in endothelial cells is stimulated by bradykinin. Figure 3.16
shows our experimental setup on the platform of an inverted microscope, which allows the micro-positioning of our NO sensors in proximity to the live cells. Scheme 1 illustrates the processes that NO may undergo after being released into a microvolume of buffer solution. NO diffuses from the cell into the buffer solution, travels toward the electrode surface where it is oxidized, and the response is recorded as an electrical signal [35].

Figure 3.16. Experimental setup on the platform of an inverted microscope. The micro-positioning of our NO sensors at proximity of the live cells.
Scheme 3.1. Representation of the likely processes that NO released into a microvolume of buffer solution. (adapted from ref. [35]).

Figure 3.17 shows a typical amperometric response after the addition of bradykinin to HUVEC with a cell density = $\sim 3 \times 10^5$ cells / ml. In Figure 3.17 (a), after HUVECs are stimulated with bradykinin, there is a sharp increase in current indicating robust NO production. About 5–25 s after the application of bradykinin, the maximum current, equivalent to a local NO concentration of approximately $250 \pm 47$ nM ($n = 6$) is achieved. This maximum current decreases gradually to the baseline after about 300s.
Control experiments are carried out to confirm that the change in measured current is due to the oxidation of NO released from HUVECs. After confirming that cell cultures are alive and active by stimulating with bradykinin, HUVEC cells are pretreated with a NOS inhibitor $\text{N}^G$-nitro-L-arginine methyl ester (L-NAME, Figure 3.17b), which should have the effect of blocking NO production in the live cells. After additional stimulation with bradykinin, the maximum currents showed a decrease of about 95% ($n=6$), indicating that NOS had been greatly inhibited and that less NO was produced. As a second control, bradykinin is added to the buffer medium without HUVEC cells (Figure 3.17c) where no signal response is measured, indicating that the response in 3.17(a) is not due to the addition of bradykinin alone, but is rather due to the stimulation of HUVEC with bradykinin.
Figure 3.17. Measurements of NO release from HUVECs stimulated by Bradykinin. (a) Typical amperometric response of NO release from HUVECs with the addition of 0.2 mM Bradykinin. (b) Typical response of NO release stimulated by Bradykinin from HUVECs pretreated with NOS inhibitor L-NAME. (c) Current response for addition of 0.2 mM bradykinin to the buffer medium without HUVEC cells. Applied potential = 0.8 V vs Ag/AgCl.
These overall experimental results demonstrate that the increase in current caused by stimulation of bradykinin is due to the oxidation of NO released from HUVECs. Even though, HUVEC cells are intensively studied to measure NO release stimulated by bradykinin, reported concentrations are varied broadly in the range of 100nM – 300nM [32]. Our finding represents the upper limits of NO released from endothelial cells.

3.2 Chapter III Part 2: Fabrication and Characterization of Non-breakable Ultra-micro Platinum Fiber Electrodes Modified with Nano-structured Ruthenium Oxide Catalyst

3.2.1 Introduction

Platinum (Pt) is extensively used as an inert metal working electrode for many processes, especially in fuel cell applications [36-39]. In addition, Pt has been widely explored as a construction element of various sensors [38]. Especially, sensing electrodes fabricated from Pt for NO determination are in steady development [25-27, 40-42]. In fact, the first reported amperometric NO probe developed by Shibuki is based on direct oxidation of NO on platinum [40, 42]. Shibuki’s prototype allowed World Precision Instruments Incorporation, (WPI) [27] to develop the a commercially available nitric oxide sensor using Pt electrode. However, similar to carbon fiber electrodes, the direct electrooxidation of NO on bare Pt electrode surfaces does not produce the highest degree of sensitivity and their selectivity is also poor.
As we already demonstrated, sensitivity and selectivity of NO electrochemical determination can be achieved through electrode surface modification using electrocatalytic platforms with high affinity for NO. It is known since the early 1960s that ruthenium modified platinum electrodes are more active than pure Pt electrodes for proton exchange membrane fuel cell (PEMFC) applications as efficient electrocatalytic platforms [43, 44]. As demonstrated in chapter II and chapter III part 1, because of the rich redox and coordination chemistry of ruthenium and the unique properties of the Ru-NO chemical bond, ruthenium oxide nanoparticles provide the right platform for the catalytic detection and quantification of NO. Electrochemical deposition of ruthenium and anodic oxidation of deposited ruthenium on Pt substrate is a well-known method for preparation of ruthenium oxide coatings [1, 2, 44].

Due to size-compatibility and non-invasive proximity to NO release sites in biological media, carbon fiber microelectrodes (CFEs) are usually the popular choice in electrochemical NO detection. However, many of these sensors are too prone to mechanical breakage, and difficulties are encountered with in vivo NO detection after prolonged periods. On the other hand, Pt fiber electrodes are flexible, yet nonbreakable, with high tensile strength that provides the proper platform for durable NO sensor fabrication.

Surface fouling is another problem encountered with long-term NO measurement in biological systems, which ultimately makes the electrodes unsuitable for these applications [25]. Pt offers the added advantage of developing reusable NO sensors because of its reproducing and renewing surface properties.
In this part of the chapter, we describe the fabrication and characterization of a flexible, nonbreakable NO sensor based on nano-structured ruthenium oxide modified Pt fiber microelectrodes. The ruthenium oxide nanoparticles are deposited on a single Pt fiber electrode by continuous cycling electrodeposition. We demonstrate how these ruthenium catalytic sites improve NO sensitivity compared to bare Pt fiber electrodes. Furthermore, we illustrate these results in terms of amperometric NO detection in standing solution.

3.2.2 Experimental Section

3.2.2.1 Materials and Apparatus

Platinum fibers (10 µm) are purchased from Goodfellow (Devon, PA). All other materials used as indicated in section 3.1.2.1. Similar experimental setup and instrumentation is used, as outlined in section 3.1.2.2.

3.2.2.2 Preparation of Integrated Single Platinum Fiber Ultra-microelectrodes

Platinum fiber microelectrodes (10 µm diameter) are prepared in-house using published procedures similar to CFE [3, 4]. Briefly, cleaned Pt fiber is mounted at the end of a copper wire and fixed with conductive epoxy. The mounted platinum fiber is inserted into a pulled glass capillary and sealed with non-conductive epoxy leaving ca. 2 mm fiber
exposed and a copper wire is then fixed to the stem of the glass tube with the non-conductive epoxy glue. The pulled end of the glass capillary is coated with Ag/AgCl ink and a layer of insulating material is placed over the body of the sensor leaving 3 mm of the Ag/AgCl exposed.

### 3.2.2.3 Electrodeposition of Ruthenium-oxide-type Catalyst on Platinum Fiber Microelectrode

The ruthenium oxides catalyst is typically preformed on the platinum fiber surface by electrochemical deposition as explained in section 3.1.2.4. However, this procedure requires optimization for Pt electrode and is described as follows. A freshly prepared Pt fiber electrode is immersed in an electrochemical cell with 10 ml of 10 mM HClO$_4$ containing 0.1mM (higher than amount used in CFE) precursors, while potential is continuously cycled between -0.85 V and +0.65 V at a scan rate of 100 V/s for a period of 15 minutes. Nafion® coating is carried out in a similar way as described in Section 3.1.2.4. After each electrode modification, the electrode is gently washed with deionized water, and allowed to dry before use.

### 3.2.2.4 Procedure

NO electrochemical analysis is carried out as described in section 3.1.2.7. Instead of CFE, bare or ruthenium oxide modified Pt fiber electrodes (PtFE) are employed as a
working electrode. The surface properties and composition of the ruthenium oxide modified PtFEs is characterized by field emission scanning electron microscopy (FESEM), and energy dispersive X-ray spectrometry (EDX). Amperometry is used to assess the performance of the modified fibers in terms of NO determination in standing NO solutions.

3.2.3 Results and Discussions

3.2.3.1 Electrodeposition of Ruthenium-Oxide-type Catalyst on Platinum Fiber Microelectrode

Figure 3.18 shows cyclic voltammograms obtained during continuous scanning modification of a typical 10 µm Pt fiber electrode in a RuCl₃ / HClO₄ solution. For clarity, only the initial portion of the modification is shown, and each trace represents 100 cyclic voltammograms.
Figure 3.18. A cyclic voltammetry for 0.1 mM RuCl₃ in 10 mM HClO₄ solution recorded at the Pt fiber microelectrode of 10 µm diameter. The potential of the electrode was cycled between -0.85 and 0.65 V at 100 V/s over 15 min. Cycles are recorded every 3 s and each trace represent 100 cyclic voltammograms.

Similar patterns of voltammograms are observed compare to CFE. During the early stages of modification the reduction current increases only in the region of the most negative potentials used, between -0.65 to -0.85 V. This triggers the initial nucleation of ruthenium-based electrodeposited material on the Pt fiber surface. After the initial steps of continuous scanning, the reduction current starts to increase rapidly at less negative potentials. The forward oxidation current also starts to increase at less positive potentials.
Therefore, as in the case of carbon fiber, after the formation of slowly growing ruthenium nuclei on the Pt surface, additional electrode processes leads to the electrodeposition of ruthenium oxides. Since the reduction of ruthenium surface oxides is a relatively slow process, [7] during the course of continuous scanning they can accumulate on the electrode surface. The capacitor-like behavior observed for electrodeposited coatings in ruthenium solutions is also a characteristic feature of ruthenium oxides [9-11].

Therefore, these voltammograms provide evidence that the modification by continuous scanning voltammetry in a RuCl$_3$ solution leads to an electrochemical deposition of ruthenium-oxide-type nanoparticles on a Pt fiber similar to carbon fiber surface. Indeed, SEM images provide the morphological evidence of the modified fiber surface.

3.2.3.2 Scanning Electron Microscopic Characterization

FESEM images of modified and bare Pt fibers are shown in Figure 3.19. The surface morphology of electrodeposited ruthenium oxides on a modified Pt fiber surface appears as “cauliflower” type nano-structures (Figure 3.19 b) and present a clear difference compared to bare Pt fibers (Figure 3.19 a).

High-magnified FESEM images (Figure 3.19 c) reveal the existence of typical nano-clusters of ruthenium oxide. These structures closely resemble the ruthenium oxide
nanoparticles grown on Pt surfaces [12, 13, 45] and provide the morphological evidence of the existence of the ruthenium oxide nanoparticles on Pt fiber electrode.

Figure 3.19. FESEM images of typical 10-µm platinum fibers bare (a), and ruthenium oxide electrodeposited (b) Pt fiber. High-magnification SEM images of Pt fiber surfaces (c) with cauliflower type nano-structured ruthenium oxide clusters.

The modification of electrodes with electrodeposited ruthenium oxide is monitored over time to determine the optimized method of deposition (Figure 3.20). As a starting point, the optimum conditions used to modify CFE were explored as a means to obtain full surface coverage. However, even after 30 minutes modification in 20 µM
RuCl₃ solution, expected coverage is not observed and some areas of the Pt fiber are uncoated, as shown in Figure 3.20b. After increasing the concentration of the RuCl₃ solution to 0.1 mM with 15 min modification time, full coverage was achieved (Figure 3.20c). Considering both voltammetric behavior and FESEM morphology, 0.1 mM RuCl₃ solutions and 15 minutes modification time were determined to be the optimized conditions for the modification process.

Figure 3.20. FESEM images of typical 10-μm platinum fibers (a) bare, (b) 30 min ruthenium oxide electrodeposited in 20 μM RuCl₃ solution, and (c) 15 min electrodeposition of 0.1mM RuCl₃ solution
3.2.3.3 Energy Dispersive X-ray Spectroscopy (EDX) Characterization

Figure 3.21 displays the EDX spectrum of modified Pt fibers with and without ruthenium oxide modification. Figure 3.21 (a) shows the modified electrode has the expected ruthenium peaks in the EDX spectrum. Signals for the Pt and Ru are closely overlapping and as indicated in zoom in image, the two most intense transitions for Ru, Lα^1 and Lβ^1 can be easily distinguished. Those peaks exactly correlate with reported values [13, 15]. As expected, only Pt peaks are observed in that region for a bare Pt fiber (Figure 3.21b).

These results confirm that electrodeposition of ruthenium oxide via RuCl₃ in perchloric acid solution results in small nucleation sites of ruthenium, ultimately grown into nano-structured clusters of ruthenium oxides on Pt fiber exactly correlate with CFE.
Figure 3.21. Energy dispersive x-ray (EDX) spectra of (a) bare and (b) ruthenium modified platinum fibers. Inset shows enlarged portion of the spectra.
3.2.3.4 Amperometric Detection of NO: Sensitivity and Detection Limit

Constant potential amperometry is used to investigate the electrocatalytic activity of nano-structured ruthenium oxides on the modified Pt ultramicroelectrodes towards nitric oxide. Figure 3.22A represents typical amperometric responses obtained for 100 nM to 6.4 µM aliquots of NO on a 10-µm unmodified (a) and ruthenium oxides modified (b) PtFEs. As clearly shown, the modified PtFE exhibits a significantly enhanced electrochemical response compared to the unmodified PtFE. As previously explained, the improved sensitivity is due to the electrocatalytic activity of ruthenium oxide nanoparticles on the Pt fiber surface that provide an enhanced kinetics of the NO oxidation reaction.

As NO decomposes rather quickly, a rapid response time is also a very important parameter for quantifying NO. Analysis of this data indicates a 90% response is observed less than 8 seconds (2-7 s) for the electrode modified with ruthenium oxides. The observed response time is fast enough to detect physiological levels of NO [23].
Figure 3.22. (A) Amperometric responses of 10-µm bare (a) and ruthenium oxides modified (b) Pt fiber electrode with successive additions of high nM aliquots of NO. The applied potential was + 0.8V. (B) The resulting calibration plots.
Figure 3.22B shows resulting calibration plots for unmodified (a) and ruthenium oxide modified (b) PtFEs in high nanomolar (nM) to low micromolar (µM) concentration range of NO. An excellent linearity is observed for the modified electrodes with correlation coefficient \((R^2)\) value of 0.9995 compare to the bare Pt fiber \((R^2=0.9834)\).

Due to the electrocatalytic ability and larger reactive surface of nano-structured ruthenium oxide, enhanced sensitivity is expected for our modified PtFE as in the case of CFE. As indicated from the slopes of calibration plots, the observed sensitivity for the ruthenium oxide modified PtFEs, 6.7 pA/ nM, is ~10 fold higher compared to bare PtFEs (0.63 pA/ nM). These results are attributed to the efficient electrocatalytic oxidation of NO by the deposited ruthenium oxide catalytic sites present on the Pt fiber surface.

Using the optimized condition of 15 min electrodeposition, which is used throughout this work, we found that we can reliably detect as low as 350 pM NO concentration, based on the analytical criterion of signal to noise ratio \(S/N > 3\), which is shown in Figure 3.23. This remarkable detection limit is a direct result of the fact that the response of our ruthenium oxide catalytic system is better and particularly enhanced at low NO concentration, as explained earlier.
Figure 3.23. Typical amperometric response of ruthenium oxides modified PtFE with the addition of 350 pM of NO. The applied potential is +0.8V vs. Ag/AgCl.

This enhanced response to nitric oxide is maintained even when compared with other systems reported for nitric oxide determination. Table 3.2.1 shows a comparison of performance in terms of sensitivity and detection limits for systems of similar geometry (cylindrical) and where the electrode size is in the same range (i.e. radii in the micrometers). More detailed comparisons of NO detection systems can be found in several review articles published elsewhere [25-27].

Our ruthenium oxide modified Pt fiber provides a significantly higher sensitivity as compared to other cylindrical Pt fiber electrodes. As indicated in part one of this chapter, our ruthenium oxide modified carbon fiber electrode shows better-normalized sensitivity. It is also worth noting that our ruthenium oxide modified PtFEs as NO-sensing platforms provide one of the best detection limits seen for similar systems.
Table 3.3. Comparison of normalized sensitivities and detection limits of few systems used as platforms for NO detection and quantification.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Modifier(s) (inner/outer layer)</th>
<th>Potential at which NO is determined (reference electrode)</th>
<th>Sensitivity (pA/ nM)</th>
<th>Normalized sensitivity (pA/ nM µm²)**</th>
<th>Detection limit</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pt/Ir (fiber (cylindrical; diameter = 50µm length = 0.5 mm)</td>
<td>None (Nafion® /WPI membrane)</td>
<td>0.86 V/Ag-AgCl</td>
<td>5.7</td>
<td>7.08 x 10⁻⁵</td>
<td>2.14 nM</td>
<td>[46]</td>
</tr>
<tr>
<td>Carbon (fiber (cylindrical; diameter = 7µm length = 2 mm)</td>
<td>None (Nafion® /WPI membrane)</td>
<td>0.86 V/Ag-AgCl</td>
<td>1.03</td>
<td>2.33 x 10⁻⁵</td>
<td>5 nM</td>
<td>[3]</td>
</tr>
<tr>
<td>Carbon (fiber (cylindrical; diameter = 7µm length = 2 mm)</td>
<td>None (Nafion/ cellulose acetate)</td>
<td>0.91 V/Ag-AgCl*</td>
<td>0.44</td>
<td>5.83 x 10⁻⁷</td>
<td>1 µM</td>
<td>[28]</td>
</tr>
<tr>
<td>Carbon (fiber (cylindrical; diameter = 7.8µm length = 0.5 mm)</td>
<td>PBPB (Nafion®)</td>
<td>0.80 V/Ag-AgCl</td>
<td>1.04</td>
<td>2.17 x 10⁻⁵</td>
<td>5 nM</td>
<td>[30]</td>
</tr>
<tr>
<td>Carbon (fiber (cylindrical; diameter = 7µm length = 2 mm)</td>
<td>Ruthenium oxide ((Nafion®)</td>
<td>0.80 V/Ag-AgCl</td>
<td>12.41</td>
<td>2.82 x 10⁻⁴</td>
<td>200 pM</td>
<td>Chapter II-Part 1</td>
</tr>
<tr>
<td>Pt (fiber (cylindrical; diameter = 10 µm length = 2 mm)</td>
<td>Ruthenium oxide ((Nafion®)</td>
<td>0.80 V/Ag-AgCl</td>
<td>6.7</td>
<td>1.1 x 10⁻⁴</td>
<td>350 pM</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Potential value converted from the SCE reference used in that work.
** Values calculated from reported sensitivities and electrode size used.
3.2.3.5 Amperometric Detection of NO: Selectivity of the Ruthenium Oxide Modified PtFEs towards NO

In addition to sensitivity, selectivity is also important for electrochemical NO determination in biological systems. As explained earlier, electrochemical determinations at low potentials are of a great advantage to avoid interference through a potential-based selectivity.

In previous reports and existing electrochemical sensors, the applied potential used to detect NO is normally between +0.85 and +1.1V vs. Ag/AgCl. While we have shown that the Pt electrode can be use to detect nM levels of NO at +0.8V vs. Ag/AgCl with great sensitivity, potentials as low as +0.5 V can also be used to determine NO with our system. In fact, Figure 3.24A shows that ruthenium oxide modified electrodes maintain even better (70-fold) increases in sensitivity for NO quantification compared to the bare Pt electrode, with a good linear response at an applied potential as low as +0.5 V vs. Ag/AgCl.
Figure 3.24. (A) Amperometric responses for bare (a) and ruthenium oxide modified (b) PtFEs with successive additions of NO, at a lower potential of +0.5 V vs Ag/AgCl. (B) The resulting calibration plots based on responses observed in (A).
3.4. Summary for Chapter III

Ruthenium oxides modified carbon fiber and Pt fiber microelectrodes have been prepared and characterized for quantification of NO. FESEM and AFM imaging techniques provided the evidence for the existence of typical nano-structured clusters of ruthenium oxide in the ca. 100 nm ranges on the modified fiber surface. Energy dispersive x-ray spectroscopy and Auger electron spectroscopy were used to determine the surface composition and coverage of the fiber surface and they show modified fiber surfaces contain 95.5% ruthenium and 4.5% oxygen.

We have particularly shown that the nano-structured ruthenium oxide acts as efficient electrocatalytic sites for the oxidation of NO. The electrocatalytic oxidation of the NO analyte is likely mediated by the Ru$^{4+}$/Ru$^{6+}$ redox couple. As a result, the NO response of ruthenium oxides modified CFEs and PtFEs are much enhanced compared to corresponding bare fiber electrodes. Under optimized conditions, the modified carbon fiber electrodes show ca. 25-fold enhanced NO response compared to unmodified electrodes while modified Pt fiber electrodes show ca. 10-fold enhanced NO response compared to unmodified electrodes.

The low detection limit (200 pM) and the high sensitivity of ruthenium oxide modified fiber ultramicroelectrodes show superior performance over other similar systems with platinum, glassy carbon, and gold electrodes in NO detection.
Also we expanded our improved NO sensor to measure NO at the level of live collection of cells, by using cell lines such as HUVEC. Measuring NO released from live single cells is critical to gaining fundamental understanding of many biological functions. The small size of our ultra-micro NO sensors are compatible with such analyses.

Finding the amount of NO released at the cellular level has the potential to shed light on the pathophysiology of this remarkable molecule. The method described here, which has been extended to other systems allows the determination of NO even in gas phase as well as in vivo systems which are described in the next chapter.

3.5. References


CHAPTER IV

ENHANCED NITRIC OXIDE SENSING ON MULTI-WALLED CARBON NANOTUBES MODIFIED WITH RUTHENIUM NANOPARTICLES

4.1 Introduction

Nanostructured Carbon-based materials, especially carbon nanotubes (CNTs), have received a great deal of attention for their possible electrochemical and catalytic applications in a wide variety of technical areas, thanks to their unique structural features, electronic properties, thermal conductivity, and electrochemical stability [1-3]. Generally, CNTs exist in two forms, namely multi-wall carbon nanotubes (MWCNTs) and single-wall carbon nanotubes (SWCNTs) as shown in Figure 4.1 [4]. SWCNTs consist of a single graphite sheet rolled seamlessly, producing a cylinder of 1-5 nm in diameter. MWCNTs can be visualized as concentric and closed graphite tubules with multiple layers of graphite sheet defining a hole with a diameter typically from 2-50 nm [5].
Due to their high surface areas, their central hollow cores and the outside walls, CNTs can be used as a superior material for highly sensitive nanoscale sensor fabrication [6, 7]. A wide range of electrochemical biosensors ranging from amperometric enzyme electrodes and DNA hybridization biosensors to various gas sensors has been reported [7-9].

In the case of sensors fabricated for quantification of NO, CNTs have been reported to have catalytic effects on the electrooxidation of NO [10, 11]. In these studies, NO sensors were fabricated by modifying the surface of glassy carbon electrodes or carbon fiber electrodes. However, the lack of solubility and difficulty of manipulation in many common solvents, which diminish the uniformity and reproducibility of the CNT film, has imposed major limitations on direct immobilization of CNTs on the electrode surface [7]. Several strategies have been proposed for immobilization of CNTs on electrochemical transducers. Some of these methods rely on dispersion of CNTs in
Poly(diallyldimethylammonium) (PDDA) [9], polyethylenimine [12], Nafion® [11, 13, 14], surfactants[10], and special solvents [15, 16].

The problems of agglomeration and poor dispersion of CNTs in ordinary solvents can also be minimized through chemical modification or functionalization of CNT surfaces [6, 17, 18]. In fact, chemical functionalization of CNT can be used to attach desired chemical species, which improve solubility and also biocompatibility of these tubes. Although such treatments cause changes in delocalized π orbital systems of CNTs, the resulting modified nanotubes are well dispersed in various solvents and form uniform and stable thin films at the surface of modified electrodes.

There have been numerous research studies on modifying CNTs [18], especially with metallic nanoparticles as catalytic sites. The simple goal of these methods is to increase the solubility and specific areas, and enhance the catalytic activities, of the electrodes. As explained in previous chapters, metal nanoparticle-modified electrodes usually exhibit high electrocatalytic activities towards compounds with sluggish redox processes at bare electrodes.

An interesting class of CNT derivatives is obtained by depositing metallic or semiconductive nanoclusters on the CNT surfaces [19, 20]. Such hybrid nanosized materials made from metals /metal oxides and CNTs are promising for a wide array of applications in nanoscale devices and nanoelectronics. Furthermore, uniform dispersion
of metallic nanoparticles immobilized on CNT surfaces can yield ideal nanocatalysts for application in chemically modified electrodes.

There are numerous studies on the decoration of CNTs with noble metal nanoparticles, due to their enhanced catalytic activity in the oxidation of methanol in fuel cells and the hydrogenation of aromatics [21, 22]. Especially, platinum (Pt) and Pt based alloys such as Pt-Ru, Pt-Ni, and Pt-Sn have been prepared and studied as possible catalysts for the electrooxidation of methanol in fuel cell applications [21, 23, 24]. CNT-supported ruthenium catalysts exhibit high selectivity in heterogeneous catalysis as compared to other carbon substrates [25]. As described in a previous chapter, we have particularly shown that ruthenium nanoparticles act as efficient electrocatalytic sites for the oxidation of NO. in this work, we explore the possibility to use ruthenium nanoparticles as catalytic site for NO detection on carbon nanotubes.

Recently, a number of approaches such as chemical vapor deposition [26], chemical reduction [27], electrodeposition [28], electrostatic force directed assembly [29], and supercritical fluid synthesis [30], have been reported to prepare metal nanoparticles deposited on CNTs. However, with the exception of chemical reduction, there is rather limited control over the surface coverage of metal nanoparticles, i.e., the density of decoration is low in many cases. In addition, metal nanoparticles are loosely bound and tend to detach from the surface of CNTs under external disturbance such as ultrasound [31]. Among the available approaches, the controllable mild condition in the
liquid-phase reduction process has advantages and facilitates the formation of a uniform layer of metal nanoparticles.

In this chapter, we describe an electrochemical sensor for NO detection using ruthenium-nanoparticle-decorated multi-walled carbon nanotubes on microelectrodes. The acid treated MWCNTs are decorated with Ru nanoparticles by chemical reduction of the corresponding metal salts using ethylene glycol (EG) as a reducing agent. These composite materials are characterized by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy dispersive X-ray spectroscopy (EDX).

As we will demonstrate the fabricated NO sensor exhibits excellent catalytic behavior towards NO oxidation as assessed by cyclic voltammetry and amperometry. This leads us to believe that the sensor can be adapted for preparation of implantable microchip NO sensors. The microchip sensor is conceptually based on a Ru-MWCNT array of microelectrodes deposited on a silicon chip substrate.
4.2 Experimental Section

4.2.1 Materials and Apparatus

MWCNT powdered cylinder cores, 25-35% MWCNT basis (I.D. × L = 2-15 nm × 1-10 μm), and ethylene glycol are products of Sigma-Aldrich Corp (St. Louis, MO). All other materials used are as indicated in section 3.1.2.1. Similar experimental set-up and instrumentation is used, as outlined in section 3.1.2.2. Bare or ruthenium oxide modified glassy carbon electrode or CFEs were used as working electrodes. Transmission electron microscopy characterization is carried out on Cu grids using Tecnai F30 ST field-emission transmission electron microscope (TEM). Scanning Electron Microscopy (SEM) characterization is carried out on uncoated or palladium-coated samples using a Hitachi S-4500 Field-Emission Scanning Electron Microscope (FESEM) equipped with energy dispersive X-ray (EDX) elemental analysis capability.

4.2.2 Ru-Nanoparticles-Decorated MWCNTs Preparation

MWCNTs are purified and oxidized using following literature protocols [9, 31]. Typically, commercial MWCNTs (250 mg of MWCNTs) are refluxed in 500 mL of 2.0 M HNO₃ for 2 days. The MWCNTs dispersion is allowed to settles overnight, and the clear solution above the suspension is then removed. The solid form (about 15 mL of suspension) is separated by ultracentrifugation (30 min at 14 000 rpm). The purified MWCNTs are further oxidized by treatment with 15 mL of 1:3 HNO₃/H₂SO₄ mixtures for
2 hours in an ultrasonic bath. The suspension is diluted 10 times with water after removal of the clear supernatant, and the resulting sample is dried under vacuum overnight.

In the ruthenium nanoparticle decoration procedure, 0.2 g of the acid-treated MWCNTs are dispersed in 100 mL of ethylene glycol, and then 0.25 g of ruthenium (III) chloride is added to this suspension. The suspension is adjusted to pH 9 with 1 M NaOH, and is heated to 150 °C for 4 h with constant stirring. After heating, the product is centrifuged, washed with deionized water and dried under vacuum. Scheme 4.1 shows an illustration of the procedures used to prepare Ru-nanoparticle-decorated MWCNTs.

Scheme 4.1. Schematic illustration of the preparation of Ru-nanoparticle-decorated MWCNTs.
4.2.3 Preparation of the Ru nanoparticle /MWCNT Composite Film Modified GC Electrode

The modification procedure is carried out by dropping 20 μL of 10 μg/mL aqueous solutions of Ru-nanoparticle-decorated MWCNTs on the surface of glassy carbon (GC) electrodes (denoted as nano-Ru/MWCNT/GCE). Prior to modification, GC electrodes are polished with 0.3 μm alumina slurries, washed, sonicated for about 1 minute, and then rinsed with water. Modified electrodes are allowed to dry for 4 hours at room temperature. The dried electrode is carefully rinsed with water prior to electrochemical measurements. The oxidized MWCNT film modified electrode (denoted as oxyMWCNT/GCE) is prepared in a similar way by dropping 20 μL of 10 μg/mL aqueous solutions of oxidized MWCNTs on the surface of GC electrodes. Scheme 4.2 shows an illustration of the preparation of the Ru nanoparticle/MWCNT composite film on GC electrodes.
Scheme 4.2. Schematic illustration of the preparation of oxidized MWCNT and Ru nanoparticle/MWCNT composite film modified GC electrodes.
4.2.4 Preparation of the Ru nanoparticle/MWCNT/Nafion® composite film modified CFE electrode

The Ru/MWCNT/Nafion® composite film is typically electrodeposited on the carbon fiber surface using a chronoamperometric technique. A total of 0.5 mg of purified Ru/MWCNTs are dispersed in 1mL 0.5% Nafion®-ethanol solution, which is used as the modifying solution. A freshly prepared carbon fiber electrode is immersed in a Ru/MWCNT/Nafion® solution, and then the 10 mV potential pulse given for 500 ms as shown in scheme 4.3 (denoted as nano-Ru/MWCNT/CFE). The oxidized MWCNT/Nafion® composite film is typically preformed in a similar procedure using 0.5 mg of purified MWCNTs dispersed in 1mL 0.5% Nafion®-ethanol solution as the modifying solution (denoted as oxyMWCNT/CFE).
Scheme 4.3. Schematic illustration of the preparation of oxidized MWCNT and Ru nanoparticle /MWCNT composite film modified CFE electrodes.

4.2.5 Procedure

A similar procedure is carried out as described in section 3.1.2.7. Nano-Ru/MWCNT/GC, oxyMWCNT/GC, nano-Ru/MWCNT/CFE, and oxyMWCNT/CFE serve as the working electrodes. The surface properties and composition of the modified
electrodes are characterized by TEM, FESEM, and EDX. Cyclic voltammetry and amperometry are the techniques used to assess the performance of the electrodes in terms of NO detection in standing NO solutions.

4.3 Results and Discussion

4.3.1 Transmission Electron Microscopy (TEM) Characterization

Figure 4.2 shows typical TEM images of oxidized MWCNTs and their corresponding Ru-decorated MWCNTs. The acid-treated MWCNTs are entangled together because of their high surface area and the van der Waals attraction between them (Figure 4.2a,c). The formation of bundles of MWCNTs derived from the self-assembly of oxidized MWCNTs is directed by the functional groups on the surface [31]. After the reduction of ruthenium chloride in ethylene glycol solution, the oxidized MWCNTs are fully covered with small ruthenium nanoparticles, which are densely packed on the surface as shown in Figure 4.2b,d.
Figure 4.2. Typical TEM images of oxidized (a,c), Ru decorated (b,c), MWCNTs.

It is well known that the functional groups on the surface of acid-treated MWCNTs not only improve the dispersion and stability of CNTs in solvents, but also act as active sites for the adsorption of metal ions and the deposition of metal nanoparticles in the reduction process [32, 33]. During the concentrated acid treatment, amorphous carbon is preferentially oxidized to highly reactive oxygen-containing functional groups, the amount of which increase as a function of oxidation time [34]. It has been estimated
that the number of acid sites is $10^{21}$ sites per gram in the boiling nitric acid-treated CNTs [34]. The adsorption of ruthenium ions and the deposition of nanoparticles could happen preferably at those positions with acid sites available, so that the deposition of nanoparticles is controlled by the density of acid sites. A high density of active sites will give rise to improved loading with ruthenium nanoparticles.

High-resolution TEM (HRTEM) images of oxidized and Ru-decorated MWCNTs, as shown in Figure 4.3, indicate ruthenium nanoparticles are very uniform with an average size of 5 nm. Ethylene glycol, acting as the reducing agent, favors the production of small metal particles [35]. In our case, ethylene glycol gradually reduces the ruthenium clusters when the temperature reaches 150 °C, and then ruthenium clusters grow into nanoparticles in the process. In this way, ruthenium nanoparticles grow dense, and assemble onto MWCNTs. The resulting Ru/MWCNT composites materials is an ideal platform for improved response to NO as analyte.
4.3.2 Scanning Electron Microscopic Characterization

The Ru/MWCNT/Nafion® and oxidized MWCNT/Nafion® composite films are typically preformed on the carbon fiber surface using chronoamperometric electrodeposion. Typical FESEM images of modified and bare carbon fibers are shown in Figure 4.4. Comparing the surface of the bare carbon fiber with that of the MWCNT/
Nafion®, it can be seen that the modified fiber is coated with a thin layer of materials that is similar to previously reported Nafion® coated CFEs (Figure 4.4b) [11, 13]. Also, it is important to note that Ru/MWCNTs are embedded in the Nafion® layer, which acts as a selective membrane for determination of NO.

Figure 4.4. FESEM images of typical 7-µm bare (a), oxidized MWCNT (b) Ru/MWCNT modified (c) surfaces of the carbon fiber electrodes. Also visible, high-magnification SEM image of carbon fiber surfaces with thin layer of Nafion®, embedded with Ru/MWCNTs.
4.3.3 Energy Dispersive X-ray Spectroscopy (EDX) Characterization

Energy dispersive X-ray spectroscopy (EDX) is utilized as a qualitative analytical tool to characterize the surface composition of the modified surface. Figure 4.5a displays the EDX spectrum of the modified surface with the expected ruthenium peaks. The two most intense transitions for Ru, Lα₁ and Lβ₁ correlate exactly with reported values [36, 37]. No peaks are observed in that region for a bare surface (Figure 4.5b).

![Figure 4.5](image-url)

**Figure 4.5.** Energy dispersive x-ray (EDX) spectra of bare (b) and Ru/MWCNT modified (a) surfaces.
4.3.4 Electrocatalytic activity of Ru/MWCNTs modified electrodes towards nitric oxide

First, we examined the electrocatalytic activity of MWCNT and Ru/MWCNT towards NO using cyclic voltammetry. Figure 4.6 shows the typical cyclic voltammograms obtained at 100 mV/s for GC electrodes modified with oxidized MWCNT and Ru/MWCNT in the presence of 80 µM NO in pH 7.0 phosphate buffer solutions. The behavior of the modified GC electrode is to be compared to the response of unmodified GC electrode in the presence of NO (Figure 4.6a).

The oxidation potential of NO on unmodified GC electrode at about +1.15 V correlates to the NO oxidation potential measured on solid graphite electrodes reported elsewhere [38]. In the presence of MWCNTs, the oxidation of NO occurs at a much lower positive potential with the onset of oxidation current starting as early as ~+0.4V vs. Ag/AgCl. As we established in chapter two, the early oxidation potential and the larger currents observed in the presence of NO are both characteristic features of electrocatalytic oxidation of nitric oxide.

Since NO is a strong π-acceptor ligand [39, 40], it is very likely that a strong binding is established between NO and the sp² hybridized electrons of the CNTs’ outer graphene layer [41]. The existence of strongly bound NO-CNT product is supported by the irreversible response also corresponding to the electrocatalytic nature of the MWCNT.
As expected, due to the superior electrocatalytic nature of ruthenium, Ru/MWCNT modified electrodes show much more enhanced response as compared to both oxidized MWCNT and bare GC electrodes. The current response observed in Figure 4.6c is due solely to the oxidation of nitric oxide added and further additions result in proportional increases of oxidative current (*vide infra*).

**Figure 4.6.** Cyclic voltammograms of unmodified (a), oxidized MWCNTs (b), and Ru/MWCNTs (c) modified GC electrodes in the presence of 80 µM NO. Potential scanned between 0 and +1.3 V. Scan rate, 100 mVs⁻¹.
4.3.5 Amperometric Detection of NO on GC Electrodes

Constant potential amperometry is also used to investigate the electrocatalytic activity of Ru/MWCNT towards nitric oxide. Figure 4.7A represents typical amperometric responses obtained for low µM aliquots of NO on unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs modified (c) GCEs. As clearly shown, the modified GC electrode exhibits a significantly enhanced electrochemical response compared to the unmodified GCE. As previously explained, the improved sensitivity is due to the electrocatalytic activity of ruthenium nanoparticles and MWCNTs on the electrode surface that provides enhanced kinetics of the NO oxidation reaction.

Figure 4.7B shows resulting calibration plots for unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs modified (c) GCEs in the low µM NO concentration range. Excellent linearity is observed for the nanoRu/MWCNTs modified electrodes.
Figure 4.7. (A) Amperometric responses of unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs (c) modified GCEs with successive additions of NO; applied potential +0.8 V. (B) Resulting calibration plots.
4.3.6 Amperometric Detection of NO on CFEs

As noted earlier, designing and fabricating an NO sensor with spatial and temporal resolution is very important for *in vivo* measurements. Due to their size-compatibility necessary to offer non-invasive/non-destructive close proximity to the site of NO release in biological media, the carbon fiber microelectrodes (CFEs) are useful in electrochemical detection of NO. Therefore, CFEs are used to further investigate the improved response of Ru/MWCNTs composites to NO as an analyte. Chronoamperometric electrodeposition is used to modify the carbon fiber surface with Ru/MWCNTs.

Figure 4.8A represents typical amperometric responses obtained for high nM aliquots of NO on an unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs modified (c) CFEs. The modified CFEs exhibits the expected enhanced electrochemical response compared to the unmodified CFE. This is a direct result of the electrocatalytic response of NO on MWCNT and embedded Ru nanoparticles in MWCNT.

Figure 4.8B shows resulting calibration plots for unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs modified (c) CFEs. An excellent linearity is observed for the nanoRu/MWCNTs modified electrodes with correlation coefficient ($R^2$) value of 0.998. In general, the observed sensitivity of nanoRu/MWCNTs for the studied NO range is around 1pA/nM at +0.8 V potential, and is at least 10 times more sensitive than unmodified CFEs.
Figure 4.8. (A) Amperometric responses of unmodified (a), oxyMWCNTs (b), and nanoRu/MWCNTs (c) modified CFEs with successive additions of NO. Applied potential +0.8 V vs. Ag/AgCl. (B) The resulting calibration plots.
Biologically more relevant lower concentrations of NO are also explored using this amperometric method. The typical staircase amperometric responses obtained for low nM concentration of NO are displayed in Figure 4.9A, along with the resulting calibration curve Figure 4.9B.

Using optimized conditions, we found that we can detect reliably as low as 500 pM NO concentration, based on the analytical criterion of signal to noise ratio, S/N > 3, which is shown in Figure 4.10. This detection limit is a direct result of the fact that the response of our ruthenium oxide catalytic system is better and is particularly enhanced at low NO concentration (Sub-saturation).
Figure 4.9. (A) Amperometric responses of nanoRu/MWCNTs modified CFE with successive additions of NO; Applied potential +0.8 V. (B) The resulting calibration plots.
Figure 4.10. Typical detection limit of nanoRu/MWCNTs modified CFE at 500 pM NO. Applied potential = +0.8V vs. Ag/AgCl.

4.4 Summary for Chapter IV

Ruthenium-nanoparticle-decorated multi-walled carbon nanotubes on microelectrodes are fabricated and characterized by SEM, EDX, and TEM. These modified electrodes showed high electrocatalytic response for NO oxidative detection. NO response of MWCNT modified electrodes is much enhanced compared to unmodified electrodes. Due to the strong π-acceptor ability of NO, strong binding is established between NO and the sp² hybridized electrons of the CNTs. The existence of strongly bound NO-CNT product is supported by the irreversible response and corresponds to the electrocatalytic nature of the MWCNT. In addition, we have shown that ruthenium nanoparticles decorated MWCNT provide further enhanced response characteristics due to electrocatalytic nature and high surface area of ruthenium nanoparticles. Under optimized conditions, the modified Ru-MWCNT modified electrodes show 10-fold enhanced response for NO compared to unmodified electrodes.
4.5 Future Direction

With recent advances in fabrication technologies, the size of microelectrodes can be further reduced down to nanometer scale. Such nanoelectrodes have shown unprecedented temporal and spatial resolution as well as extremely high sensitivity [6, 7]. For instance, a nanoelectrode array based on vertically aligned multiwalled carbon nanotubes (MWNTs) embedded in SiO$_2$ is used for ultrasensitive DNA detection, Figure 4.10 [42].

Figure 4.11  SEM images of MWCNT nanoelectrode arrays: (a) 3×3 electrode array; (b) array of MWCNT bundles on one of the nine pads; (c) and (d) arrays of MWCNTs at UV lithography and e-beam patterned Ni spots, respectively. The scale bars are 200, 50, 2 and 5 µm, respectively. Reprinted from [42]
As we discovered, due to the efficient electrocatalytic nature of nanoRu/MWCNTs for the oxidation of NO and their nano-scale size, Ru nanoparticles and MWCNTs composite materials can be easily adapted to fabricate ultra-small microchip electrodes for sensitive quantification of NO. The proposed microchip sensor is conceptually based on a Ru-MWCNT array of microelectrodes deposited on a silicon chip substrate, similar to aforementioned DNA sensor.

4.6 References


CHAPTER V

RUTHENIUM OXIDE COLLOIDS AND NANOWIRES AS ELECTRO CATALYTIC PLATFORMS FOR GAS PHASE NO DETECTION

5.1 Introduction

Nitric oxide is a highly diffusible radical gas and has been known for many years to be a toxic pollutant in car exhaust fumes, fossil fuels and cigarette smoke. Environmental Protection Agency has established national ambient air quality standards that require oxides of nitrogen (NO and NO₂) level to be below 0.053 ppm (annual average) [1]. NO gas can be oxidized in the air into NO₂, which can be toxic to lung tissue and is a major contributor for ground-level ozone formation. Extended inhalation of high concentration of nitric oxide can lead to hypotension, sepsis, hemorrhage and other adverse conditions [1-3].
As we outlined in chapter one, the discovery of the biological functions of NO in the 1980s came as a complete surprise and caused high activity in research. NO is produced by mammalian cells and can be detected in the exhaled air at the ppb level [4]. The level of exhaled nitric oxide is elevated in a number of diseases related to airway inflammation due to induction of iNOS gene [5]. NO is a well-known vasodilator. Therefore, potential therapeutic use of inhaled nitric oxide as a selective pulmonary vasodilator has been extensively explored in recent years [6, 7]. Because nitric oxide can also serve as an inflammometer (inflammation marker) in conditions like asthma, there has been increasing interest in the use of exhaled nitric oxide as a breath test in diseases involving airway inflammation [8, 9].

Inhaled nitric oxide therapy is typically administered using mechanical ventilators. The levels of delivered NO and side product NO₂ must be continuously monitored to ensure safety/effectiveness of the NO therapy and to prevent potential toxic effect of NO₂ [10]. In addition, measurement of exhaled NO can be an easy, non-invasive procedure that helps diagnosing disease conditions such as asthma [9, 11], airway infections, allergic rhinitis, and bronchiectasis [1, 3, 12].

Due to aforementioned reasons, there has been an increasing demand for accurate, stable and long-lasting measurement devices for nitric oxide gas at and below the ppm level. Currently, chemiluminescence is widely used for determining NO concentration in gas phase [13-15]. However, some of the limitations associated with chemiluminescence
are bulky instrumentation, amount of time required, cost, and the need of multiple reagents.

Electrochemical methods are also reported for the measurement of NO in gas phase. According to the operating principle, three main classes of electrochemical gas sensors; namely, amperometric, potentiometric, and conductometric sensors have been developed to measure NO. Amperometric methods are most commonly used. A modified Clark-type gas sensor, solid polymer electrolyte (SPE) such as Nafion, reticulated vitreous carbon (RVC) combined with Nafion, Au/SPE, Pt/SPE have been employed as amperometric nitric oxide gas sensors[16-18]. However, sensitivity, selectivity, response time, and signal stability of currently available methods have not yet been fully addressed. Thus, improvements in NO gas sensors are still needed.

In this section, we will describe two types of NO gas sensors. The first approach is based on RuO₂ colloids modified carbon paste electrode. As described in chapter II, RuO₂ colloids modified CPE towards NO measured in solution phase is adapted to NO determination in gas phase. RuO₂ colloids as catalytic sites for NO detection are capitalized on to fabricate improved NO gas sensor.

The second method consists of state of the art new generation of NO gas sensor based on clusters and arrays of ruthenium oxide nanowires. Nanostructures, such as nanowires, nanotubes, and nanorods, of various materials have attracted considerable attention from the scientific community because they exhibit unique electrical [19-21]
and optical [22, 23] properties that can be exploited for nanoscale device fabrication. Among them, the electronic and sensing properties of nanowires have been widely studied because of their enormous surface-to-volume ratio and high density of surface sites. Specially, the nanowire arrays of metallic materials have attracted great interest for gas sensing applications [24].

Various nanowire fabrication methods are in steady development. These emerging technologies can be based on template synthesis, microlithography, scanning probe lithography (AFM nanomachining), chemical vapor deposition, and electrochemical techniques [25]. Electrodeposited nanowire synthesis can overcome the limitations of other methods due to the relative ease of fabrication and surface modification. A wide range of sensing materials can be deposited by electrodeposition, including metals, alloys, metal oxides, semiconductors, and conducting polymers [26]. Electrodeposition allows a high degree of specificity in location and chemical identity of a deposit, as well as control of thickness [26]. Electrochemical step edge decorating technique pioneered by the Penner group shows great promise in nanowire fabrications [27-30]. They reported formation of many kinds of nanowires on the step edges of highly oriented pyrolytic graphite (HOPG) and their application as gas sensors [31].

Using a similar approach, we developed ruthenium oxide nanowires on HOPG surface with the aid of fast scanning cyclic voltammetry and chronoamperometry techniques. Clusters and arrays of these nanowires are then explored as a potential NO gas sensor.
5.2 RuO₂ Colloids Modified CPE Based NO Gas Sensor

5.2.1 Materials and Apparatus

Compressed NO gas (99.5%) and 1 ppm NO balanced with N₂, are purchased from Air gas (Burr Ridge, IL). All other chemicals and apparatus are used as previously outlined in section 2.2.1.

5.2.2 Experimental Designs

An amperometric NO gas sensor is fabricated in-house out of polypropylene centrifuge tubes for use with the CPE, and is fitted with an Ag/AgCl reference electrode as well as a Pt auxiliary electrode immersed in the pH 7.00 phosphate buffer solution (Figure 5.1). The loosely packed carbon paste is directly exposed to gases to be measured and is in contact with a Cu wire as a current collector.

Vertically aligned Polypropylene centrifuge tube is used to house counter/reference electrodes and electrolyte. The prefabricated CPE is placed tightly against a Cu ring that is connected to a Cu wire. The working electrode is in contact from one surface with the electrolyte solution. Teflon tubing is used as inlet and outlet for the gas chamber on the opposite side.
**Figure 5.1.** Schematic drawing of an amperometric gas sensor assembly. Dimensions are not proportional to actual sizes and are for illustration purpose.

### 5.2.3 Results and Discussion

#### 5.2.3.1 Amperometric Detection of NO

Constant potential amperometry is used to investigate the response characteristics of CPEs towards NO in gas phase. The gas sensor is conditioned by polarizing at the
+0.8 V (vs. Ag/AgCl) potential under a N$_2$ stream. When the purging gas is switched to NO, both unmodified (a) and RuO$_2$-modified (b) CPEs show increasing current responses, Figure 5.2. After introducing N$_2$ gas again, response gradually goes back to the original baseline indicating that the observed increased responses are solely due to the NO gas.

The modified CPE exhibits a significantly enhanced electrochemical response compared to the unmodified CPE. This is indeed a direct result of the electrocatalytic response of NO on embedded RuO$_2$ particles on the carbon paste.

![Typical amperometric response curves of unmodified (a) and RuO$_2$-modified (b) CPE NO sensor. The sensor was polarized under a N$_2$ gas, applied voltage of 0.8 V (vs. Ag/AgCl).](image)

**Figure 5.2.** Typical amperometric response curves of unmodified (a) and RuO$_2$-modified (b) CPE NO sensor. The sensor was polarized under a N$_2$ gas, applied voltage of 0.8 V (vs. Ag/AgCl).
The magnitude of the current response is directly proportional to the concentration of the NO gas. Figure 5.3 shows a typical response curve of RuO$_2$-modified CPE gas sensor for varying concentration of NO. The response time seemed to vary from 10-25 s depending on the concentration of NO and individual sensors. In general, the observed sensitivity of NO gas sensor for the parts per millions (ppm) range of NO is around 32 pA/1ppb, Figure 5.3B.
**Figure 5.3.** (A) Typical amperometric response curves of RuO₂-modified carbon paste NO sensor with successive additions of NO. The sensor was polarized under a N₂ gas, applied voltage of 0.8 V (vs. Ag/AgCl). (B) Resulting calibration plots based on amperometric responses recorded in (A).
As we noticed in solution phase, much larger sensitivity is observed for lower ranges of NO concentrations, and similar trend is observed for NO in gas phase as well. Figure 5.4A represents response measured for NO in ppb levels and enhanced sensitivity calculated from the dose-response curve (Figure 5.4B) is 53 pA / ppb.

The lower detection limit is estimated to be 20 ppb, based on the analytical criterion of signal to noise ratio S/N > 3, which is shown in Figure 5.4C. The sensitivity and detection limit achieved by our NO gas sensor is not as good as some recently published gas sensors [16]. However, the fabrication of RuO$_2$-CPE based NO sensor is simple, low cost, and it produces consistent quality in measuring NO.
Figure 5.4. (A) Amperometric responses of RuO$_2$-modified NO gas sensor to NO in the ppb range at 100, 200, 400, ppb of NO (B) Calibration plot that results from the dose-response amperometric curve. Applied potential is +0.8V. (C) Typical detection limit of NO sensor at 20 ppb NO.
5.3 Clusters and Arrays of Ruthenium Oxide Nanowires Based NO Gas Sensors

5.3.1 Materials and Apparatus

Highly Oriented Pyrolytic Graphite (HOPG) surfaces (ZYA and ZYH Grades) are obtained from GrafTech (Cleveland, OH). Compressed NO gas (99.5%) and 1 ppm NO balanced with N₂, are purchased from Airgas. All other chemicals are used as previously outlined in section 2.2.1.

Cyclic voltammetry and chronomperometry (CA) measurements are performed using BAS-100B electrochemical workstations. Scanning Electron Microscopy (SEM) characterization is carried out with Hitachi S-4500 Field-Emission Gun Scanning Electron Microscope (FESEM) equipped with energy dispersive X-ray (EDX) elemental analysis capabilities. AFM was performed with a Agilent pico-SPM using the MAC mode interfaced with a PicoScan controller.

5.3.2 Fabrication of Ruthenium Oxide Nanowires on HOPG Surface

Ruthenium oxide nanowires are obtained by electrodeposition from an aqueous plating solution onto the step edges present on the surface of HOPG as an electrode (Scheme 5.1). The electrodeposition was performed in perchloric acid solution containing 20 µM RuCl₃, with continuous scanning (cyclic voltammetry) or stepping (chronoamperometry) the potential of the underlying electrode.
5.3.3 Results and Discussion

5.3.3.1 Electrodeposition of Ruthenium Oxide Nanowires

The synthesis of ruthenium oxide nanowires using electrochemical step edge decoration (ESED) involved the step edge selective electrodeposition of ruthenium oxide on HOPG electrode surfaces, as shown in Scheme 5.1. The nanowire synthesis method employed here based on a similar method carried out by Penner and co-workers [28]. We adapted this ESED method to prepare Ru nanowires using cyclic voltammetric continuous scanning and chronoamperometric approaches, Figure 5.5. As shown in
Figure 5.5A, CVs for a HOPG electrode immersed in the plating solution is virtually similar to the CVs reported for electrodeposition of ruthenium oxide nanoparticles on CFE and PtFE (section 3.1.3.1 and 3.2.3.1). This, typical CV response is characteristic of the capacitor-like behavior observed for ruthenium oxides coatings electrodeposited from ruthenium solutions on various surfaces [32-34]. After the two cycles of fast scanning process (100 V/s in our case) ruthenium oxides nanowires are formed on the edges of the HOPG surface.

**Figure 5.5.** Typical cyclic voltammograms (A) and chronoamperometric response (B) recorded during the modification of the HOPG surface.

The preferential growth of the ruthenium oxide nanowires on the step-edges of the HOPG may have at least two origins: First, it is widely accepted that the kinetics of electrochemical processes (electrodeposition in our case) are much faster in step-edges...
compared to basal plane [35]; second, mass transport at the relatively nanoscopic-wide step-edges tends towards radial diffusion [36]. This is translated into faster mass transport per unit area, and thus faster growth of nuclei along the step edges. These nuclei tend to coalesce with adjacent ones along the step edge to form nanowires as evidenced by SEM and AFM (see later). However, significant amounts of ruthenium oxides nanoparticles also deposited on the basal planes with CV method as noticed from AFM. Therefore, chronoamperometry (CA) is used to achieve more control and preferential growth of nanowires on the step-edges of HOPG.

CA is used as an electrochemical method for ruthenium oxides deposition, and simultaneously, as a technique suitable for controlled electrochemical nucleation. Figure 5.5B shows typical CA response obtained for nanowire formation in perchloric acid solution containing 20 µM RuCl₃ as precursor of the underlying HOPG electrode. In CA experiments, the potential is stepped from the open-circuit potential to the potential at which the deposition of ruthenium oxides would occur. Under these conditions, the system made a transition from no reaction to the steady-state reaction, controlled by the rate of mass transfer of ruthenium ions toward the electrode surface.

In the case of heterogeneous systems under diffusion control, nuclei formed on the surface contribute to the active surface area available for reaction [37], the step-edges of the HOPG in our case. Initial current increase for heterogeneous systems is due to the increase of surface area whenever the nucleation is involved and as nucleation progresses, the nuclei will begin overlapping. Each nucleus will define its own diffusion
zone through which ruthenium ions have to diffuse, representing the mass-supplying mechanism for continuation of growth [38]. Within the diffusion zone, growth of already-established ruthenium oxides nuclei can continue, or additional nucleation can be initiated on various sites preferentially on the step edges, both governed by the steady state conditions.

5.3.3.2 AFM Characterization of Ruthenium Oxide Nanowires

Figure 5.6 shows the typical two dimensional AFM image and line profile analysis of ruthenium oxide nanowires obtained by continuous scanning electrodeposition from RuCl₃/perchloric acid solution onto the step edges present on the surface of HOPG. AFM analysis indicates that ruthenium oxide nano-structured colloids on the step-edges combined to form continuous nanowires. According to profile analysis, the nanowire thickness is in the order of 10-15 nm. However, significant amounts of isolated nanoparticles of ruthenium oxides are also present on the basal planes with CV electrodeposition method.

Longer and continuous nanowires are obtained by chronoamperometric electrodeposition as shown in Figure 5.7a. It is worth noting that clear distinction of nanowires roughly parallel to each other could be observed for the sample prepared by CA electrodeposition method. The length of the nanowires varied from hundreds of nanometers to several microns. A high-magnification three dimensional AFM image of a
single nanowire also is reported in figure 5.7b. In this case, the nanowire is 50 nm in diameter and ~25 nm in height.

**Figure 5.6.** (a) AFM image indicating continuous nanowires of ruthenium oxides. (b) Line profiles of the nanowires reported in image (a).

**Figure 5.7.** AFM images indicating continuous nanowires (a). High-resolution 3-D image of a single nanowire (b).
5.3.3.3 SEM Characterization of Ruthenium Oxide Nanowires

A low-magnification SEM image of a HOPG surface with nanowires growth using chronoamperometric electrodeposition technique is shown in Figure 5.8. An important characteristic of the nanowires prepared by electrochemical step edge decoration is their length [28]. This image shows many nanowires with lengths exceeding 200-400 µm. In general, these nanowires are aligned parallel with one another because of the nature of the step-edges on the HOPG surface.

Figure 5.8. SEM image of an HOPG surface after the deposition of ruthenium oxide nanowires. The step edges present within individual grains on the HOPG surface are oriented parallel to one another.
Nanowires varying in diameter from 15 to 200 nm are obtained. As seen from Figure 5.9, the diameter of these wires depends not only on the method of preparation, but also on the size of the step-edges present in the HOPG substrate. From the SEM images shown in Figure 5.9b and 5.9c, it is apparent that diameters of 25 nm and 100 nm of these nanowires can be obtained by varying the pulse width of the CA methods. It is worth noting that these SEM images clearly show that ruthenium oxide nanowires possess a high degree of uniformity.

**Figure 5.9.** Typical low-magnification (a), (c) and high-magnification (b), (d) SEM images indicating uniform/continuous nanowires with varying diameters.
**5.3.4 Future Direction (Work in Progress)**

Work in progress is exploring the use of Ru nanowires for NO gas sensing. Freshly deposited ruthenium oxide nanowires are transferred from the graphite electrode surface onto a glass slide coated with Nafion. When the Nafion film is hardened (2 hours), arrays of nanowires are contacted with silver epoxy from two sides. Only nanowires long enough to span these distances were involved in sensor function. NO gas measurement is carried out in a closed glass chamber. The sensing layer is to be faced against the gas inlet at a continuous flow of NO or N\textsubscript{2} gas in the chamber. The current to voltage (I-V curve) is then obtained for N\textsubscript{2} and NO gas passed through the measuring chamber.

While this work is still underway, preliminary results in our hands confirm the Ru nanowires can be used to discriminate few 100s of ppm of NO using I-V curves.
5.4 Summary for Chapter V

We successfully developed two type of gaseous NO sensors. The first approach is based on RuO₂ colloids modified carbon paste electrode. As we noticed in solution phase, enhanced response is observed for NO gas in ppb levels and improved sensitivity calculated from the dose-response curve is 53 pA/ppb. The lower detection limit is estimated to be 20 ppb, based on the analytical criterion of signal to noise ratio S/N > 3. The fabrication of RuO₂-CPE based NO sensor is simple, low cost, and it produces consistent quality in measuring NO.

The second method consists of state-of-the-art new generation of NO gas sensors based on clusters and arrays of ruthenium oxide nanowires on Nafion modified electrodes. These nanowires are fabricated and characterized by SEM and AFM. Their use after lift off from graphite surface is being explored as a potential NO gas sensing platform.

5.5 References


23. Niemeyer, C.M., and Ceyhan, B. (2001). DNA-Directed Functionalization of Colloidal Gold with Proteins This work was supported by Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. We thank Prof. D. Blohm for helpful discussions and generous support. Angew Chem Int Ed Engl 40, 3685-3688.


