Bioelectrochemical Regeneration of Enzymatic Cofactor Coupled to CO_2 Reduction in a Redox Flow Cell

A Major Qualifying Project Report

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Abstract

Concerns about emissions and climate change have expedited research into alternative energy sources and methods. Fuel cells are an alternative energy conversion device of growing interest in the scientific community. Often compared with combustion engines, fuel cells are emerging as a dependable low- to zero-emissions alternative. While combustion engines often lose some energy in the form of heat in the exothermic reaction, fuel cells convert the chemical energy of fuels directly into electrical energy, making them a much more efficient alternative. Among fuel cells, polymer electrolyte membrane fuel cells (PEM) are of particular interest because they are compact, lightweight, and operate at lower temperatures than other contenders.

For this project, we explored the bioelectrochemical regeneration of an enzymatic cofactor coupled to CO_2 reduction in a redox flow cell. To do this, a rhodium complex was immobilized onto a carbon paper coated with multi-walled carbon nanotubes (MWCNTs). Combining PEM fuel cell technologies and a redox flow technique, the NADH cofactor was electrochemically regenerated via mediation of the covalently immobilized rhodium complex. The reaction was assessed using cyclic voltammetry and amperometry in the flow reactor.

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1.0 Introduction

The continuously increasing global demand for energy has exacerbated greenhouse gas emissions and thus expedited climate change. To overcome these challenges and to curb our reliance on fossil fuels and coal, much recent research has focused on alternative or "green" energy production methods. Alternatives include renewable resources, such as wind and solar power, as well as clean resources, such as fuel cells and nuclear energy. Fuel cell technology is an emerging hot topic in the science community due to its high reliability, scalability, and relatively low-cost of operation.[2] Fuel cells use the chemical energy of hydrogen or other fuels to efficiently produce electricity, making them a dependable low- to zero-emissions alternative to combustion engines.[3]

There are many types — polymer electrolyte membrane, direct methanol, alkaline, phosphoric acid, molten carbonate, solid oxide, and reversible — of fuel cells being researched and explored.[4] For this project, we focused on polymer electrolyte membrane fuel cells (PEM), interchangeably also called proton-exchange membrane fuel cells. PEM fuel cells are made up of three primary components: two electrodes (cathode and anode) and a conductive electrolyte. The electrodes are typically saturated in an electrocatalyst and the electrolyte material serves as an ionic conductor and is typically a polymeric membrane.[5]

To operate, hydrogen gas is input at the anode. The hydrogen atoms are separated into individual protons and electrons on the surface of the catalyst, then the protons migrate through the membrane to the cathodic side while the electrons travel externally through a circuit to generate electricity.[6] Most PEM fuel cells currently use platinum catalysts. Our experiment explores fuel cells using enzymatic cofactors as catalysts as opposed to traditional chemical catalysts.

A cofactor is a non-protein chemical compound or metallic ion that is used to catalyze a reaction. In the field of biochemistry, cofactors are often used to assist in biochemical transformations. Cofactors are categorized into inorganic ions and complex organic molecules also called coenzymes. For this project, we are working with nicotinamide adenine dinucleotide (NAD) + hydrogen (H), a coenzyme central to metabolic function in all living organisms. NAD^+ is the oxidized version of NADH, and NADH is the reduced form of the molecule.

The cofactor pairing of these nicotinamide adenine dinucleotides, NAD^+ and NADH, appear in many oxidation-reduction "redox" chemical reactions.[7] NADH is of particular interest due to its enantioselectivity,[8] meaning it prefers to interact with the chiral sector of one enantiomer over another.[9] This quality is especially valuable in the pharmaceutical industry, as drug-protein interactions must be predicted in order to understand therapeutic properties and toxicity levels in drug development.[10]

Unfortunately, NADH production is expensive. Therefore, scientists are exploring methods to produce NADH via reductive regeneration from its oxidized form, NAD^+. Cofactor regeneration is the process of restoring the cofactor to its stable form which allows it to be reused.[11] This process is used on an industrial scale within the pharmaceutical industry and to complete organic synthesis.[12]

Regeneration of NADH can be completed through the use of 4 different methods: chemical, photochemical, enzymatic, or electrochemical. Chemical regeneration of NADH requires a cofactor with a high redox potential to complete the reduction to NAD^+.[13] This method has a low total turnover number (TTN), produces a lot of waste, and requires a large feed stream.[14] Photochemical regeneration utilizes light to facilitate the reduction of NADH, this method is very cheap but is not efficient, especially in the visible light range.[14] Enzymatic regeneration of NADH utilizes enzymes to facilitate the reduction, this is the most common and favorable process used in industry as it has high efficiency and TTN.[11] Electrochemical regeneration of NADH is a process facilitated by the continuous transfer of electrons through the use of an electrode.[11] This process has a wide range of applications but a low TTN and the potential for electrode fouling.[14]

This project also has a major focus on the bioconversion of CO_2 into a usable form and creating energy with very minimal or without CO_2 emissions, adding an environmental benefit to this project, in addition to the economic benefit. Finding new ways of producing energy and not emitting CO_2 has never been more important. If this process was used on a large scale, the carbon emissions would be limited and energy could be produced at the same or a similar rate.

2.0 Background

2.1 Electrochemistry Overview

Electrochemistry is the study of electron movement during chemical reactions. Reduction and oxidation are two of the main phenomena that are covered within electrochemistry and are applicable to this study. Reduction occurs when an atom increases by one electron in a chemical reaction, always occurring at the cathode, the positively charged electrode within the cell. Oxidation occurs when an atom decreases by one electron and always occurs at the anode, the negatively charged electrode within the cell. In the cell that this study is focused on, nicotinamide adenine dinucleotide phosphate (NADH) is being oxidized to nicotinamide adenine dinucleotide (NAD^+), and carbon dioxide (CO_2) is being reduced to formate (CHO_2).

The difference in the cell potential dictates the movement of electrons within a cell. The flow of electrons moves from the higher energy potential area to the lower energy potential areas. In this cell, the flow moves from anode to cathode. These cycles are important to the context of this fuel cell, as NAD^+ is the active cofactor that is being primarily used for this cell due to its ability to behave as an electron carrier.[15]

2.2 Fuel Cell Overview

In light of the climate crisis, fuel cells are being researched for their potential to replace traditional energy systems, often powered by fossil fuels and coal. Preliminary research has indicated that a combination of fuel cells and other "green" energy sources, such as wind, solar, nuclear, and geothermal, may be a promising alternative solution to curb greenhouse gas emissions.[16]

Fuel cells work differently than typical combustion engines. Fuel cells convert the chemical energy in fuel directly into electricity. In combustion reactions, some energy is lost to the exothermic reaction in the form of heat. As fuel cell reactions omit this step, they are typically more efficient than combustion engines.[2]

2.2.1 Fuel Cell Basics

Researchers are hopeful that fuel cells will be the "green" energy replacement to rechargeable batteries and thermal engines.[16] Currently, there are a plethora of types of fuel cells being researched. All are composed of the same components — two electrodes, separated by a membrane, typically a solid or liquid electrolyte — the electrolyte material differentiates the types of fuel cells.

To operate, a gas fuel, typically hydrogen, enters at the anode. This oxidizes at the entrance, splitting the hydrogen to produce cations and electrons. The cations pass through the membrane, while the electrons diverge on a separate path through a circuit to create an electric current, prior to returning to the cathode. The electrons and cations recombine at the cathode to reproduce hydrogen. Typically, oxygen gas also flows through the system. The oxygen and hydrogen atoms combine to form water, the only waste product of the system.[16]

The anodic substance, or fuel gas, can be any material that is able to give up electrons, but hydrogen is the preferred material, as it reacts well with catalysts and has a high energy density. Oftentimes, it is paired with oxygen as the oxidant, as it exists naturally in the air and works well in reduction reactions.

Most current research focuses on optimizing and increasing the quality of the electrolyte material. Ideally, a membrane should only allow positive ions to pass through to the cathode, as the electrons are forced through an external circuit to generate electricity. The strength of the electrolyte is determined by its ability to block electrons as they try to move toward the cathode.

2.2.2 Fuel Cell Components

As mentioned earlier, the many types of fuel cells — proton-exchange membrane, direct methanol, alkaline, phosphoric acid, molten carbonate, solid oxide, and reversible — all contain the same baseline components. All fuel cells consist of an anode, a cathode, and an electrolyte.[17] Oftentimes, a catalyst is also added to reduce the activation energy needed to begin the reaction. In addition to these parts, fuel cells also have other components used to insulate the system and ensure proper gas flow, as seen in *Figure 1* below.

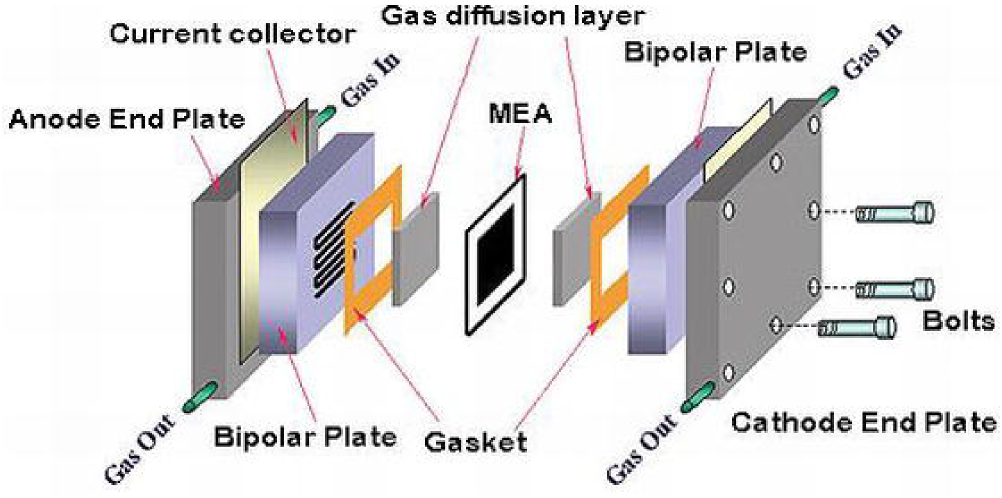


Figure 1: Fuel Cell Components [16]

The end plates keep the system together and prevent gasses from leaking out. The bipolar plates are composed of the current collector and a separator plate. They're typically made of metal or a carbon composite, built with channels to aid in uniform gaseous fuel flow to the electrodes. The channels are made in different shapes, as seen below in *Figure 2*.[16]

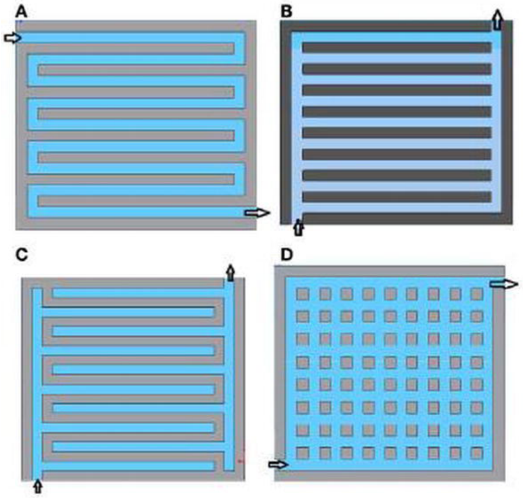


Figure 2: Bipolar Plate Designs [16]

Due to most fuel cells being assembled in stacks, the bipolar plates also help conduct current between adjacent cells and also help dissipate any heat produced during the reaction. The gaskets are used to prevent leakage of the fuel or reactant. Some fuel cells operate at high temperatures, therefore the gasket must withstand this. The gas diffusion layers are conductive pieces of carbon felt, with the purpose of transferring the reactants between the bipolar plates and layers of catalyst. The membrane electrodes assemble (MEA) is made up of the electrodes, the electrolyte, and the catalyst. The electrolyte is the membrane within the fuel cell responsible for preventing electrons from passing through to the cathode. The positive ions should pass through this membrane, while electrons are blocked and diverged through an outer circuit to produce electricity. Therefore, the membrane must be made of a semi-permeable material. Lastly, catalysts are often used to decrease the reaction energy barrier and increase the rate of the reaction. Catalysts are often chemically recovered from the reaction mixture.[16]

2.2.3 Proton-Exchange Membrane (PEM) Fuel Cells

For this project, we are focusing on proton-exchange membrane (PEM) fuel cells. PEM cells are compact, lightweight, and operate at lower temperatures than other contenders. PEM cells have a membrane that is proton-conducive, which doubles as the electrolyte and the separator.[18] Therefore, the design of a PEM cell differs a little from a typical fuel cell. A schematic can be seen below, in *Figure 3*.

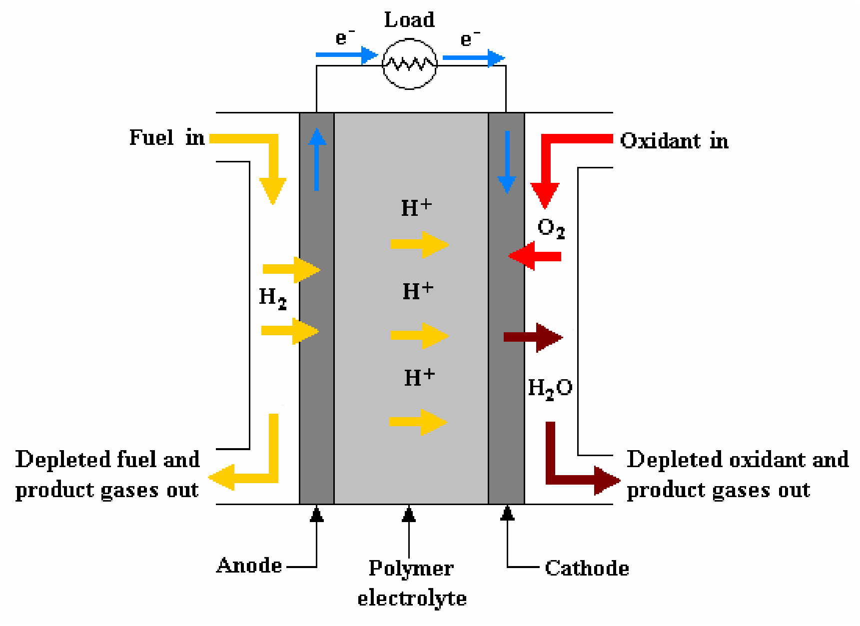


Figure 3: Proton-Exchange Membrane Cell Design [16]

This project is focused on using an enzymatic cofactor as a catalyst. Furthermore, this project explores the bioelectrochemical regeneration of this enzymatic cofactor, NADH, to reduce the cost of fuel cell production.

2.3 Half Fuel Cell

Within a fuel cell, two half-reactions occur at each electrode in a reduction-oxidation "redox" reaction. The oxidation half-reaction occurs at the anode, where electrons are released, and the reduction half-reaction occurs at the cathode, where electrons are accepted.[18] This reaction completes a full circuit when each half-reaction occurs. Each electrode has an electrochemical reaction, which entails an electric potential. This is referred to as the half-cell potential, and the combination of these potentials gives the electrochemical reaction a positive cell potential.[7] For this experiment, the half-reactions are producing chemicals, not energy.

2.4 Reaction Cycles Within the Cell

A reaction cycle refers to the continuous flow of chemicals in a reaction that allows for the reaction to repeat until the limiting reactant is all converted to the product. In this reaction cycle, hydrogen flows through the cell and through a mediator membrane. This membrane is made up of multi-walled carbon nanotubes (MWCNT) with the immobilized rhodium complex ($[Cp^*Rh(bpy)Cl]^+$), an electrochemical mediator. This cell operates with the presence of a phosphate buffer solution (PBS), which allows for the cell to be maintained at a pH of 7.2 which has previously been determined to be the optimal level for the reactions to occur. Formate dehydrogenase (FDH) is the enzyme used to facilitate these reactions. The enzyme utilizes the presence of CO_2 and NADH to create a reaction cycle that converts NADH to the active form of NAD^+, which is recycled by the presence of the Rh complex, and takes present CO_2 and reduces it to formate as a product. This cycle is shown in *Figure 4*.

Figure 4: Flow Cell Diagram
showing the flow of electrons and the reduction of CO2 to formate as a product and the regeneration of NADH via immobilized Rh complex.

The above figure shows the reaction that is occurring within the flow cell. The end goal of this process is ultimately to convert all CO_2 to formate as a final product, which can be easily converted to salts, esters, or formic acid. This cycle also allows for the regeneration of NADH as a cofactor.

2.5 NADH Regeneration

2.5.1 NADH Cofactor

Nicotinamide adenine dinucleotide (NADH) is a coenzyme that is critical to metabolic function in all living organisms. In the scientific community, NADH is studied for its role in redox reactions, as its original form combined with its reduced form, NAD^+ makes it a "redox couple," meaning that the oxidizing and reducing agent exists within the same atom or molecule.[7] For the reaction in this project, the NAD^+/NADH coenzyme is used as an electron carrier. However, NADH is expensive, so this project also explores methods to regenerate NADH within the reaction.

2.5.2 Regeneration Methods of NADH

Regeneration of NADH can be completed through the use of 4 different methods: chemical, photochemical, enzymatic, or electrochemical, as seen below in *Table 1*.

***Table 1:** Comparison of Coenzyme Regeneration Methods*
Advantages and Disadvantages of the Four Strategies for Coenzyme Regeneration Adapted from Methods for the Regeneration of Nicotinamide-coenzymes [14]

Method	**Advantages**	**Disadvantages**
Enzymatic	● Environmentally friendly ● High TTN ● High selectivity ● Combination of target products synthesis with in situ-regeneration processes	● Enzyme instability ● High enzyme cost ● Complexity of product isolation
Chemical	● Use of inorganic salts, H_2, coenzyme analogues as reducing agents and O_2 as oxidant ● Low cost	● Low TTN, low selectivity ● Pollution emissions when inorganic salts are used ● Low atom economy
Electrochemical	● Use of clean, renewable electrical energy ● Broad applications	● Low TTN, low selectivity ● Electrode fouling ● Mediator dependent
Photochemical	● Use of cheap, abundant, clean solar energy ● Broad applications	● Low efficiency in particular in visible light region ● Requirement for photosensitizers and mediators

Electrochemical regeneration is the chosen method for this project. The electrochemical method based on electron transfer requires the use of a mediator, in this case, a rhodium complex that assists the regeneration of the active form of the cofactor.

2.5.3 Importance of the Electrochemical Regeneration of the Cofactor

The application of this specific regeneration cycle is beneficial from an economic stance. NADH is an expensive enzyme that makes the investigation of how to reuse and regenerate the enzyme of high priority. This cycle allows for the regeneration of this enzyme by converting NADH into its active form NAD^+ and then back to NADH by using the Rh complex. This extends the use of this cofactor allowing them to invest more time on other projects to further their research and not hinder the process of interest.

2.5.4 Coupling Enzymatic Reactions to Regenerated NADH

This process allows for the bioconversion of CO_2 to formate and the regeneration of NADH. Utilizing the addition of formate dehydrogenase as the enzyme to facilitate both reactions allows for NADH to be used as an electron carrier and donate the electron to the enzyme thus moving forward the bioconversion process. This allows for the mediator to return the cofactor to its active state, with an extra electron, and continue being an electron donor to the enzymatic reaction. Coupling these two reactions is ultimately very beneficial in terms of efficiency, time, and money, as this cycle can optimize the desired outcome being the regeneration of NADH and the bioconversion of CO_2.

2.6 Flow Chemistry

2.6.1 Flow Chemistry Basics

Flow chemistry refers to the continuous operation of the apparatus. Pumps move the reactants into the apparatus, in this case a fuel cell, then at a conjunction, the reaction occurs. The concentrations of the input reagents are known, and the flow rates are controlled by a mass flow controller, enabling easy setup of the parameters for the experiment. Flow chemistry is often used in reaction scale-up.

2.6.1 Differences Between Flow and Batch Processes

Flow and batch processes are often compared, as they each introduce advantages and disadvantages for a chemical reaction. Batch processes are dependent on the ratio of the concentrations and volume of the reactants, as opposed to flow processes, which rely on concentration and flow ratios.[21] Batch processes operate in a specific sequence, often linked together in sequence with other batch processes. Flow processes allow a process to operate seamlessly and simultaneously. For this project, a flow process is used.

2.6.3 Electrochemical Reactions in Flow

In electrochemistry, the ability to set the continuous flow rate parameters allows better control over the flow of electrons through a process.[21] Furthermore, electrochemical flow processes can be analyzed via spectroscopy and UV-Vis technologies.

At their core, fuel cells convert the energy from fuel into electricity. They operate by a continuous input of a fuel gas, hence the chosen flow processing method. Theoretically, fuel cells would be able to continue operating endlessly and indefinitely, provided continuous fuel input.[16]

Inside the fuel cell studied for this project, CO_2 is converted into formate through an electrochemical reaction involving the reduction of NADH to NAD^+ via the rhodium complex. In this fuel cell, hydrogen is one input, and the other is the NAD^+ solution. The two reactants combine and react at the proton-conducting membrane with the rhodium complex to produce NADH. Lastly, the NADH reacts with the formate dehydrogenase and CO2 to convert it to formate.

2.7. Objective

For this project, the two goals were to investigate the electrochemical regeneration of the cofactor and to couple the regeneration of the cofactor to the reduction of CO2 mediated by formate dehydrogenase. Additionally, this project is aimed at coupling these two reactions to reach a high bioconversion of CO_2 while maintaining a stable environment as regeneration takes place. Coupling these two reactions to create a cycle within the flow cell is beneficial as regeneration of NADH can act as an electron donor to the enzymatic ration thus making this the main focus of this project.

3.0 Materials and Methods

3.1 Graphite Felt Electrode Pretreatment

A 4 cm x 4 cm x .4 cm graphite felt piece was cut and used as a cathode within the reactor. This felt undergoes treatment to make the felt less hydrophobic which would limit the distribution of solution within the reaction. This treatment, described in **Appendix A.1**, includes washing the felt with iterations of ethanol-water solution beginning with pure ethanol and ending with pure water.

3.2 Carbon Paper with MWCNT (CP-MWCNT) Preparation

In a glass container, 10 mg of multi-walled carbon nanotubes (MWCNT) is combined with 50 mL of ethanol, then sonicated for 5 hours. Carbon paper is cut into either 5 cm x 5 cm x 0.05 cm squares, or into a circle with a 10 cm diameter. Once the ethanol-MWCNT solution was ready, the suspension was decanted and vacuum filtered through, using the carbon paper as the filter. The carbon filters were then dried in a 60℃ oven overnight. This process was repeated to prepare bucky papers, where the MWCNT suspension was decanted and filtered through a PVDF membrane filter.

3.3 Carbon Support Electrode Functionalization with [Cp*Rh(bpy)Cl]$^+$

Following the protocol laid out within the literature, the carbon support was functionalized. 1 mM of 4-amino-2,2-bipyridine mixed with 2 mM of sodium nitrite in 0.5 M of HCl was gently stirred for 5 min producing 2,2-bipyridyl diazonium cations. Two cyclic voltammogram cycles were performed to apply a negative potential to the system. These cycles were run between 0.4V and -0.8 V with a scan rate of 20 mV/s. Following this, the electrode was rinsed with pure water and left out to dry.

The functionalized 2,2-bipyridine moieties electrode was then soaked in a DCM solution with 0.15 mM $(RhCp*Cl2)_2$ gently being stirred for 4 hours. The functionalized rhodium complex electrode was then rinsed with DCM for 5 mins, to remove any residue on the electrode that was left unreacted.

3.4 Enzymatic Carbon Paper with MWCNT (CP-MWCNT-FDH) Preparation

Carbon paper with multi-walled carbon nanotubes was prepared following the procedure found in **Appendix A.2**. 50 µL of a suspended formulation of formate dehydrogenase enzyme was deposited onto the prepared paper and then stored in a fridge with a temperature of 3°C for 2 hours.

3.5 Electrochemical Reactor

3.5.1 Gas Diffusion Electrodes

For the reactor, a platinum/carbon gas diffusion electrode with a platinum concentration of 0.2 mg/cm^2 was used as the anode. This electrode is attached to the graphite bipolar plate, grooved with 1 x 1mm^2 channels, aiding the movement and dispersion of hydrogen through the anode chamber. The gas diffusion electrode ensures continuous and uniform production rates of both the electrons and protons at a constant hydrogen flow rate of 20 mL/min.

3.5.2 Electrochemical Reactor Schematic

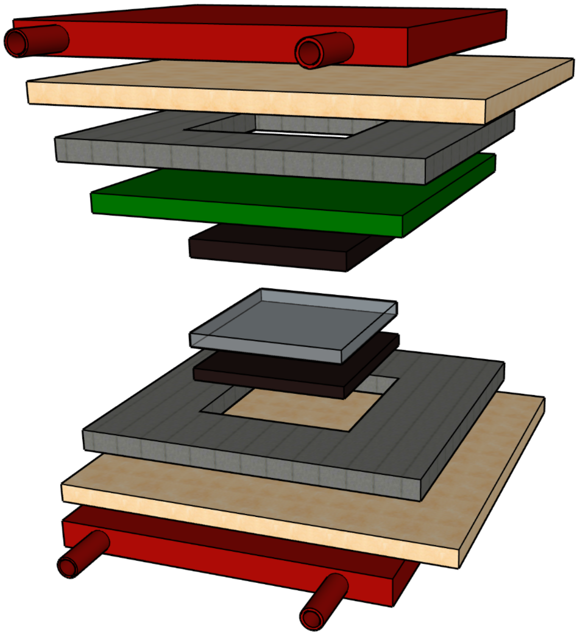


Figure 5: Schematic View of the Flow Electrochemical Reactor

Hydrogen enters at the bottom and NAD^+ solution enters at the top (red top and bottom pieces). A proton-conducting Nafion membrane is placed next to the gas diffusion electrode (translucent gray) to promote hydrogen oxidation from the graphite felt electrode. The graphite felt (black) is placed into the gaskets (dark gray) to provide the correct compression for the system. The polymeric grid (green) is used to hold the graphite felt in place and ensure no solution leaks. This allows for NADH production by electrocatalytic reduction of NAD^+ with $[Cp^*Rh(bpy)Cl]^+$ complex. Electrons move from the anode (bottom copper plate) to the cathode (top copper plate).

3.5.2 Electrochemical Reactor Process

A 50 mM phosphate buffer solution and various amounts of NAD^+ are introduced into a glass storage tank. Assuming room temperature, the pH is then adjusted to 7.2. The solution is fed into the fuel cell via a peristaltic pump through a graphite felt piece. Graphite felt is useful as it is a good electrical conductor with homogenous solutions and works as a three-dimensional cathode in this system. In this process, the graphite felt is first used as a cathode with the rhodium complex. Then, it was used in the second half of the reaction as a conducting material, as it touches the CP-MWCNT functionalized with the rhodium complex. A Nafion membrane was placed between the two electrodes to prevent solution and to aid in the uniform transport of protons between the electrodes. The two copper ending plates are used to quantitatively measure the potential within the cell during the process. Nitrogen is continuously sparged into the solution within the glass container to prevent evaporation of NAD^+. The nitrogen and hydrogen flow rates are controlled by mass flow controllers.

4.0 Safety

Safety within the lab is important to ensure the wellbeing of those within the lab and for the integrity of the data. This project involves heavy use of MWCNT and Ethanol (98%). When using the MWCNT it is necessary to wear a mask at all times to avoid the inhalation of carbon. When using ethanol it is necessary to use goggles to avoid any contact with your eye and potential irritation. When in the lab it is always a good idea to use gloves and lab coats to protect yourself and your clothing. Wearing gloves in the labs limits the potential for irritation of the skin and stops any oils from your skin from entering the system. Additionally, proper lab attire will always be worn (long pants, closed-toe shoes).

5.0 Results and Discussion

5.1 Immobilization of $[Cp^*Rh(bpy)Cl]^+$

A two step process is used to immobilize the rhodium complex onto the carbon paper. In the first step, 4-amino-2,2-bipyridine reacts in solution to produce bipyridyl diazonium cations. Next, cyclic voltammetry is used to release dinitrogen to promote the generation of aryl radicals onto the graphite paper. Lastly, the rhodium complex covalently is immobilized after the complete reaction of $(RhCpCl_2)_2$ with the functionalized electrode.

5.1.1 Selection of the Electrode Material

To improve the electro-enzymatic efficiency, it's crucial to choose an electrode material with appropriate properties. One method is to enhance the electroactive surface area. In this experiment, this is done by dispersing MWCNT onto a carbon paper and immobilizing the rhodium complex on this surface. This method was chosen as a combination of fuel cell technologies and redox flow techniques, and the MWCNT surface proved more efficient than bucky paper with graphite felt electrodes.

This experiment was tested using cyclic voltammetry, with parameters set to 0.4 V to -0.8 V vs Ag/AgCl. An irreversible wave was seen at -0.35 V vs Ag/AgCl. This corresponds to the grafting of 2,2-bipyridine and the release of N2. Then, a lower reduction peak was observed in the second scan, indicating the electrografting step was successful. Lastly, the CP-MWCNT electrographted with the 2,2-bipyridine moieties and was soaked in a $(RhCpCl_2)_2$ solution to immobilize the rhodium complex.

Next, this was evaluated using cyclic voltammetry at a flow rate of 20 mL/min of the buffer solution at a pH of 7.2 A graphite felt piece was place in the system to ensure uniform coverage of the solution in the cathodic compartment of the cell and to maintain good conduction with the graphite bipolar plate.

Without NAD^+, the cathodic current was smaller. The addition of NAD^+ between the concentrations of 1 to 4 mM led to an increase in the cathodic current and slightly shifted potential. The maximum current wass observed around 3.5 mM NAD^+. A prior work showed that under high flow conditions, the immobilized system became unstable, corroborating the results.

NADH was fully regenerated after 50 minutes of electrolysis and showed an average faradaic efficiency of 71% using the CP-MWCNT-bpy-Rh cathode. By integrating the cathodic peak data, the concentration of the rhodium complex was approximately 4.125 $nmol/cm^2$. From this, the turnover frequency was calculated to be 1.7 s^{-1}, corroborated by literature values in prior publications. The ratio between dissolved rhodium complex and NAD^+ could not exceed 40, as the turnover frequency would stay around 300. This also led to a regenerated NADH solution that did not have any leftover or trace rhodium complex.

To check the results, a bucky paper functionalized by the rhodium complex was also put through the same experiment, yielding a conversion rate of 92% after 50 minutes and a faradaic efficiency of only 56%. Small amounts of $(RhCpCl_2)_2$ were still found in the solution and could not be removed by dissolution of the solvent. Therefore, the data confirms that carbon paper with with a layer of MWCNT and immobilized with $[Cp^*Rh(bpy)Cl]^+$. (CP-MWCNT-bpy-Rh) was the best option for the regeneration of the NADH cofactor.

5.1.2. Stability and Sustained Activity

A cell containing the gas diffusion electrodes, Nafion membrane, and the CP-MWCNT-bpy-Rh was pressed together under 40 bars of pressure. This was done to improve the contact within the whole cell. It was tested at different times under the same flow conditions after the electrode preparation: 4 hours, 12 hours, 24 hours, and 120 hours. The constant cathodic current that was observed shows that the electrode maintained working for more than 5 days. This data confirms the stability of the rhodium complex, giving promise to long-term operation of the electrode. After this, NADH regeneration and its application in electro-enzymatic biosynthesis was further explored.

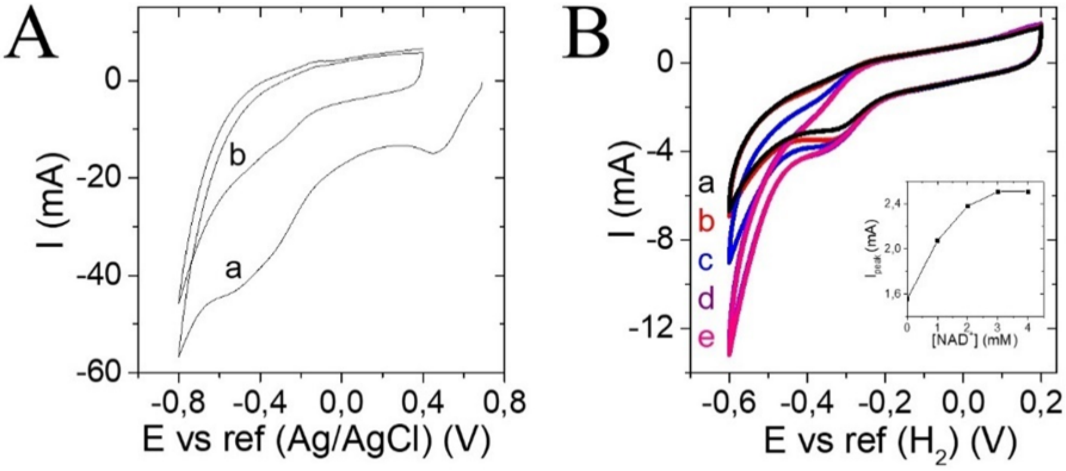


Figure 6: Cyclic Voltammetry Results [1]

A: *(a) first and (b) second cyclic voltammogram for the reduction of diazonium cations generated 'insitu' from 1 mM 4-amino-2,2'-bipyridine and 2 mM* $NaNO_2$ *in 0.5 M HCl, as recorded on CP-MWCNT electrode at 20 mV/s.*

B: *Cyclic voltammograms recorded at a potential scan rate of 5 mV/s (Scan 4) using a CP-MWCNT-bpy-Rh electrode in 50 mM PBS buffer at pH 7.2 under nitrogen and in the presence of increasing concentrations of* NAD^+ *(a) 0 mM (b) 1 mM (c) 2 mM (d) 3 mM and (e) 4 mM . The geometric surface area of the electrode was 16* cm^2 *and hydrogen and solution flow rates are 20 mL/min.*

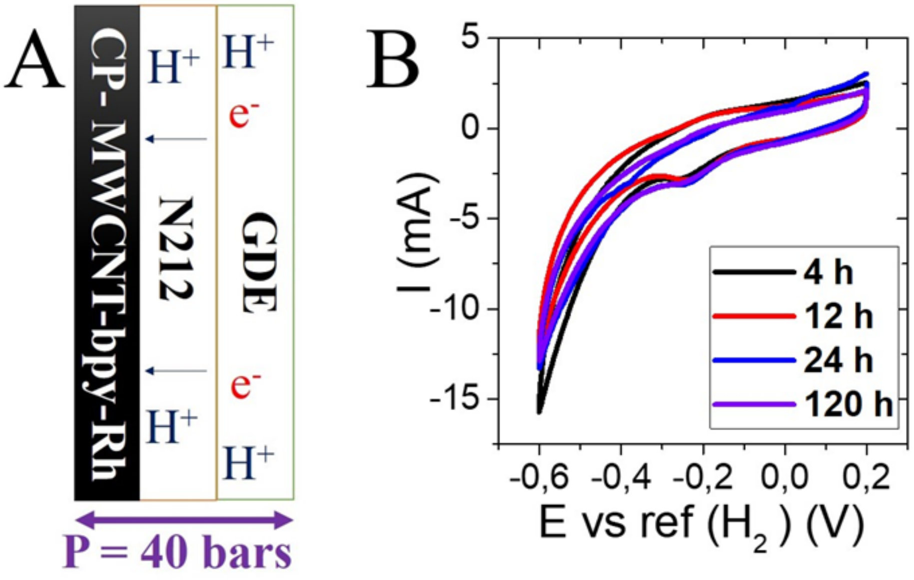


Figure 7: System Under 40 Bars of Pressure [1]

A: *Schematic representation of GDE-N212-(Cp-MWCNT-bpy-Rh) system pressed together at 40 bars.*

B: *Cyclic voltammograms recorded at a potential scan rate of 5 mV/s using a* $[Cp^*Rh(bpy)Cl]^+$ *functionalized CP-MWCNT (using the pressed system in Figure 7A) electrode in 50 mM PBS buffer at pH 7.2 under nitrogen after 4 h, 12h, 24 h, and 120 h of electrode preparation. The geometric surface area of the electrode was 16* cm^2 *and hydrogen and solution flow rates are 20 mL/min.*

4.2 Application of Regenerated NADH in an Enzymatic Reaction

4.2.1 Parameters of Optimization for the Efficient Bioconversion of CO_2

The active form of NADH is produced by the redox flow reactor with the presence of an enzymatic reaction. This also allows for the regenerated NADH to act as an intermediate in the catalytic hydrogenation of CO_2.

Regeneration of NADH on a CP-MWCNT-bpy-Rh electrode coupled with the bioconversion of CO_2 has been shown to be a successful cell construction. This environment allows for the current collector and the active electrode to be connected with the catalytic layers not exceeding 50 μm. The CO_2 and the NADH are circulated in the cathodic compartment where the Rh complex works to regenerate NADH. This regeneration of NADH allows for the bioconversion of CO_2 to formate.

Using small amounts of the NADH cofactor (10 μM) as an active mediator in the bioconversion of CO_2 is economically desirable. This allows the concussion to be drawn that stability of NADH is consistent, in both the oxidized and reduced form, as time passes. Low volumes of cofactors have also been shown to reach a high TTN which is desirable for the objective of the system.

6.0 Conclusion

Coupling the regeneration of NADH and the bioconversion of CO_2 is a very efficient way to run a fuel cell and regenerate the cofactor. The investigation of the electrochemical regeneration of NADH coupled with the bioconversion of CO_2 is ongoing but thus far has been very promising. This cell is running with high efficiency and meets all of the desired qualities. The cycle that is created allows for the bioconversion of CO_2 to be run to completion, creating formate, which on a larger scale could help with CO_2 emissions. Both of the original objectives of this project have been met by the cell in question which will allow for further investigation to be done.

7.0 Recommendations

Within research, processes can always be improved upon, both to ensure accurate and precise results, as well as to create replicable procedures. Recommendations can range from additional steps in processes to reduce errors and recommendations for equipment upkeep. The addition of steps to complete a process more thoroughly may likely add to the total run time for an experiment. However, the additional time investment should improve the accuracy of the result, reflecting in thorough and honest research results.

One recommendation that can be implemented into the process is error mitigation. Oftentimes when weighing samples, it was unclear if the scale had been calibrated recently, as the mass value seemed to fluctuate outside of a reasonable range, resulting in potential instrumentation error reporting an incorrect mass. Therefore, we would recommend adding a daily calibration step, logged in an annotated notebook, to increase the accuracy of all measurements using the scale.

Additionally, when the carbon papers are being prepared, a significant amount of the carbon nanotubes are lost in the process. Theoretically, we begin with a solution of 10 milligrams of carbon nanotubes in 50 mL of ethanol. The vacuum filtration process is split in two ways. The first method is much more accurate, as the apparatus is tightly fit onto the square-sized piece of carbon paper to prevent leakage. The carbon nanotubes-ethanol solution is filtered through once, and the nearly-clear ethanol left in the vacuum flask indicates almost all of the carbon nanotubes have been deposited onto the carbon paper. The second method is much less accurate, as the carbon paper is freehandedly cut into a circle and placed in a circular unfitted filter. Due to the leakage, as it is not tightly fitted, we would repeat the filtration process three times to increase the amount of carbon nanotubes that were deposited onto the paper. However, there was no way to ensure all of the carbon nanotubes were deposited on the paper, and this was reflected in the leftover solution in the flask used for filtration. As seen in the photos below, the first method using the tight-fitted filtration apparatus resulted in a nearly-clear solution post-filtration. The second method using the unfitted filter resulted in poor filtration, as there are still visible carbon nanotubes in the solution post-filtration.

Table 2: *Filtration Apparatuses*

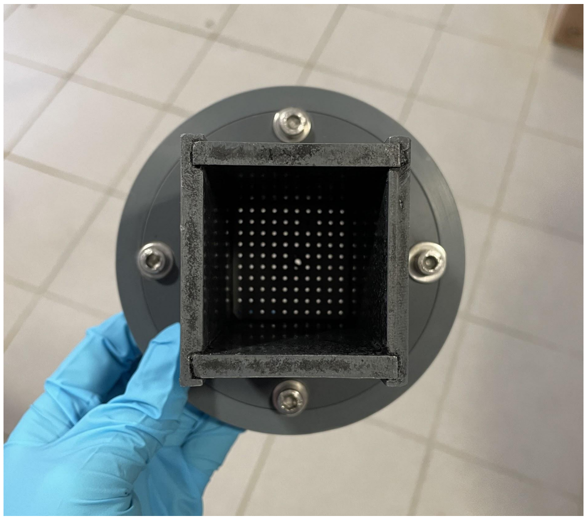
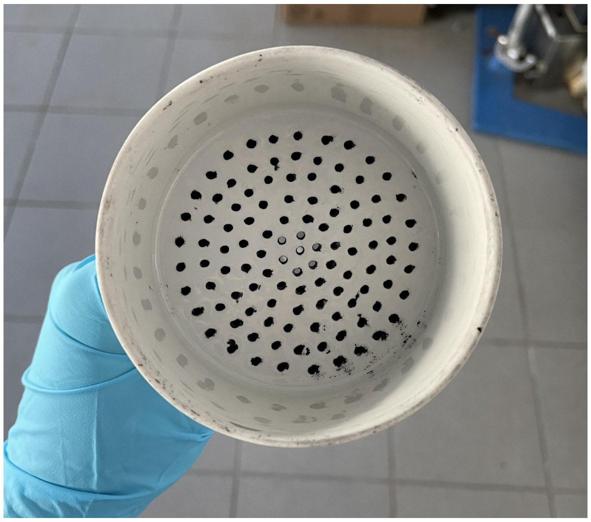
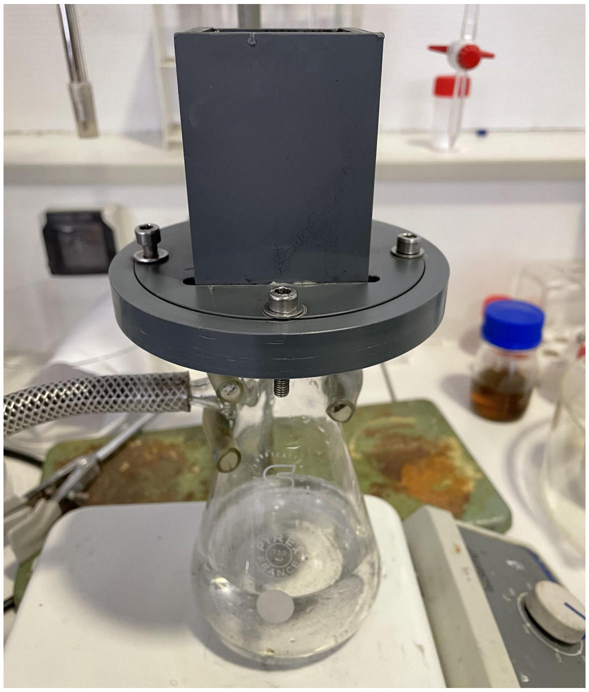
First Method: Tightly-Fitted Apparatus	Second Method: Unfitted Filter
	
	

Table 3: *Remaining Solution Post-Filtration Comparison of Both Apparatuses*

First Method: Tightly-Fitted Apparatus	Second Method: Unfitted Filter
	

Our recommendation to standardize the filtration process would be to weigh the carbon papers prior to carbon nanotube deposition, then weigh them after the filtration process to know how much of the carbon nanotubes were deposited on each paper from the original 10 milligrams. Making changes like these will allow for an increase in the accuracy and precision of the research so that trials can be repeated with minimal uncertainty.

For the purpose of certainty, it would be interesting to look into the potential for contamination during ethanol recycling. Though filter papers are being utilized to remove carbon that was emulsified in the ethanol, there is the potential for the ethanol to still be contaminated. Reusing ethanol without ensuring that there is no contamination could cause outcomes that cannot be replicated. As this is the main solvent being used all trials should be completed with the certainty that there is no contamination.

Though we are new to this particular lab, oftentimes important lab equipment (beakers, bottles, wrenches, etc.) would go missing without a backup readily available. To save on lost time looking for this equipment, it would be ideal to invest in multiples of important pieces of equipment. This will allow everyone working in the lab to complete their work with the equipment that is required.

Finally, we have a recommendation to add an organic solid waste container into the laboratory. As organic materials can be harmful to the environment and those who come in contact with it, having a separate waste container specifically for solid organic material is important. In the process of recycling ethanol, a filter paper with carbon nanotubes and traces of ethanol is produced and needs to be disposed of making this recommendation important to consider if this process is to be continued.

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Appendix

Appendix A.1: *Graphite Felt Electrode Pretreatment*

Equipment & Materials

- Graphite felt (GFD4.6EA, SGL, Germany)
- Scissors
- 200 mL beaker
- Water
- Ethanol (96% purity)
- Glass 250 mL container + cap
- 50 mL volumetric flask
- Sonicator bath

Procedure

1. To begin, pour a small amount of ethanol, < 20 mL, into the 200 mL beaker. Swirl the solvent around the glassware to coat it with ethanol, then dump into the appropriate waste container. This step ensures the cleanliness of the glassware prior to beginning the experiment. Next, fill the beaker with ethanol, approximately 150 mL. Pour a small amount, < 20 mL, into the 250 mL glass container. Pour waste into the appropriate waste container. Lastly, pour ethanol from the 200 mL beaker into the 50 mL volumetric flask, swirl around and dump the waste. Once all the glassware has been rinsed with ethanol, pour ethanol from the beaker into the volumetric flask until it is filled to the 50 mL line. Transfer this volume to the 250 mL glass container.
2. Submerge carbon felt in the ethanol and cover bottle with a screw top
3. Sonicate for 15 mins
4. Add water to the glass bottle to create a 80:20 ethanol-water solution
5. Sonicate for 15 mins
6. Repeat steps 4 and 5 with 60:40, 40:60, 20:80, and 0:100 ethanol-water solution

Appendix A.2: *Carbon Paper with MWCNT (CP-MWCNT)*

Equipment & Materials

- Multi-walled carbon nanotubes
- 200 mL beaker
- Ethanol (96% purity)
- Four (4) glass 250 mL containers + caps
- 50 mL volumetric flask
- Scale
- Sonicator bath
- Carbon paper 5 cm x 5 cm
- Weighing paper
- Funnel and filtration apparatus (PVDF membrane filter)
- 250 mL Erlynmeyer flask
- Small plastic funnel
- Glass plates, diameter ≈ 10-15 cm
- Vacuum
- Ring stand
- Screwdriver
- ⅝ wrench
- Screws
- Nuts & bolts
- Electric drill

Procedure

1. To begin, pour a small amount of ethanol, < 20 mL, into the 200 mL beaker. Swirl the solvent around the glassware to coat it with ethanol, then dump into the appropriate waste container. This step ensures the cleanliness of the glassware prior to beginning the experiment. Next, fill the beaker with ethanol, approximately 150 mL. Pour a small amount, < 20 mL, into the 250 mL glass container. Repeat the above step, swirling the ethanol to clean and coat the glass container. Pour waste into the appropriate waste container. Repeat with all four glass containers. Lastly, pour ethanol from the 200 mL beaker into the 50 mL volumetric flask, swirl around and dump the waste. Once all the glassware has been rinsed with ethanol, pour ethanol from the beaker into the volumetric flask until it is filled to the 50 mL line. Transfer this volume to the 250 mL glass container. Repeat this step with all four glass containers, then cover each container with a tightly screwed on cap.
2. Next, wearing a mask and using a scale, measure 0.0100g ± 0.0005g of carbon nanotubes onto a weighing boat. Uncap the glass containers with 50 mL ethanol and carefully pour the carbon nanotubes into the solvent, then recap it. Repeat for the remaining containers.

3. Move the containers with the ethanol-carbon nanotube solution into a bath and sonicate for 5 hours.
4. Cut a 5 cm x 5 cm square piece of carbon paper.
5. Assemble the filtration apparatus by placing the carbon piece of paper to cover the filtration holes. Manually place nuts and bolts into the top and bottom pieces to clamp and sandwich the carbon piece in-between. Use the ⅝ wrench to hold the bottom nut in place and use a drill to tighten the screws into all the openings.
6. Turn on the vacuum pump and place a plastic filter with a filtration paper inside on top, then balance the filtration apparatus on top.
7. Take one of the glass containers with the ethanol-carbon nanotube solutions and shake it to aggravate the carbon nanotubes. Open the cap and pour the solution into the filter apparatus. Wait 2-3 minutes and check that there is no excess liquid yet to be filtered. Once only a sludge-like solution remains, turn off the vacuum and disassemble the filtration apparatus.
8. Gently remove the carbon paper and move it to a glass plate.
9. Store the carbon paper in a 60℃ oven overnight.

Appendix A.3: *HPLC Dilutions to Make a Standardized Curve*

Equipment & Materials

- Sodium phosphate monobasic monohydrate
- Sodium phosphate dibasic heptahydrate
- Sodium formate
- Three (3) 50 mL plastic tubes + caps
- 1000 mL volumetric flask
- Fifteen (15) 100 mL plastic tubes + caps
- 1mL micropipette + tips
- Fourteen (14) HPLC glass vials
- Refrigerator

Procedure

1. Uncap three 50 mL plastic tubes and individually measure 0.34g of sodium phosphate monobasic monohydrate, [weight] of sodium phosphate dibasic heptahydrate, and [weight] of sodium formate, placing the compounds directly into the tubes.
2. Prepare a sodium phosphate solution by combining the sodium phosphate monobasic monohydrate and sodium phosphate dibasic heptahydrate into the 1000 mL volumetric flask. Fill with water, place on a stir plate and allow the solution to mix for 15 minutes.
3. Measure the pH of the sodium phosphate solution. The acceptable range is 7.1-7.4 pH.
4. Measure 50mL of the sodium phosphate solution and place in a separate 100 mL plastic container. Add the sodium formate to the solution and mix. This will be the 100mM sodium phosphate-sodium formate solution we dilute to create a standardized curve.
5. Use a micropipette to measure 1 mL of the sodium phosphate-sodium formate solution into a HPLC glass vial.
6. To begin making a series of solutions, label the two containers as phosphate buffer solution and sodium phosphate-sodium formate solution. Next, fill a 100 mL plastic tube most of the way with the sodium phosphate buffer solution.
7. Using a micropipette, measure appropriate amounts of phosphate buffer solution and sodium phosphate-sodium formate solution to create proper dilutions of 100mM, 80mM, 60mM and 50mM (see table below) into individual 100mL plastic tubes, labeled with their corresponding concentration. Pull 1mL of each solution with the micropipette and place into HPLC glass vials, making sure to label each one in descending order (e.g. #1 = 100mM, #2 = 80 mM, #3 = 60 mM, etc.)
8. Next, using the 50mM solution instead of the 100mM solution, continue diluting in increments of 40mM, 30mM, 20mM, 10 mM, 5mM, 4mM, 3mM, 2mM, 1mM, and 0.5mM (see table below) into individual 100mL plastic tubes, also labeled with their corresponding concentration. Measure 1mL with the micropipette and place into the corresponding HPLC vial.

9. Place the 100 mL plastic containers into a refrigerator to keep the solutions cool. Bring the HPLC vials to the room with the HPLC instrument.

Dilutions Table

Concentrations calculated using $C_0V_0=C_1V_1$

Sample #	**Concentration** (mM)	**Volume** $_{\textbf{formate sol'n}}$ (mL)	**Volume** $_{\textbf{buffer sol'n}}$ (mL)	**Total Volume** (mL)
1	100	~50	0	~50
2	80	4	1	5
3	60	3	2	5
4	50	12.5	12.5	25

*swap 50 mM concentration with 100mM concentration and continue dilutions
(e.g. for 40mM, use 4 mL of formate solution from 50 mM and 1 mL of buffer solution)

5	40	4	1	5
6	30	3	2	5
7	20	2	3	5
8	10	1	4	5
9	5	0.5	4.5	5
10	4	0.4	4.6	5
11	3	0.3	4.7	5
12	2	0.2	4.8	5
13	1	0.1	4.9	5
14	0.5	0.05	4.95	5