ELECTROCHEMICAL MODULATION OF NITRIC OXIDE RELEASE USING DIAZENIUMDIOLATED SPECIES

BY

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UNDERGRADUATE THESIS

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ABSTRACT

Nitric oxide (NO) has many different potential applications in healthcare. It functions as a primary vasodilator, which helps to widen arteries and allow unrestricted blood flow. Nitric oxide has also been confirmed to kill microbes in the body (bacteria and fungi). Further, NO is known to enhance wound healing and is a potent antiplatelet agent (i.e., prevents blood from clotting). This research project focuses on developing a method to control/modulate the release of NO effectively to a given site in the body in small, concentrated amounts for various purposes (e.g., killing of microbes, preventing thrombosis, or killing cancer cells). The method is based on electrochemical modulation of NO release from well known proton-driven NO donors, namely diazeniumdiolates. Nitric oxide release from these molecules is proton dependent. Hence, by oxidizing water at an electrode surface, the local pH of a layer adjacent to the electrode will decrease, greatly increasing the rate of NO release from a diazeniumdiolate solution reservoir in contact with the electrode. Our initial goal is to devise a small silicone rubber catheter with electrodes and a diazeniumdiolate reservoir within the lumen, and allow NO to pass through the tubing wall to the surrounding solution area. Such an approach can yield a combined thromboresistant and bactericidal intravascular catheter design.

To Francis Soehnel

ACKNOWLEDGEMENTS

I would like to thank numerous people that made this life experience possible. I wholeheartedly appreciate all of the time, money, and support that my advisor Professor Mark E. Meyerhoff has given to me. Without him, none of this research would have been possible, nor would my current passion of chemistry exist. Also, Bo Peng, who has been more than a tremendous help to me by teaching me basically everything I need to know to survive and how to think like an analytical chemist. My group members who constantly gave me support and help, especially Gary Jensen and Natalie Crist. Finally, to my parents, grandparents, and friends who give me ceaseless encouragement for what I love doing the most in my life.

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INTRODUCTION

Nitric oxide (NO) is one of the most well known biological gases that is produced inside the body[1]. It is a polar, radical molecule produced physiologically by Nitric Oxide Synthases (NOSs). There are three types of NOS: nNOS, eNOS, iNOS. All of the aforesaid synthases use the amino acid L-arginine, along with oxygen, to produce gaseous NO as well as L-citrulline. Upon production of the NO, and due to its instability, it is easily scavenged by many molecules in the body (e.g., hemoglobin, oxygen, superoxide), which can lead to oxidative/nitrosative stress (Fig. 1). Also, NO can only travel one-cell length before it is diluted over 200 times its original concentration[2]. Thus macrophages that generate NO must produce high concentrations and be in very close proximity to the targeted area/species (e.g., microorganisms) to be effective.

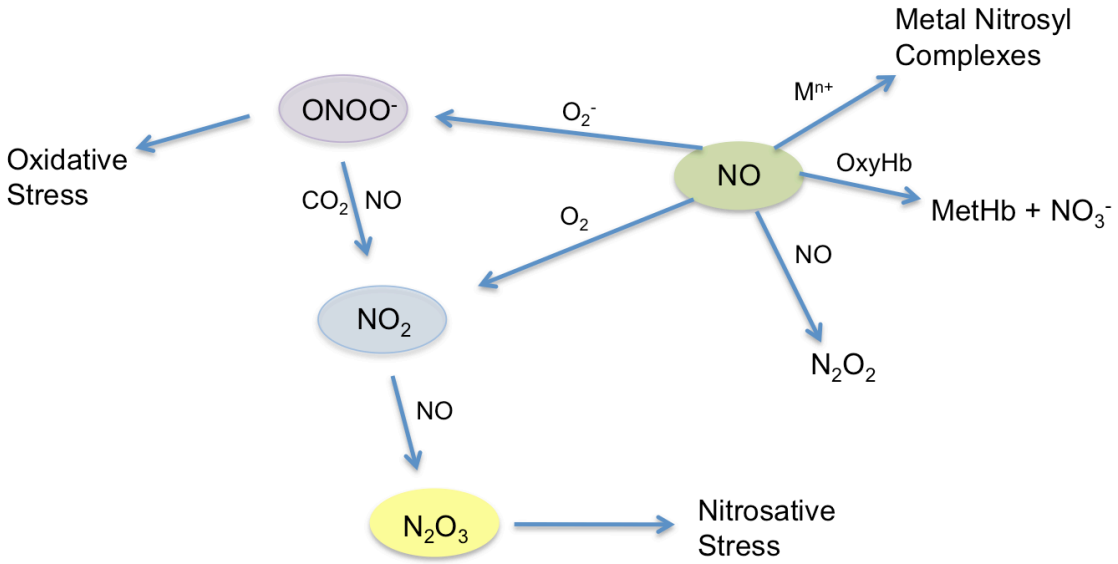
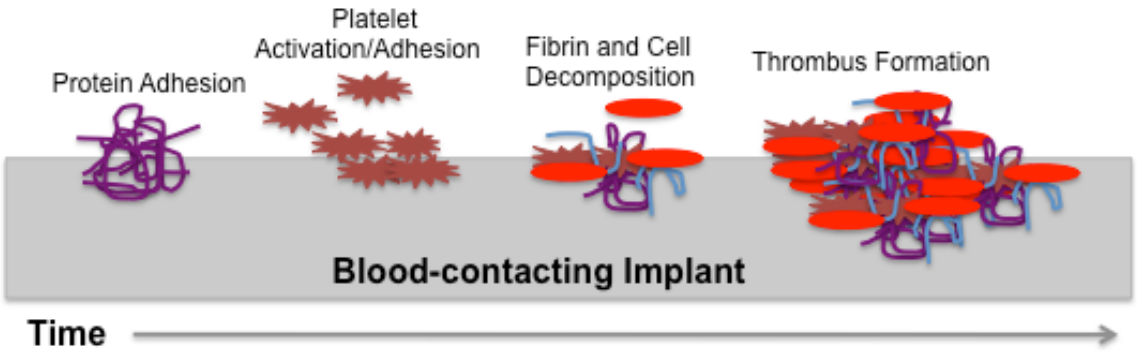


Figure 1. Common physiological reactions of NO.

There are many roles that NO can perform inside the body depending on its propinquity and locus. In daily medical procedures, catheters, vascular grafts, etc., are implanted or inserted into patients. Over time, these objects become coated with proteins,

platelets, and fibrin, leading to clotting (Scheme 1). A catheter would have to be removed and re-inserted, leading to discomfort for the patient and time for the nurse. However, upon coating the catheter with an NO-releasing agent (e.g., diazeniumdiolates, S-nitrosothiols), platelet adhesion significantly decreases, and risk of infection and biofilm formation is also likely to be reduced[4].



Scheme 1. Thrombus formation process on graft or catheter in the body[3].

Along with its anti-thrombotic activity, NO also is a bactericidal agent. Everyday all sorts of bacterial organisms build up on our skin. In a hospital setting, however, this is hazardous due to the already abnormal health condition of the patient along with open skin access into the body (e.g., catheter sites), thereby, increasing infection risks. Biofilm formation can be prevented through a constant flux of NO from a catheter, thus, diminishing the bacteria around the high-risk site on the patient[3]. Vasodilatation is another key characteristic of NO[1]. Upon release from eNOS /iNOS, nitric oxide is able to permeate into smooth muscle and vascular cells causing a relaxation process. Blood vessels are dilated, thereby increasing blood flow to necessary areas (e.g., hypoxic environments). This primary aspect of NO has been utilized for many reasons, one being for sensitizing cancerous tumors to chemotherapy[5]. Chemotherapy is a widely used process for aiding patients in ridding malignant cells. Malignant tumors constrict blood vessels in the local area, which allows for a significant decrease of the chemotherapy drugs in the locus. However, by treating with both NO and chemotherapy, the drug is able to reach the tumor unimpeded. Vasodilatation through NO release has also been extensively researched to aid in penile erections[6]. eNOS is able to produce NO, which

diffuses into smooth muscle tissue. Upon entering the tissue, an increase in the activity of guanylyl cyclase occurs, which elevates cGMP levels. This growth in cGMP levels triggers a reduction of cytoplasmic Ca^{2+}, thereby relaxing the corpora cavernosa.

Concentration is a crucial facet of NO that when modified even slightly can cause a complete reversal in the role of the radical molecule (Fig. 2). NO can be produced in a myriad of amounts in the body. Macrophages produce NO in the body in high concentrations, through the aid of iNOS. Such high levels of NO can lead to nitrosative stress and thus apoptosis. However, it has been shown that even at such high levels of NO (1 μM), nitrosative stress might not be the only outcome. Increased concentrations of NO can lead to nitration of transferrin receptors, the receptors that control iron uptake, leading to a decrease in iron transference into the cell. Low levels of iron, prevent apoptosis from occurring and thus has the exact reverse effect of nitrosative stress[7]. On the other hand, lower levels of NO are produced by both endothelial and stromal cells. Unlike macrophages, which can produce micromolar concentrations of nitric oxide, skin cells use eNOS, which can produce only nanomolar amounts of NO. Under these conditions, NO has different properties, most of which promote angiogenesis, cell proliferation and progression. Concentrations as low as 1-30 nM can promote cell growth[2]. Cell growth however includes both normal body cells, along with cancer cells. Tumors are able to grow under nanomolar concentrations, mostly due to the angiogenic properties of NO[8]. The combination effect of NO by activating cyclooxygenase-2 (COX-2), thereby releasing growth factors such as eicosanoids and prostanoids, promote angiogenesis[9,10].

There are numerous NO donors that have been prepared synthetically that can be used for the required purpose, but the most important donors for this research are diazeniumdiolates (NONOates). There are different types of NONOates (e.g., half-lives, structures), but the two that are extensively studied in this research are NONOated PEI and diethylamine (DEA/NO) (Fig. 3). One aspect that most NONOates have in common is their stability in alkaline solutions.

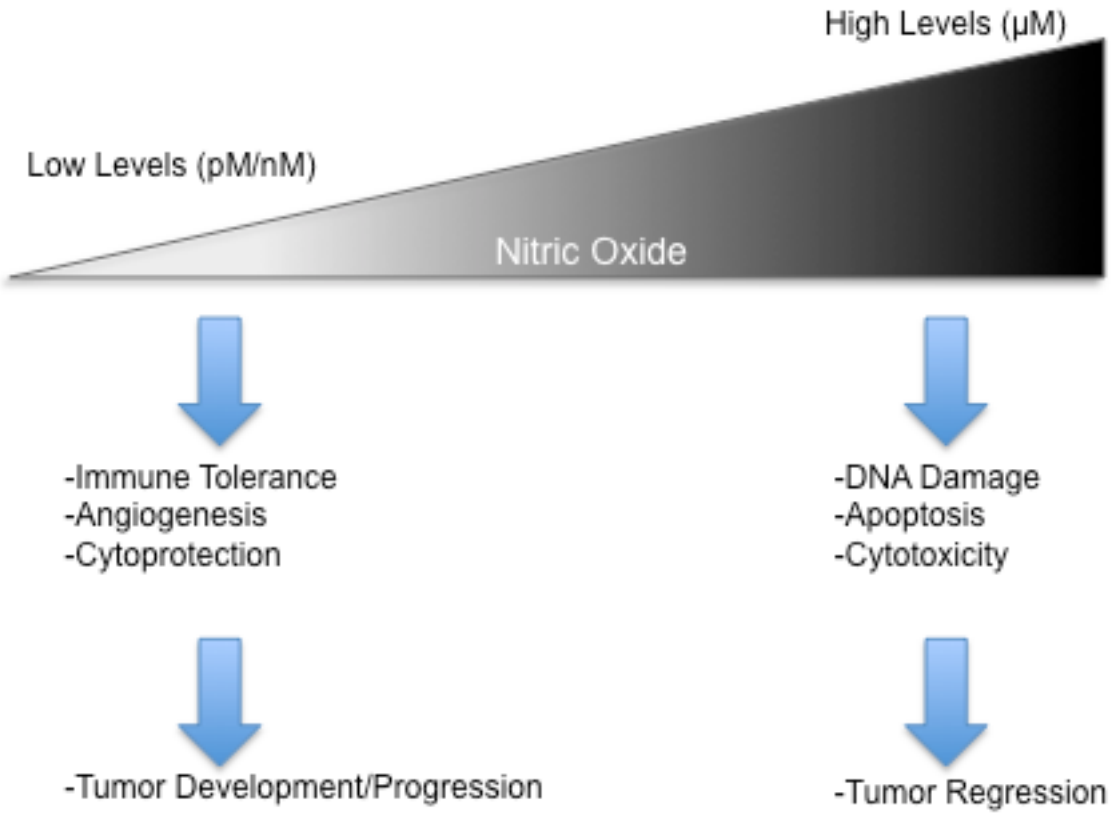


Figure 2. Illustration of physiological effects of various amounts of NO[8].

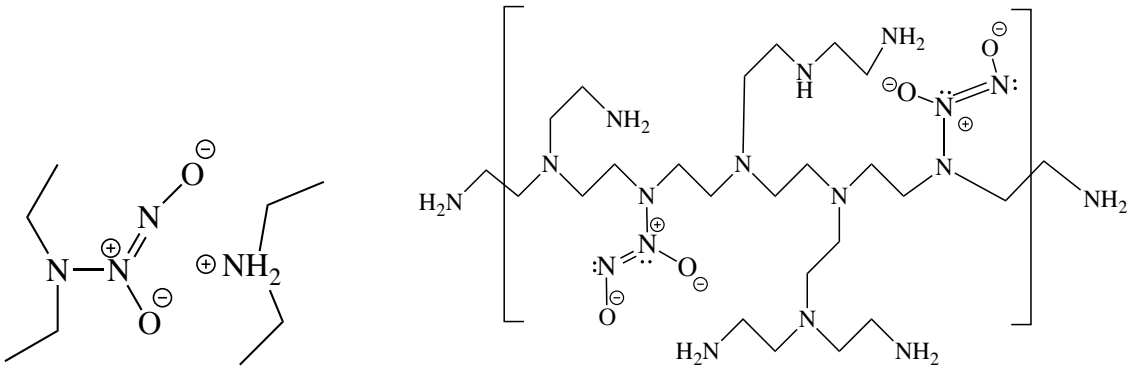
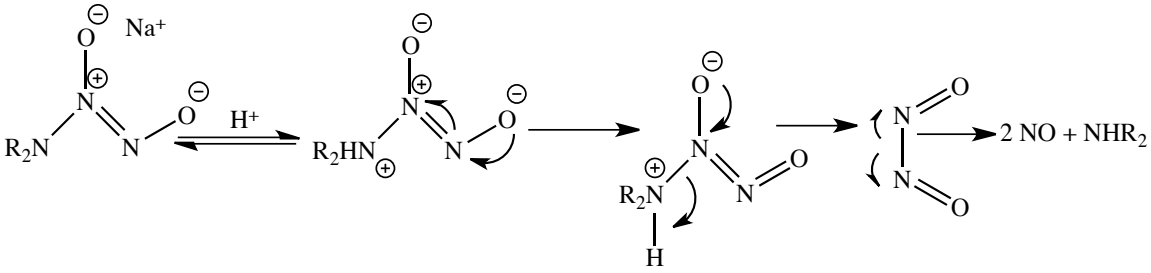


Figure 3. Diethylamine (left) and polyethyleneimine (right) NONOates.

NONOates release NO upon reaction with a hydrogen ion (Scheme 2), so under basic conditions where the amount of available hydrogen ions is extremely low, the concentration of the NONOates remains relatively constant. The half-lives of the NONOates not only highly depend on pH, but also the buffer that is employed[11]. Disodium diazen-1-ium-1,2,2-triolate (OXI/NO), for example, is affected by nitrite that is in solution. Every NONOate has its own half-life that is predetermined by its structure. NONOated spermine (SPER/NO) ($t_{1/2}$ = 5-50 min. at pH 7.4[12]) dimerizes as the concentration increases, thus, increasing the half-life since the stability of the dimer occurs[13]. DEA/NO ($t_{1/2}$ = 2 min. at pH 7.4[12]) is a small molecule, compared to other larger NONOates. For any particular research that is done, the best NONOate comes from its stability in high pH, along with its rate of NO release at physiological pH of 7.4



Scheme 2. Mechanism of NO release from hydrogen ion driven reaction.

The goal of this research is to create a system that can release NO electrochemically using the protons produced upon the oxidation of water. Diazeniumdiolates in alkaline solutions are stable NO reservoirs. Near the electrode's surface, the pH drops significantly due to proton production, and NONOates in the vicinity will release their NO. Mass transfer in the solution will allow for more NONOates in the bulk solution to reach the electrode's surface, which creates a constant release of NO until the potential is turned off thus causing a stop to proton production. Upon creation of such a system in a catheter model, the NO produced can diffuse through the walls and enter into the necessary area (e.g., malignant tumor). The benefits of such a system allow for a stable reserve of NO, and a continuous release of it when required. An on/off switch for NO release would then be properly created.

MATERIALS AND METHODS

Materials

18.2 MΩ Millipore water was used to prepare all buffer solutions. Sodium methoxide was a product of Fluka (St. Louis, MO). Silicone tubing [I.D:.020 in. O.D: .0370 in.] was purchased from HelixMark (Carpinteria, CA). High (~25,000 g/mol) and low (~800 g/mol) M.W. polyethylenimine (PEI), sodium trimethyl silanolate (95%), methanol, sodium ascorbate, sodium formate, guanine, pyridoxal, resorcinol, and sodium dithionite were from Sigma-Alrich (St. Louis, MO). Tetrahydrofuran (THF) and diethyl ether were from Thermo Fisher Scientific Inc. (Wayne, MI). Spermine/NO, diethylamine/NO were from Cayman Chemical Company (Ann Arbor, MI). Teflon coated 0.0055'' diameter gold wire from A-M Systems (Carlsborg, WA), Teflon coated 0.250mm diameter silver wire from Medwire (Mount Vernon, NY), 100-mesh gold 2.5 cm x 3.5 cm gauze from ALS (Tokyo, Japan) were utilized. A pyrolytic graphite carbon rod, a 3 mm diameter gold electrode, a 3 mm diameter platinum electrode, and a Ag/AgCl electrode were all purchased from CHI Industries (Austin, TX). All of the chemicals' purities are as supplied by the company unless otherwise stated.

Preparation of Diazeniumdiolated Polyethylenimine (NONOated PEI)

High M.W. PEI was dissolved in a 25% w/w sodium methoxide in methanol solution. THF was added once the PEI dissolved. The solution was then transferred to a high-pressure NO reactor. The reaction vial was purged with argon to remove any oxygen. Once removed, NO was loaded to ~70 psi and held constant for three days. On the third day, the solution was a white/yellow liquid, which was washed with diethyl ether to precipitate the NONOated PEI and then subsequently washed with THF/ether to remove any unreacted PEI. A vacuum pump was used to dry the product for 24 hours. The solid white/yellow product was stored in the freezer near -20°C where it remains quite stable for long periods of time.

Characterization of NONOated PEI

Two methods were used to ensure that the diazeniumdiolate was indeed generated from the reaction: UV-Vis absorbance and chemiluminescence. A Lambda 35 UV-Vis instrument was used to observe a highly resolved NONOate peak at ~252 nm[14].

Chemiluminescence was then performed to determine the amount of NO release from the NONOated PEI on a Siever 280i Nitric Oxide Analyzer (NOA). Small aliquots of the NONOated PEI were injected into 0.183 M H_2SO_4 in a nitrogen-purging environment. The diazeniumdiolate was acidified, and the produced NO was carried into the instrument[15]. A plot of NO release (ppb) vs. time (min.) was generated. Using a calibration constant, the amount of NO released in the injection could be calculated in moles of NO.

Electrochemical Release Methods

CH Instruments Electrochemical Analyzer (Austin, TX) and Gamry (Warminster, PA) Reference 600 were used as the potentiostats for electrochemical testing. All given potentials are vs. Ag/AgCl unless otherwise noted. iR compensations were performed before each required run to ensure no shortages or malfunctions would occur in the experiment.

Oxidation of NO Overtime During Electrochemical Release

A 0.5 mg/mL of NONOated PEI was dissolved in 10 mM carbonate buffer with 100 mM NaCl. One-milliliter of this solution was kept on the benchtop over night as a control, while 10 mL were used for electrochemical testing. A potential of +0.80 V was applied for 17 hours, while measuring the NO release using the NOA. The working, reference, and counter electrodes are as follows respectively: gold mesh (S.A. = 8.75 cm^2), commercial Ag/AgCl, pyrolytic graphite rod. After the electrochemical generation of NO, two 15-μL aliquots of both the benchtop and electrochemically used solution were injected into 0.183 M H_2SO_4 to fully release all of the NO.

Titration of PEI and NONOated PEI

A 2 mg/mL solution was made of PEI (20 mL solution) in 100 mM NaCl. HCl (0.1 M) was added drop-wise and the amount added was recorded until the pH dropped to about 2.5, which was measured using a calibrated glass pH electrode. A plot of pH vs. amount of HCl was generated using Microsoft Excel.

RESULTS

Characterization of NONOated PEI

In order to prove that the PEI has been successfully diazeniumdiolated in the NO reactor, UV and chemiluminescence data were utilized. From the UV spectra (Fig. 4), the characteristic diazeniumdiolate peak was located at 252 nm[14].

Integration of the peaks from chemiluminescence (Fig. 5) yielded a total release of $9.71 \times 10^{-9} \pm 3.94 \times 10^{-10}$ mol NO from 50 μg of NONOated PEI, thus giving an average diazeniumdiolation of 8%. This implies that for every gram of the synthetic NONOated PEI, only 80 mg of the solid is diazeniumdiolated.

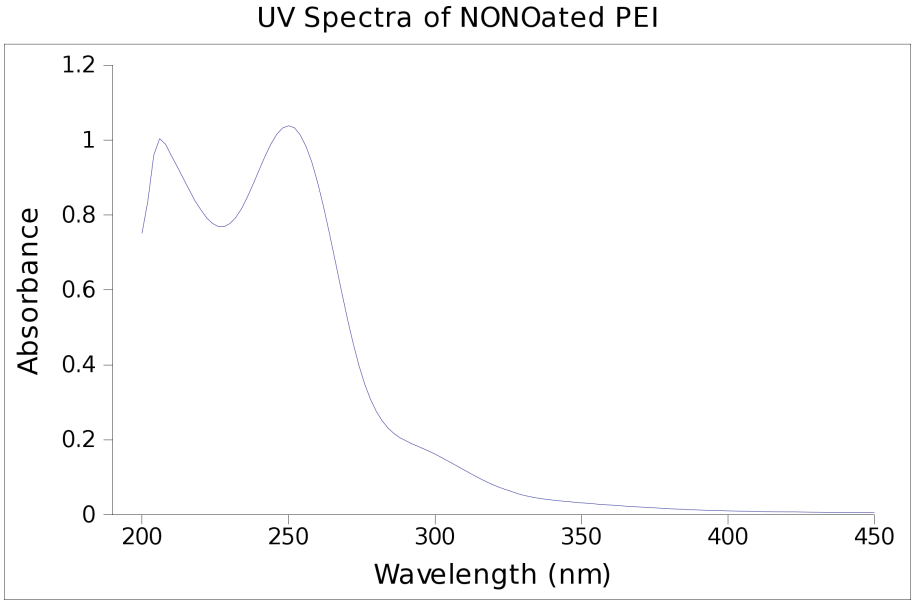


Figure 4. Ultraviolet Spectra of NONOated PEI with NONOate peak at 252 nm.

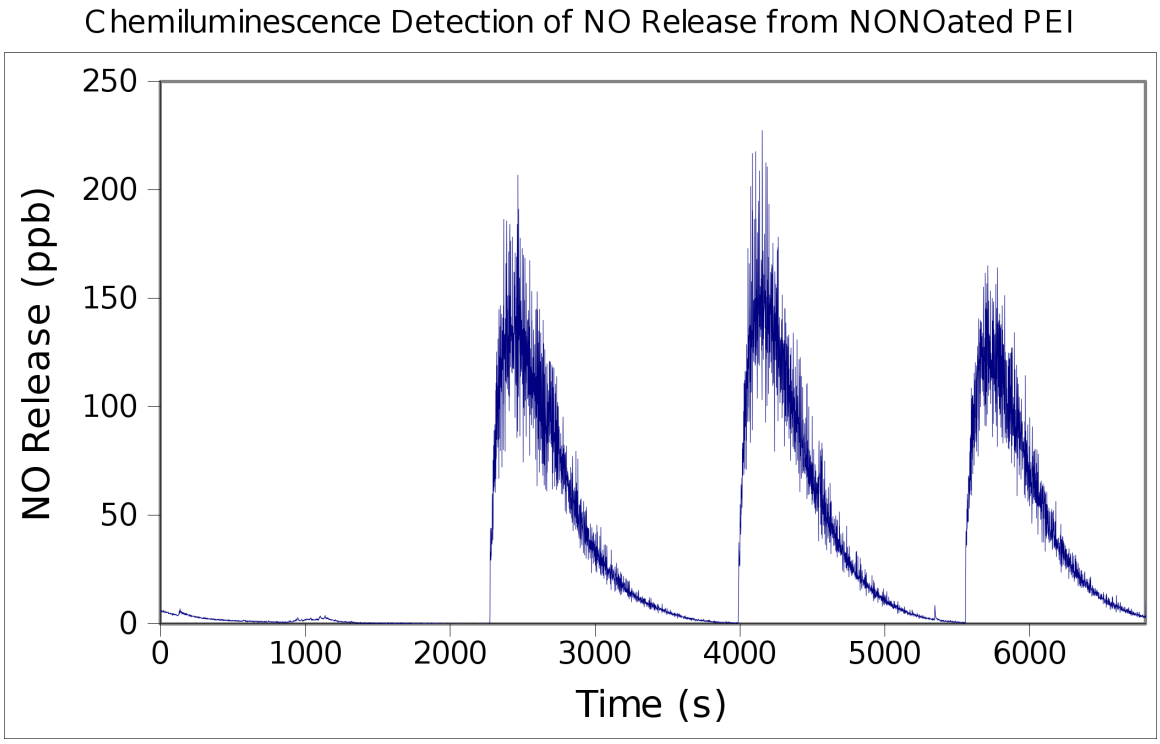


Figure 5. Release of NO from 3-50 μL injections of 1 mg/mL NONOated PEI dissolved in 10 mM PBS (pH= 10). Acidic solution was 0.183 M H_2SO_4.

Optimization of Electrochemical Release Using Various Working Electrodes

Different optimum applied potentials were obtained for various electrochemical systems (Table 1). For gold, platinum and carbon electrodes, the optimum potential for the highest, constant NO release was +0.80 V, +0.87 V, and +1.0 V, respectively, using a Ag/AgCl reference electrode (see Supporting Information section for exact experimental results).

System	Working	Counter	Optimum Potential
1	Gold mesh	Pyrolytic Graphite Rod	+0.80 V
2	Platinum Coil	Pyrolytic Graphite Rod	+0.87 V
3	Pyrolytic Graphite Rod	Platinum Coil	+1.0 V

Table 1. Various electrochemical systems for generating NO via NONOated PEI.

Borate Buffer vs. PBS Buffer

Using the same electrochemical setups, NO release was measured using the NOA while having the gold mesh as the working, commercial Ag/AgCl in 3 M NaCl as the reference, and a platinum coil as the counter. The solution was a 2 mg/mL of NONOated PEI dissolved in 10 mM PBS (Fig. 6) and a separate solution containing the same concentration of diazeniumdiolate in 10 mM borate buffer (Fig. 7). A potential of +0.80 V was held for 3-ten minute intervals.

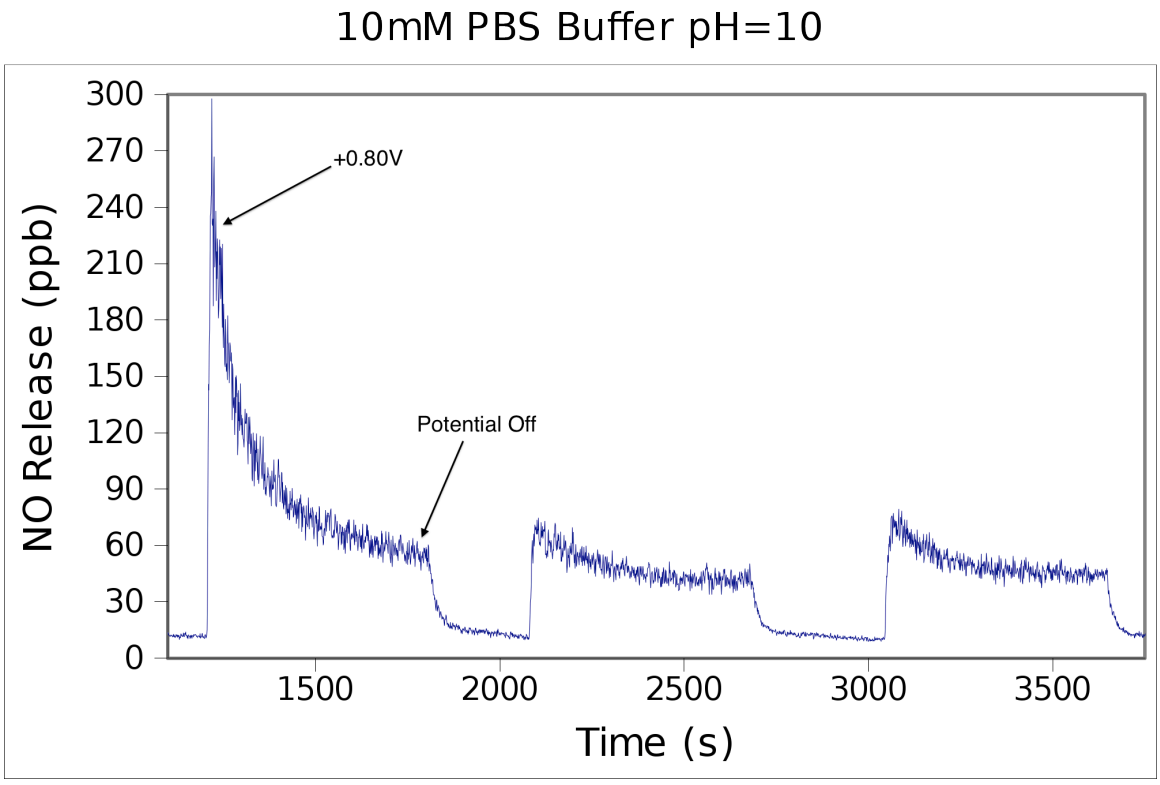


Figure 6. NO Release from 2 mg/mL NONOated PEI dissolved in 10 mM PBS (pH=10).

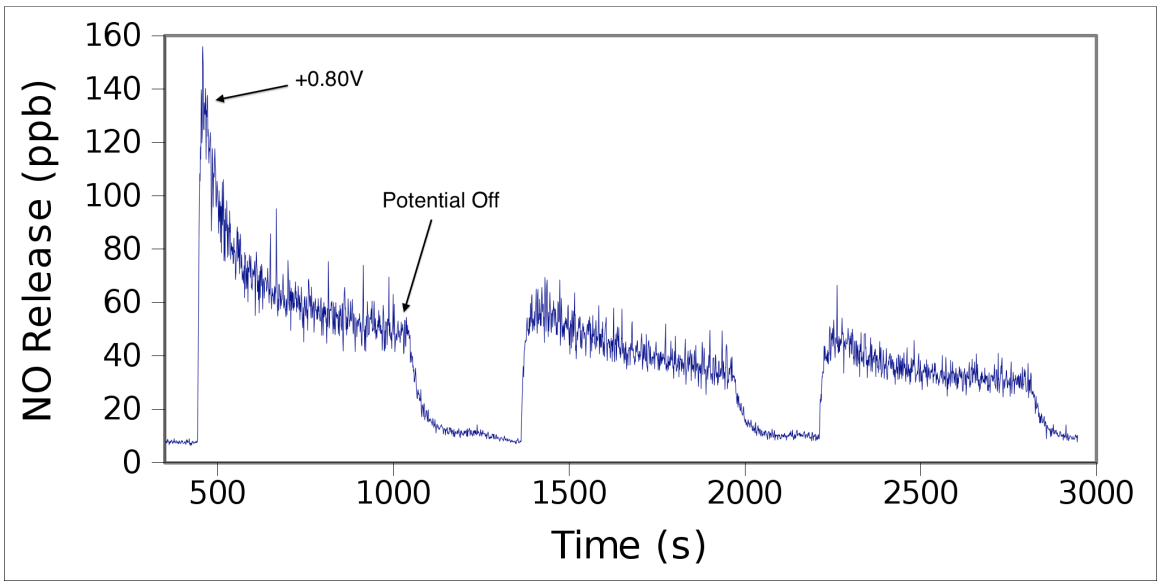


Figure 7. NO Release from 2 mg/mL NONOated PEI dissolved in 10 mM borate (pH=10).

Stability of NONOated PEI in PBS vs. Carbonate Buffer (Control Experiments)

A 2 mg/mL sample was created in 10 mM PBS and in 10 mM carbonate buffer both at pH 10.30. After connecting to NOA cell, the following NOA graphs were obtained for PBS (Fig. 8) and carbonate (Fig. 9).

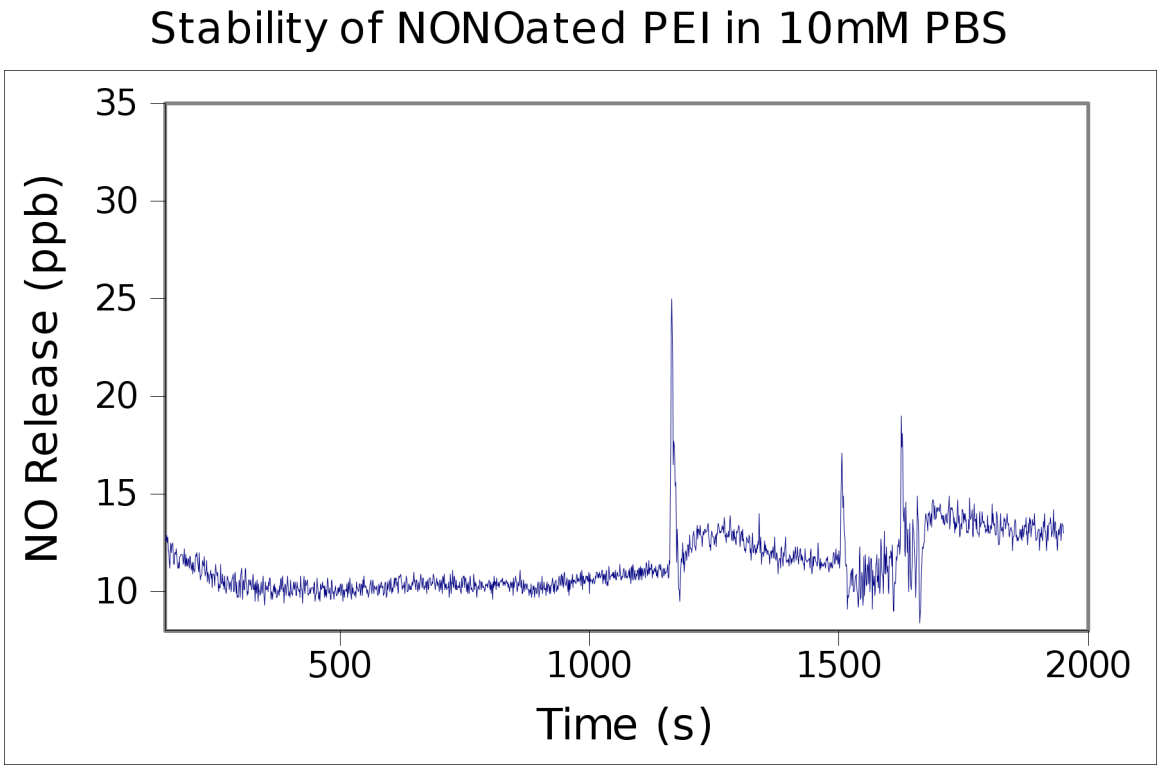


Figure 8. Baseline in solely 10 mM PBS solution (pH=10.30).

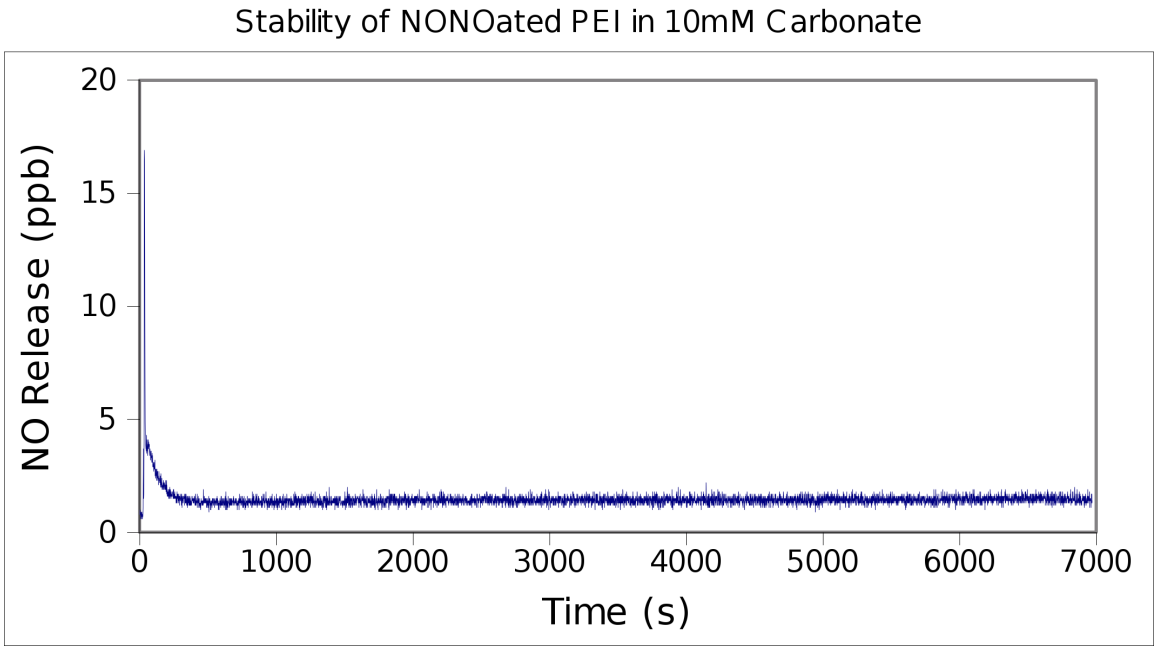


Figure 9. Baseline in 10 mM carbonate (pH=10.30) with no external bias.

Oxidation of Gold Electrode Under Various NaCl Concentrations

A commercial gold electrode with a determined electrochemical surface area of 1.57×10^{-2} cm^2 was employed as the working electrode in 0, 10, and 100 mM NaCl solutions (Fig. 10). The oxidation peak (~0.63 V) and reduction peak (~0.27 V) both shift between 10 and 75 mV depending on the salt concentration.

Holding at a potential of +0.80 V for 10 minutes produces a larger reduction peak (Fig. 11) around +250 mV with a peak current of 2.8 μA. The potential hold for 1 minute at +0.80 V gives a reduction peak height of 2.05 μA.

The peak at +0.60 V is Au oxide formation, while the peak at +0.25 V is the reduction of Au oxide to Au. The shift of the Au oxide reduction peak to +0.30 V in Fig. 10 is a result of the large concentration of NaCl utilized (100 mM).

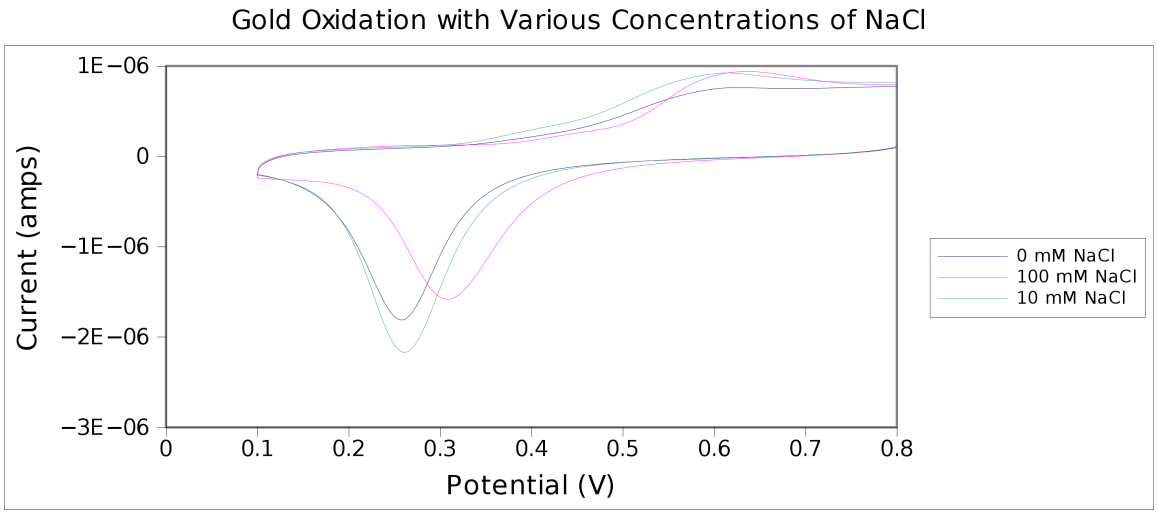


Figure 10. Oxidation of Au in 10 mM carbonate buffer (pH=10) with scan rate of 10 mV/s and a potential hold at +0.80 V for 60 s.

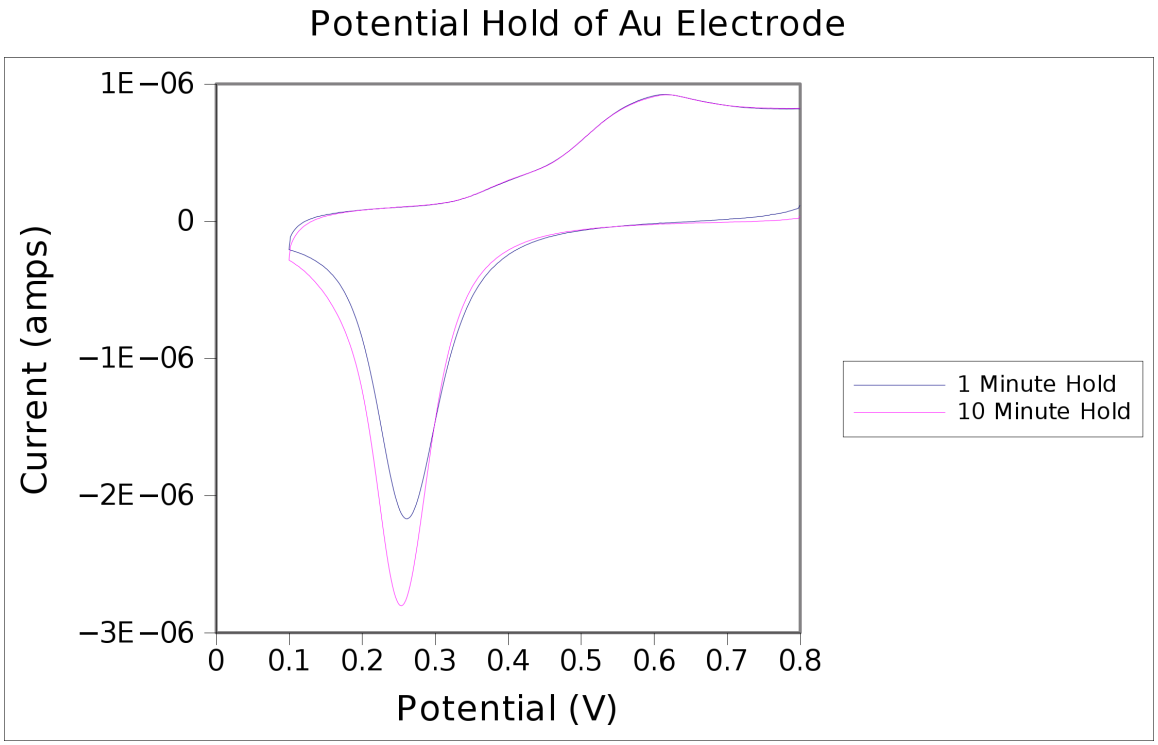


Figure 11. Potential hold at +0.80 V for 60 s and 10 minutes in 10 mM carbonate buffer and 10 mM NaCl. Scan rate is 10 mV/s.

Study of Oxidation of NO Using NOA as Detector

In order to determine the amount of produced NO lost via its electrooxidation, the following experiment was performed. After integration of the observed peaks after injecting aliquots of the NONOated PEI solution (both electrochemically used and the control), total moles of NO produced for the control and electrochemically used solutions respectively were: 6.684×10^{-6} mol and 5.5056×10^{-6} mol. The total amount of NO produced from electrochemical processes was 9.624×10^{-8} mol. Thus 1.083×10^{-6} mol of NO was lost. Efficiency for NO release of 9% from an 11 mL solution of 0.5 mg/mL NONOated PEI was obtained, along with a theoretical maximum electrochemical run time of 4.4 days using the gold mesh (S.A. = 8.75 cm^2) as the working, a pyrolytic carbon rod as the counter, and a Ag/AgCl reference electrode.

Optimization of Buffer Concentration and Electrode System

The buffer (10 mM carbonate in 100 mM NaCl) and Low M.W. PEI solution (1 mg/mL) were monitored for pH changes after applying +0.800 V using Ag/AgCl as the reference electrode, gold mesh as the working, and varying counter electrodes (Table 2).

Solution	Counter	Time Span (hrs.)	Initial/Final pH	ΔpH
Buffer	Carbon Rod	13	11.29/10.15	1.14
Buffer	Pt. Coil	13.5	11.14/9.92	1.22
Low M.W. PEI in Buffer	Carbon Rod	14	11.26/9.67	1.59
Low M.W PEI in Buffer	Pt. Coil	13.5	11.12/9.77	1.35

Table 2. The effect of water oxidation on bulk solution pH in 3-electrode system.

Two-electrode systems were also utilized when looking at pure buffer and Low M.W. PEI solutions. Gold mesh was the working electrode followed by either a quasi Ag or a Ag/AgCl reference electrode. The pH change was monitored as well (Table 3).

Solution	Counter	Time Span (hrs.)	Initial/Final pH	ΔpH
Buffer	Ag/AgCl	12.5	11.21/10.04	1.17
Buffer	Quasi Ag	12.5	11.11/10	1.11
Low M.W. PEI in Buffer	Ag/AgCl	13	11.04/9.50	1.54
Low M.W. PEI in Buffer	Quasi Ag	12.5	11.05/9.61	1.44

Table 3. The effect of water oxidation on bulk solution pH in 2-electrode system.

Titration of PEI and NONOated PEI

The titration curves for a 2 mg/mL of PEI can be found below (Figs. 12,13). The p*K*a range of PEI is determined to include a wide range of pH values across the spectrum.

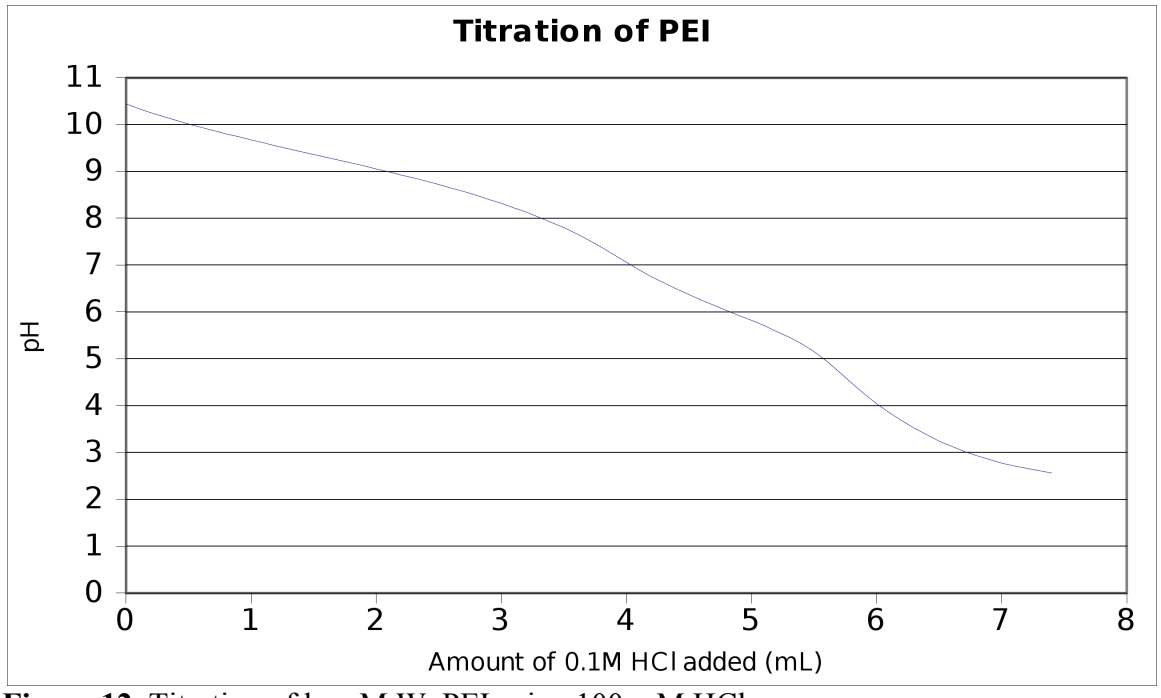


Figure 12. Titration of low M.W. PEI using 100 mM HCl

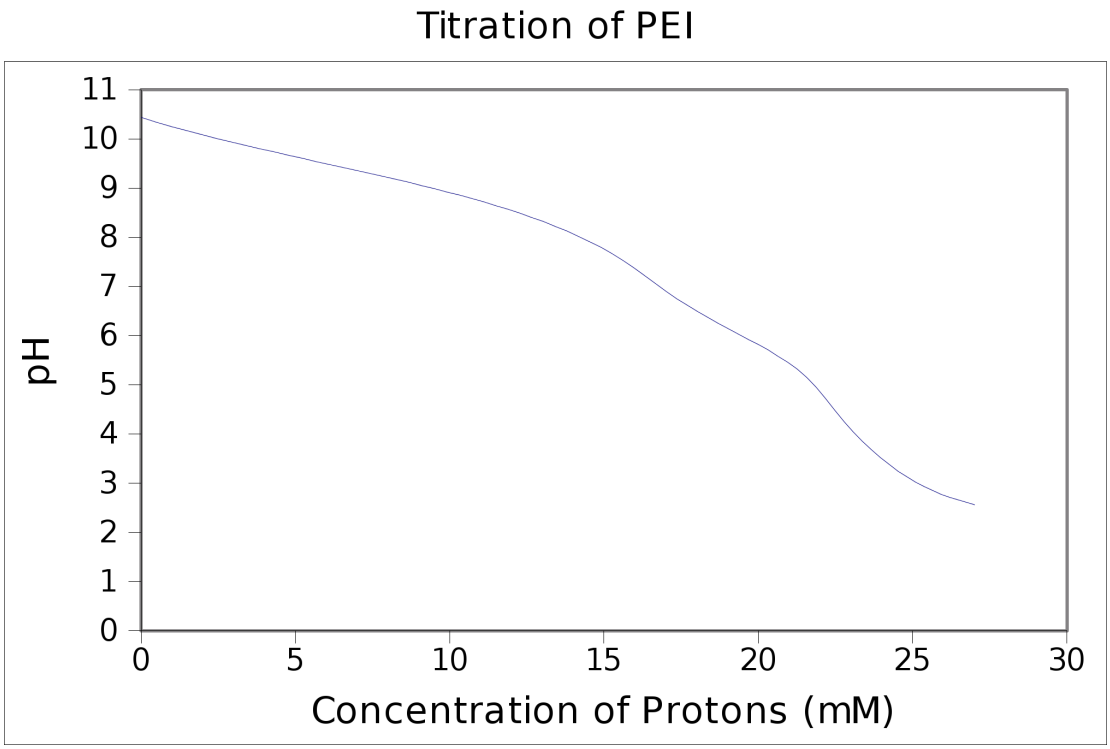


Figure 13. Titration of PEI in terms of the concentration of H^+.

DEA/NO Release of NO in Mini-Solutions

Using a 500 μM DEA/NO solution, NO was released overnight using a 300-μL solution (Fig. 14) electrochemically [working = gold coil (S.A.=.0719 cm^2), reference/counter = Ag/AgCl wire (S.A. = 0.315 cm^2)].

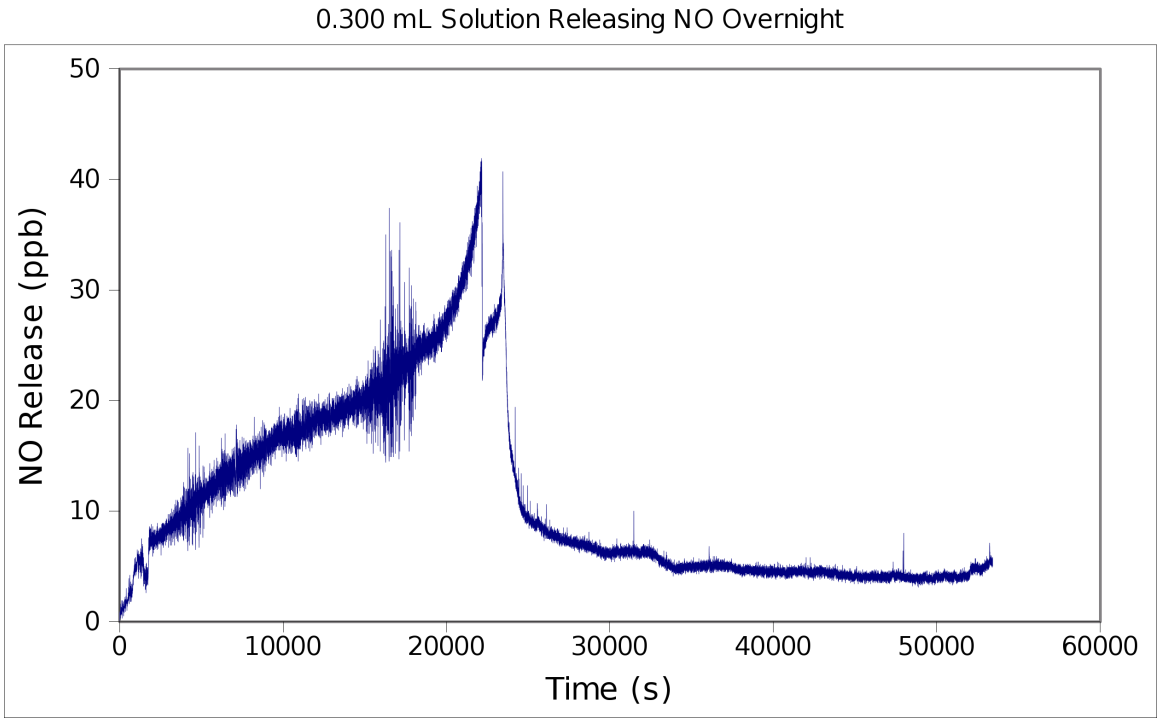


Figure 14. NO release overnight from 300-μL aliquot of 10 mM carbonate and 100 mM NaCl solution (pH=11).

Testing of Various Proton Donors for Releasing NO

A variety of proton donors were tested for their proton-producing properties upon electrochemical oxidation (e.g., ascorbate, pyridoxal, guanine). Many did indeed contain reproducible voltammetric peaks (Fig. 15,16,17,18) in which protons were produced by monitoring the drop in pH overtime (Table 4). All samples underwent cyclic voltammetry (CV) in 10 mM carbonate and 100 mM NaCl to identify the oxidation peaks (i.e., oxidation of water to produce protons).

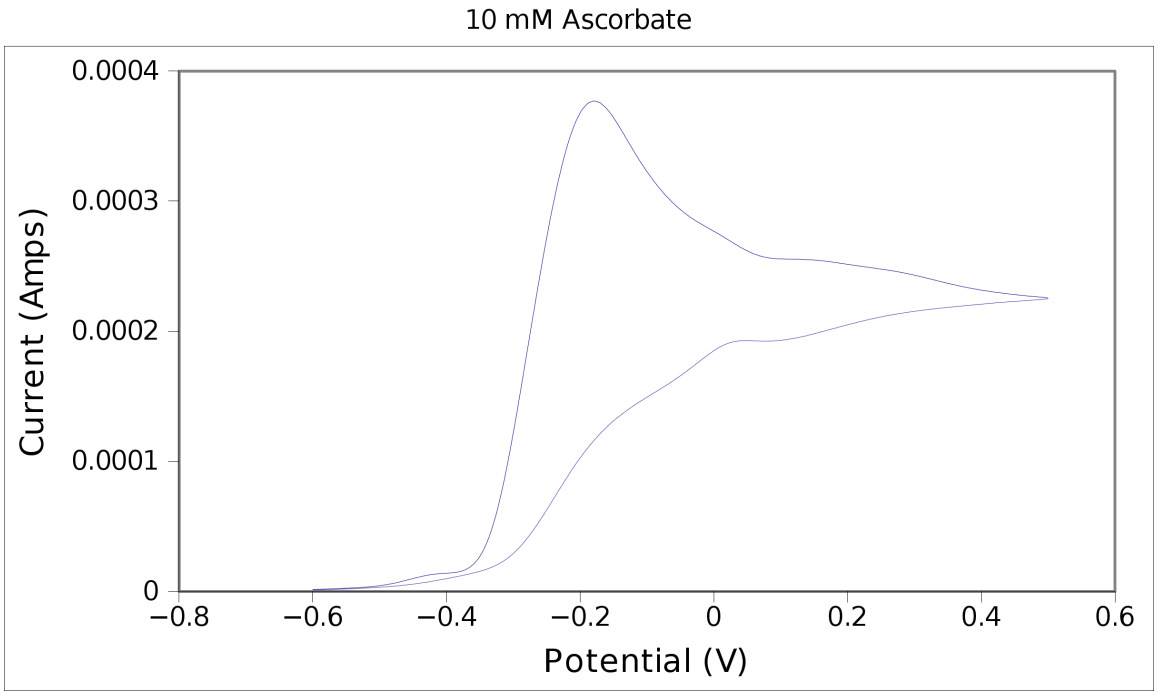


Figure 15. CV of 10 mM ascorbate at 10 mV/s (pH=11.40).

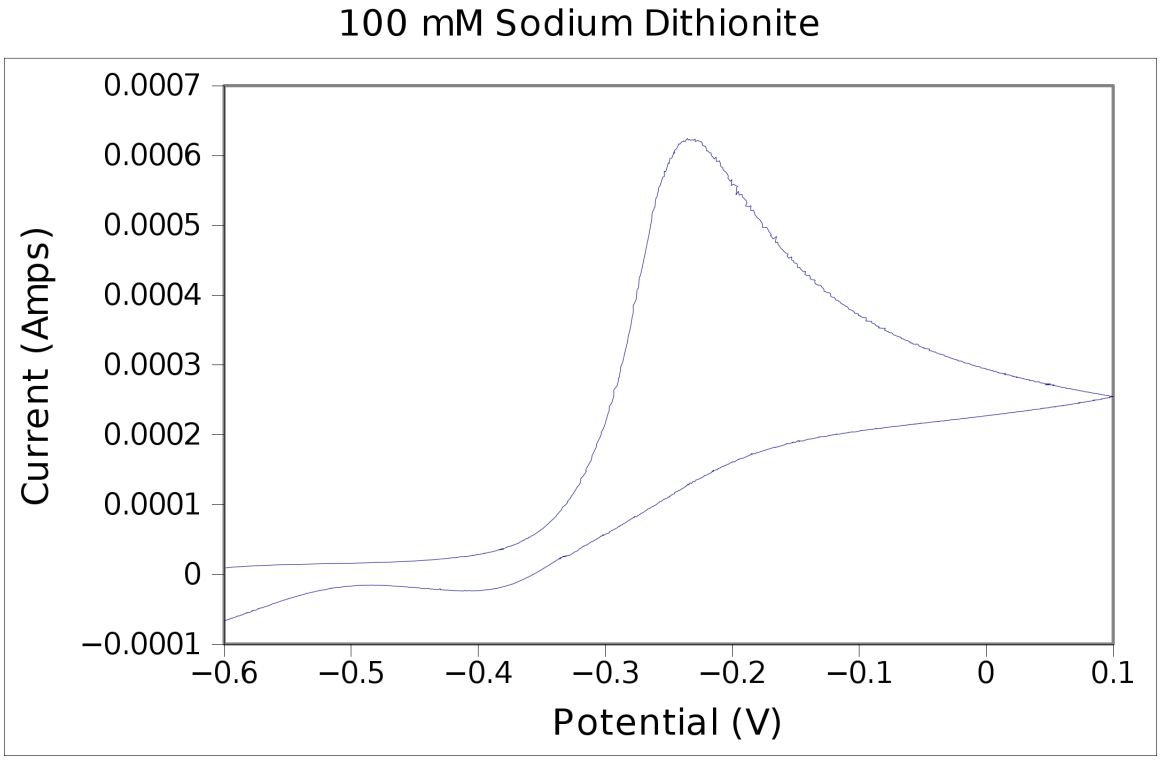


Figure 16. CV of 100 mM sodium dithionite at 50 mV/s (pH=11.14).

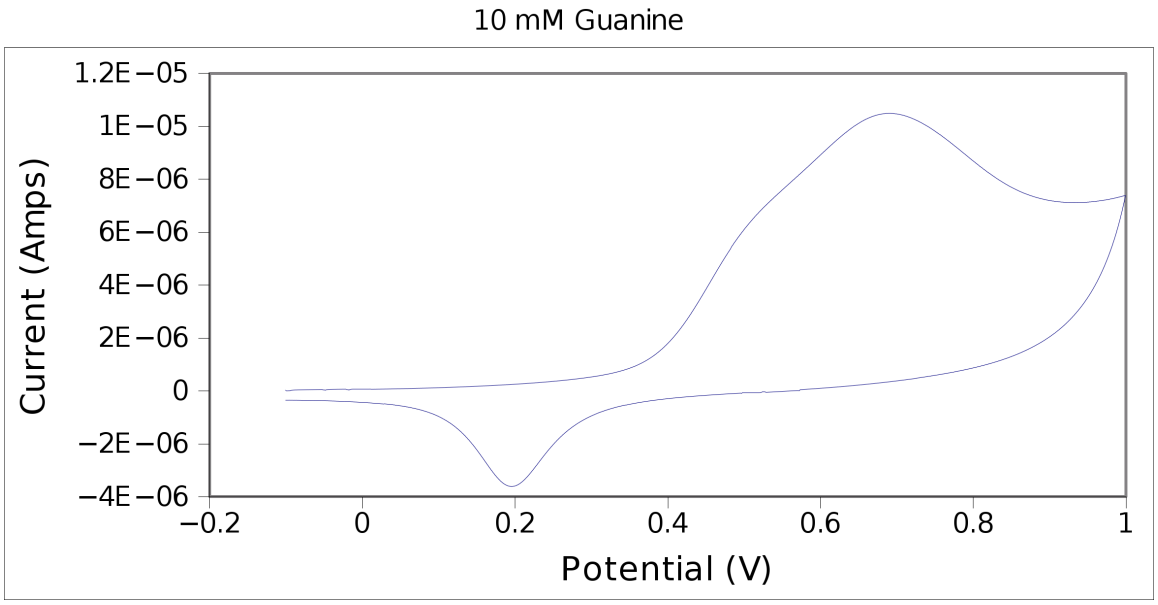


Figure 17. CV of 10 mM guanine at 25 mV/s (pH=11.01).

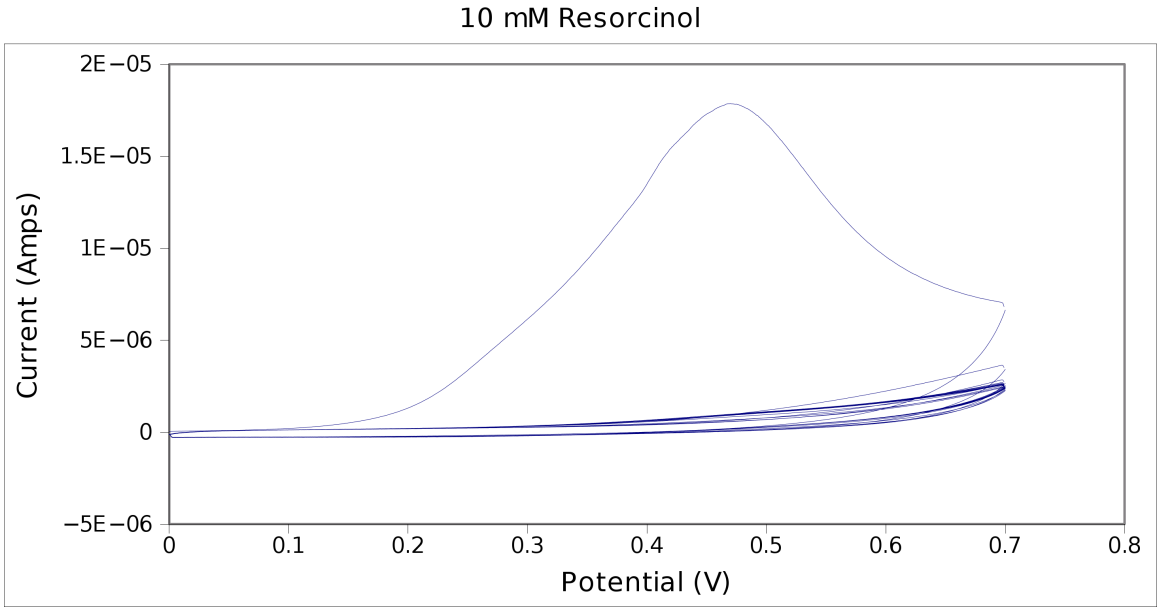


Figure 18. CV of 10 mM resorcinol at 25 mV/s (pH=10).

Proton Donor	Time Span	Potential (V)	Initial/Final pH	ΔpH
Ascorbate	2 hrs	+0.20	11.1/10.68	0.42
Sodium dithionite	0.5 hrs	-0.6-0.1	11.14/7.56	3.58
Guanine	2 hrs	+0.50	10.94/10.85	0.09
Resorcinol	16 hrs.	No potential	9.96/9.96	0

Table 4. pH shifts over time. Sodium dithionite was solely tested during the CV runs. Resorcinol was tested for its auto oxidation in air only.

DISCUSSION

Polyethylenimine is a large polymeric species with many secondary amine sites, which makes it a prime compound to form diazeniumdiolates. Characterization of it proves that NO can indeed be loaded onto it, and a diazeniumdiolation of 8% can be reached (where diazeniumdiolation yield is the percentage of the total secondary amines diazeniumdiolated in the compound). The low diazeniumdiolation is mainly due to the polymer matrix. The steric hindrance disallows NO to migrate to all the secondary amine sites and attach covalently. Branched PEI's abundant side chains supports this suppostion[16] .

Electrochemical release of NO using NONOates has yet to be extensively researched. The use of electrochemistry to liberate NO potentially allows for a constant, controlled release of NO when necessary from a reservoir of NO that remains stable for weeks. From the electrochemical studies done thus far, NO release has been successfully generated on pyrolytic graphite, gold, and platinum electrodes using +1.0, 0.80, and 0.87 V (vs. Ag/AgCl), respectively. The gold mesh was able to give a more constant NO release at the lowest potential. Being able to oxidize water at +0.80 V saves not only energy, but allows for a greater opportunity of not oxidizing the nitric oxide itself.

A major concern for being able to keep a stable NONOate solution in which the bulk pH doesn't change, but the local pH changes only near the electrode surface, is the buffer. Too weak of a buffer will allow the protons produced from water oxidation to change the entire bulk solution's pH, while too strong of a buffer will not permit the lowering of the local pH near the electrode's surface. The p*K*a of the buffer is what is truly important. The pH that is necessary appears to be between pH 10 and 11. PBS, borate, and carbonate all at 10 mM concentration were examined to discern their capabilities of being buffers for a system such as this.

NONOated PEI in both borate and PBS buffers were studied for their constant, controlled release of NO over time. According to Figures 6 and 7, PBS has a 20% higher NO release over the borate buffer. This further proves that the buffer can drastically change the NO releasing capabilities. Borate has a p*K*a of 9.23, while PBS has a p*K*a of

12.33. This means that at pH 10, the borate buffer has a better buffer capacity and acts as a good buffer, thus leading to lower proton concentrations near the electrode surface and in the bulk solution. The buffer is consuming most of the protons produced from the oxidation of water, thereby, making less protons available for the NONOates to consume, which in turn lowers the NO release. The PBS is a poor buffer at this pH and thus allows for a dispersion of protons throughout the solution, which liberates even more NO. PBS may release more NO during the electrochemistry, but the downside is that it releases more NO even without any external bias. According to Fig. 8, when solely the NONOate was dissolved in PBS without any electrochemical modulation, the baseline of NO release was fluctuating constantly between 10 and 15 ppb. As mentioned above, the PBS is a poor buffer in the pH 10/11 areas, and therefore it is inefficient at controlling the solution protons, which leads to a high baseline of NO release. The PBS buffer is not an effective buffer for long-term studies.

In order to ensure that a loss of NO isn't occurring when the potential is turned off, carbonate buffer was tested for its proficiency. After comparison of the results for the PBS and carbonate system, carbonate buffer proved to be much more effective. Figure 9 depicts the stability of the NONOated PEI in the buffer showing it only has a 2 ppb release of NO. The invariability in the concentration of the NONOate in carbonate buffer stems from the fact that its p*K*a is 10.33. This allows the NONOate solution of pH 10/11 to have sufficient stability when using carbonate as the medium.

Another important factor is the electrodes that are used and the chemical processes that occur on them at the given applied potentials. Gold has been comprehensively researched for decades on its functions in various pH solutions along with different applied biases. The results are the formation of a sundry of gold oxidation states/products[18]. Pourbaix diagrams support that in high pH solutions under a potential of +0.80 V, $Au(OH)_3$ is created[17]. Upon the production of $Au(OH)_3$, three moles of H^+ are released, thus lowering the pH near the electrode surface. The initial spike that is first formed in Figures 6 and 7 that disappears in the following potential holds could be due to the passivation of the gold electrode. A larger concentration of protons are created in the first ten minutes, and over time it decreases until the gold electrode is fully oxidized, thus leading to the constant release of NO in the 2nd and 3rd potential holds. A 1-minute

potential hold has a smaller reduction peak than the 10-minute hold (Fig. 11), thus supporting that over time the oxidation layer grows. As +0.80 V is applied to the NONOated solution, protons are not only coming from the oxidation of water, but also from the oxidation of the gold electrode.

The chloride concentration also influences the gold surface. As the concentration of chloride reaches 100 mM, the reduction peak on the gold electrode starts to decrease (Fig. 10). $AuCl_4^-$ is not forming since the pH is above 9, so the only possible explanation is the adhesion of the chloride onto the surface of the gold electrode. There are active sites that the chloride can bind to, thereby increasing the passivation of the surface and reducing the cathodic peak[18]. This is important when considering the concentration of chloride that needs to be added to the buffer to support a 2-electrode system setup.

As commonly known, NO is easily oxidized in oxygen rich environments, however, it can also be electrochemically oxidized. In PBS (pH=7.4), the oxidation of NO occurs at +0.72 V on a gold electrode[19]. The oxidation potential of NO fluctuates between +0.675 V and +1.05 V depending on the pH of the solution and the electrode that is used[20-24]. Using the Nernst equation, a thermodynamic value of NO oxidation can be calculated. Using the standard reduction potential of +0.957 V vs. Ag/AgCl in 100 mM NaCl and a pH of 11, a thermodynamic potential of +0.3713 V was obtained for the oxidation of NO to NO_3^-.

In order to find out how much NO is lost due to its electrooxidation at the electrode, chemiluminescence measurements were utilized. By using a standard control of the NONOated PEI not being electrochemically perturbed, in concert with a sample solution being electrochemically modulated, an efficiency of NO release can be obtained. Injecting both the electrochemically tempered and the control into an acid solution and measuring the NO release obtained from both, a percentage of NO lost can be calculated. If no NO was unaccounted for, the amount of NO released from the electrooxidation of water should account for the difference in the two aliquots. However, there were 1.083 x 10^{-6} moles of NO unaccounted for, which can only be assumed to be lost due to the electrooxidation of NO since the overpotential was so high in order to perform water oxidation. The efficiency of the electrochemical production of NO is about 9% (obtained from dividing the total amount of NO produced from electrooxidation of water by the

amount of NO that was unaccounted for). There are options for increasing this number by changing the active sites on the electrode that inhibit the oxidation of NO. Oxides on the surface of the electrode have proven to promote the oxidation of NO[25]. By removing this oxide by cycling the potential or using different electrodes that do not oxidize as much at such high potentials (e.g., Pt) the probability of NO oxidation can be decreased. The overall goal, however, would be to find a water oxidation catalyst that can lower the overpotential. If water oxidation can occur below NO oxidation, then this would be a much more efficient application for releasing NO. Both IrO_x and Co (II) oxides have proven to be good candidates for such an electrode surface[26-27].

Although a low NO release efficiency was obtained for its electrochemical generation, 9% could be enough depending on the total amount of NO available. Thus, the ultimate design of a catheter must be employed to assay its capabilities. In order to reach this point, the effects of various 2 and 3-electrode systems needed to be examined. In a catheter model, a 2-electrode system will be the most effective due to the availability of space for the electrodes. Measuring the drop in pH using water oxidation for the different systems is the most effective means for determining their future capabilities. It is important to note that the bulk pH should not change in real applications, and that the local pH near the electrode's surface is what should be altered. The measured drop in bulk pH using a large gold mesh electrode is to show that proton production is occurring. Table 2 and 3 show the change in pH over similar time intervals for a 3-electrode and 2-electrode system, respectively. Samples with solely the buffer were tested, along with samples including the low M.W. PEI that is used to make the NONOates. This is necessary because upon release of NO, the resulting byproduct is PEI, which contains secondary amines that can be protonated in lieu of using those protons to release NO. The most promising result obtained is that both the 2-electrode and 3-electrode system have the relatively the same drop in pH. This is extremely important since this mean that water oxidation in a 3-electrode system should occur around the same rate and produce the same amount of protons as that in the 2-electrode system. That is, the catheter design has promise for releasing NO from using only 2 electrodes. However, the other interesting fact from these experiments is the further drop in pH from the low M.W. PEI solutions. One would think that the pH would not drop as much due to the extra buffer capacity

resulting from the secondary amines on the PEI chain. This peculiarity prompted further testing of the PEI chain itself.

To see if the PEI is protonated or deprotonated before it is dissolved in solution, the p*K*a of it needs to be discerned. A titration using HCl ensued yielding 2 graphs (Figures 12-13). The low M.W. PEI has a range of p*K*a values, which agrees with what has been previously reported in the literature[28]. There is a range in lieu of an exact p*K*a due to the large amount of monobasic secondary amines that are in very dispersive and in divergent locations. Under the basic conditions that were used to generate Tables 2 and 3, the PEI would be deprotonated as soon as it is dissolved. The greater drop in pH that occurs when PEI is in solution could be explained possibly by the increased conductivity of the solution. An increase in conductivity would lead to a lower resistance, allowing water oxidation to occur much more efficiently and leading to a greater ΔpH. Further studies are required to fully confirm this phenomenon.

The solution volume inside the catheter is ca. 2 μL, thereby increasing the total volume to electrode surface area contact ratio. The electrode area to solution size ratio is extremely important. Having a small electrode in a large solution will not be able to produce an appreciable amount of NO. DEA/NO is a faster NO release diazeniumdiolate, compared to NONOated PEI, with a half-life of 2 minutes as previously reported by Keefer[12]. The benefit for using a NONOate such as DEA/NO is the ability to more rapidly release its NO. The diffusivity of the NO through the catheter walls will slow down the NO, thus, having a faster rate of NO production will be beneficial in order to have an adequate flux at the outside surface of the catheter. A 300-μL solution of DEA/NO was tested for its capabilities for NO release in a small volume. Fig. 14 depicts the increase in release of NO over time. The drop off of NO around 25,000 seconds (~7 h.) is due to the solution evaporating since the NOA cell is being purged with a constant stream of nitrogen gas. This proves that using catheter size electrodes and DEA/NO, NO can be released from micro sized solutions, further supporting the potential success of a catheter design.

Upon testing of the actual catheter design, a significant flux of NO from both NONOated PEI and DEA/NO was not acquired. This is most likely due to the oxidation of NO in the small volume. NO isn't able to diffuse away from the electrode fast enough

since the catheter solution cannot partake in stirring. Mass transfer is a big limitation for such a design. A control release of NO from the catheter design would only be possible if the NO could diffuse away from the electrode and through the walls of the tubing fast enough before it was electrooxidized. Until then, further research needs to be performed on what can be changed to accommodate such a requirement.

Electrooxidation of NO due to the high overpotential required for water oxidation has been a large obstacle in electrochemically releasing NO from diazeniumdiolates; therefore, discovering another proton donor that has a lower overpotential, compared to water, is of significant importance. A list of different proton donors was tried such as: ascorbate, guanine, sodium dithionite, resorcinol, catechol, pyridoxal, and formate. All of these have been reported to have the ability to be oxidized at potentials lower than +0.60 V, and as low as -0.20 V, which is significantly lower than the +0.80 V required to oxidize water in alkaline solutions[29-35]. Figures 15 and 16 show the cyclic voltammograms of ascorbate and sodium dithionite, respectively. These show very good and reproducible oxidation peaks at about -0.20 V. Both have low potentials for proton production; however, due to the autooxidation of sodium dithionite in air and ascorbate's ability to reduce NO and oxidize itself, many obvious problems can arise[36-37]. Both resorcinol and catechol also have problems with being oxidized in air, but the biggest concern for the oxidation of resorcinol is the formation of a polymeric film on the electrode's surface. Fig. 18 shows a good oxidation peak of resorcinol around +0.47 V. However, as the cycles continue, the current drops drastically. This is due to the fact that the initial scan is on a polished, gold electrode, but the rest of the scans now have the polymeric oxidized form of resorcinol on the surface of the gold[35]. Pyridoxal, guanine, and formate all are stable in air, and have relatively low oxidation potentials. The one issue is the change in pH that does not occur. Table 4 shows the change of the solutions pH upon holding a constant potential over time. Guanine's ΔpH, along with pyridoxal's and formate's not shown above (see Supporting Information section for data), is extremely low. This means that the kinetics of proton production at the electrode's surface is poor. Until a new proton donor is discovered, the high overpotential for water oxidation will be a primary concern for the electrochemical release of NO from NONOates.

CONCLUSION

From the results of this study, a polymeric diazeniumdiolate can be created reproducibly (NONOated PEI), which allows for a larger reservoir of NO. Using a gold electrode, the optimum electrochemical release of NO can occur from the NONOated PEI. The reproducibility of the electrochemical production supports the potential application of such a system. Optimization of the system requires a buffer with an excellent buffer capacity to allow for local NO release, but prohibits any constant flux of NO without applying any potential. Phosphate, borate, and carbonate buffers were all tested and carbonate proved to be an optimal buffer due to its higher p*K*a value. The electrode also needs to be modified properly to allow for the lowest possible potential for water oxidation to occur. Upon studying various metal electrodes, gold had the lowest potential that could release the highest amount of NO at constant flux via the oxidation of water to produce protons. However, an electrode that is not oxidized would be preferential since the oxidized layer promotes electrooxidation of the NO, so an even more suitable electrode than gold is desired. However, even with an oxidized layer, it has been shown NO can be released in high concentrations from even a 300-μL solution. This supports the catheter design model, which would allow for *in vivo* use of such a system. The overarching concern is still the high overpotential for water oxidation. Until this problem is resolved by finding a new proton donor or an electrocatalyst for water oxidation, the practical, long-term electrochemical release of NO is not feasible for the systems described in this thesis.

FUTURE WORK

Discovering proper proton donors for electrochemical generation of NO is a pressing matter. Further research can be done by testing various organic compounds such as porphyrins and porphines, which release protons upon coordinating with metal complexes. This allows for the same process to occur via an electrochemical modulation. Other compounds include conjugated rings such as hydroxypteridine, which can release protons upon oxidation of the ring[38]. Electrocatalysts should be extensively researched that allow for lower potentials of water oxidation. IrO_x and Co^{II} oxides have been shown to promote water oxidation using smaller biases than the uncatalyzed process. The formation of the oxide and its stability on a gold electrode are key aspects that need to be examined. Finally, finding a possible agent to bind to the surface of the gold electrode to hinder the oxidation of NO that would allow for water oxidation to readily occur would produce a solution for the NO redox problems. The fact that surfaces such as gold oxide can promote NO oxidation, further supports the idea that the kinetics of NO can change[25]. A compound that reduces the rate of NO oxidation would be as beneficial as an electrocatalyst for water oxidation in this project.

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SUPPORTING INFORMATION

The information in this section further support the data provided in the thesis.

Applied Potential (V)	NO Release (ppb)
+0.60	17
+0.70	30
+0.80	30
+0.85	30
+0.87	22
+1.00	31
+1.20	31

Table S1. The optimization results for pyrolytic graphite rod electrode for the most efficient NO release from a 2 mg/mL of NONOated PEI in 10 mM PBS (pH=10).

Applied Potential (V)	NO Release (ppb)
+0.60	9.0
+0.70	12.5
+0.75	14.0
+0.80	16.0
+0.83	16.5
+0.87	20.0
+0.90	16.0
+1.00	17.0

Table S2. The optimization results for platinum coil electrode for the most efficient NO release from a 2 mg/mL of NONOated PEI in 10 mM PBS (pH=10).

Applied Potential (V)	NO Release (ppb)
+0.78	87
+0.80	94
+0.82	80
+0.85	92
+0.90	63

Table S3. The optimization results for gold mesh electrode for the most efficient NO release from a 2 mg/mL of NONOated PEI in 10 mM PBS (pH=10).

Proton Donor	Time Span	Potential (V)	Initial/Final pH	ΔpH
Pyridoxal (1 mM)	0.5 hrs	-0.8-0.8	11.15/11.15	0
Formate (1 mM)	0.5 hrs	-0.1-1.0	11.03/11.00	0.03

Table S4. pH shifts over time. Pyridoxal and formate were solely tested during the CV runs.