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Evaluation of redox potential as a novel biomarker of oxidative stress, inflammatory response, and shock using nanoporous gold electrodes

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Table of Contents

List of Tables, Figures, Abbreviations, Formulas and Symbols

Abbreviations: CV = cyclic voltammogram, OCP = open circuit potential, ECMO = extracorporeal membrane oxygenation, CPB =cardiopulmonary bypass, SIRS = systemic inflammatory response syndrome, MODS = multiple organ dysfunction syndrome, NP Au =nanoporous gold, DL = double layer, SL = single layer

Equations:

Equation 1: Redox Reaction

$$Ox + e^{-} \rightleftharpoons Red$$

Ox = Electron acceptor, known as oxidant, oxidizing agent, or pro-oxidant
e^{-} = Electron
Red = Electron donor, known as reductant, reducing agent, or anti-oxidant

Equation 2: Nernst Equation

$$E = E_o + \frac{RT}{zF} \times \log(\frac{Ox}{Red})$$

E =redox potential
E^o=standard reduction potential
R=gas constant 8.31 J mol^{-1} K^{-1}
T=absolute temperature (in K)
z=number of electrons transferred
F=Faraday constant, 96,485 J $V^{-1}mol^{-1}$
log=common logarithm

Abstract

EVALUATION OF REDOX POTENTIAL AS A NOVEL BIOMARKER OF OXIDATIVE STRESS, INFLAMMATORY RESPONSE, AND SHOCK USING NANOPOROUS GOLD ELECTRODES

By Matthew C. Ellenberg

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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Director: Bruce Spiess MD, Professor, Department of Anesthesiology.

Background: Redox potential is a chemical species' affinity for electrons. Increased oxidant concentration is associated with disease[1,2], yet there is not a way to measure systemic redox status.[3] Redox potentiometry uses metal electrodes that do not work in blood because protein molecules adhere on the metal surface, blocking electron exchange.

Methods: Nanoporous gold electrodes have large surface areas that allowed electron exchange to continue in blood.[4] Redox potential was measured in blood with ascorbic acid, in cardiac bypass patients and pigs undergoing hemorrhagic shock and resuscitation.

Results: Blood redox decreased with ascorbic acid addition, both in vitro and in vivo. It was more positive in patients undergoing cardiac surgery compared to healthy volunteers.

Conclusions: Preliminary studies were limited, but appear to show correlation to disease processes and medical therapies. More work needs to be done to further examine the relation of redox to disease and treatment.

Introduction

Circulatory shock is a physiologic condition defined as inadequate blood flow to tissue, known as hypoperfusion, causing cell damage and ultimately death of the organism. Recognition and early management of shock is essential for decreasing morbidity and mortality. One of the primary reasons circulatory shock is so insidious is because it operates as a positive feedback mechanism.[5]

Hypoperfusion and hypoxia cause peripheral cell and tissue damage which leads to local inflammation. Inflammation is an important physiologic response that increases blood flow to the damaged area. Inflammation is important for wound healing and the body fighting pathogens, but can become overabundant and lead to an inability of the circulatory system to meet the demands created by the peripheral vasculature.[6] This is known as systemic inflammatory response syndrome, or SIRS, and is rapidly followed by multiple organ dysfunction syndrome (MODS) then death.[7]

Positive feedback loops are pathologic when they do not have a braking mechanism and eventually reach a tipping point, where there is a rapid shift into a new stable condition of and recovery to the old stable state may not be possible.[8,9] The new stable condition in shock is decompensation leading to death. As a result, early identification of shock is invaluable in emergency and critical care medicine.[10]

Shock resuscitation is titrated based on data and feedback from the patient; the sicker the patient is, the more aggressive the therapy needs to be. If clinicians are provided with better data, then more appropriate treatment can be provided, resulting in decreases in morbidity and mortality. There is not a single test that will determine a patients' level of shock and inflammatory response, but using multiple tests that look at multiple aspects of shock physiology and analysis of trending data can allow clinicians to predict outcomes and prevent death.[11]

A mainstay in healthcare is the simple non-invasive hemodynamic monitoring of the heart rate, respiratory rate, pulse oximetry, and blood pressure, called the "vital signs". Clinicians closely monitor

these and observe trending behavior in order to help recognize clinical deterioration.[12] While non-invasive and invasive hemodynamic monitoring is effective in discerning late stages of shock, it is not predictive early enough in the disease process and the patient is often at or beyond the tipping point in those stages.[13] It has been repeatedly shown that blood pressure does not correlate with adequate local perfusion and the early stages of shock.[14] The human body is very good at compensating, or adjusting to early stages of shock, but once it reaches the tipping point, there is rapid clinical deterioration and mortality or morbidity is imminent.[15] By the time the body shows significant hemodynamic changes, it has already undergone a great deal of insult and the clinician must act quickly and aggressively in order to successfully resuscitate the patient and have a positive outcome.[16]

"Point of Care" blood analysis is another tool that is employed to help assess circulatory status. "Point of Care" is a term used to describe lab tests that are instantly run at the patient's bedside that give immediate results and allow clinicians to make decisions in real time as opposed to sending samples off to a lab and receiving the data up to days later.[17] There are many different tests for many disease processes, but one very prominent test used to assess circulatory shock is blood lactate levels.

Lactate is a metabolic product that comes from anaerobic metabolism and the breakdown of pyruvate. It was traditionally thought that elevated lactate meant anaerobic metabolism was occurring because of local hypoxia.[18] As a result, it began to be used as a biomarker of inadequate perfusion.[19-23] Soon, clinicians realized that patients who were improving based on other physical findings showed increasing lactate.[24] It is now believed that when epinephrine is released, either endogenously and exogenously, Beta-2 receptor activation causes excess pyruvate to be available from upregulation of glycolysis.[25] The overabundant pyruvate that cannot run through the traditional, aerobic, citric acid cycle is converted to lactate. This phenomenon demonstrates that lactate is a marker of sympathetic response as well as inadequate perfusion. Blood lactate levels can increase with shock or increase with

the body's response mechanism to shock. While lactate is a useful indicator of adrenergic surge, it is clearly not an ideal biomarker because it may increase when the patient is worsening or getting better, which can be misleading.[26] Trending lactate is still used in trauma management, cardiac surgery management, and septic shock management. Early Goal Directed Therapy is a sepsis protocol that focuses a great deal on numeric guidelines and trending lactate, despite this, it has not been shown to decrease mortality.[27]

The concept of making decisions based on data is not flawed, but perhaps better or more pieces of the puzzle are necessary for making the correct decisions about resuscitation. It is important to look at multiple aspects of shock physiology in order to effectively manage that and one aspect of shock physiology that is often overlooked is the oxidative stress aspect.[28]

Oxidative Stress is defined as oxidant/antioxidant imbalance and is associated with ion channel dysfunction[29], DNA breakdown, and systemic inflammatory response syndrome.[2] At the organism level, oxidative stress is associated with myocardial hypo-contractility, vascular hypo-reactivity, and catecholamine breakdown.[30] Oxidants are defined as electron acceptors and classified as reactive oxygen species or reactive nitrogen species.[2] Oxidants such as superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite are produced during the inflammatory response to disease by neutrophils.[31,1] During the inflammatory response, these oxidants are important in fighting infection and are normally kept in balance by reducing agent scavengers such as glutathione, superoxide dismutase, and catalase.[32] When there is too much oxidant release from neutrophils or the antioxidant scavengers are depleted, the cell and surrounding tissue enter a state of oxidative stress and oxidants begin to react with structures necessary for normal function. Reactive oxygen or nitrogen species oxidize, that is they take electrons away from, specific amino acid residues on the ion channels.[29] This redox reaction (equation 1) is constantly occurring and keeping it in balance is crucial to life.[28]

Many of the intracellular processes and components exist only intracellularly and only for a very short time period. Reactive oxygen and nitrogen species are by definition reactive, that is many of them react and are no longer found in that state.[33] Many do not cross the cell membrane into the plasma, but some (hydrogen peroxide and peroxynitrite) can and go on to oxidize heme proteins or cations in blood such as Fe^{2+}. The oxidants that do not cross the cell membrane can oxidize other molecules that do cross into the bloodstream. Both of these processes result in an increased concentration of oxidants in the bloodstream, which to this point has not been effectively measured.[34]

Recent clinical trials have shown positive outcomes with high dose parenteral ascorbic acid administration in septic shock.[35] Ascorbic acid is a powerful antioxidant that humans have lost the ability to produce. Patients given high dose parenteral ascorbic acid demonstrated clinical improvements and had a lower mortality. All of the patients analyzed in that study had lower than average blood ascorbic acid concentrations prior to therapy, possibly indicating that their endogenous antioxidants had been depleted. Additionally, parenteral ascorbic acid is being used in cardiac bypass clinical trials[36,37], burn clinical trials[38] and in multiple animal model experiments with apparent success.[39,40] Ascorbic acid is a powerful antioxidant and may have a beneficial effect due to restoring the oxidant/antioxidant or redox balance.[41]

Despite the strong association with pathologic conditions, there is not an ideal biomarker of oxidative stress that is measured in the clinical setting.[42] An ideal biomarker should be easily obtainable, minimally invasive, and predictive of condition severity before the condition reaches the tipping point, where there is irreversible damage and rapid clinical deterioration occurs. Redox potentiometry is an established technique in analytical chemistry that measures increased oxidant concentrations, but it has not been effective in the biologic setting due to technologic challenges.[43-45]

Potentiometry involves the measurement of potential difference between two electrodes, a reference electrode and a working indicator electrode. The reference electrode is inert and non-polarizable in solution, meaning that it has a fixed potential. The standard hydrogen electrode ha a potential defined as 0V under standard conditions but is not often used experimentally. Commonly used reference electrodes are silver chloride coated silver electrodes and saturated calomel electrodes.[46]

The working electrode interacts with the electrolyte solution, by either donating electrons to a solution if the solution has more oxidants (creating a positive, cathodic, or reduction current) or accepting electrons from solution if there is an excess of reductants in solution (creating a negative, anodic, oxidation current). These working electrodes interact with the solution they are in and develop an electric potential based on the redox reactions occurring at the electrode-electrolyte interface. Working electrodes are often gold, platinum, or carbon because they are relatively inert and do not react in most solutions, or in carbon's case, the redox reactions just occur very quickly. In order to have a functioning working electrode, the interface has to be large and clean to effectively allow electron transfer.[46]

Blood is a complex, heterogeneous solution with large, amphipathic proteins such as albumin. These proteins adhere to the metal surface of the working electrode and prevent further electron transfer by any electroactive species in the solution.[47] Herein is the current difficulty with measuring redox potential in blood and why it has not been effectively used as a biomarker to date.[48]

It has been previously shown that nanoporous gold dramatically reduces the impact of protein adhesion on metal redox electrodes and does it without the use of chemical modification or application of a very high reducing potential.[4] Small redox active species are able to interact with the electrode within the pores while large protein molecules such as albumin and fibrinogen cannot fit. Not only do these electrodes have a much larger surface area than their planar equivalents, but due to the porosity,

the majority of the electrode's electroactive area is not reachable by protein molecules that would normally cause electrode dysfunction.

Increased oxidant concentration is well linked to cellular dysfunction, inflammatory response, progressive shock, and mortality, but there has not been an effective way to measure it.[49] Nanoporous gold electrodes can effectively measure redox potential where traditional electrodes have fallen short.[50] Therefore, it is the goal of this research to evaluate blood redox potential measured by nanoporous gold redox electrodes. This was done by measuring blood redox potential in animal model shock experiments and in patients with known pathologies where they may have increased oxidative stress.

Objectives:

- Fabricate and characterize nanoporous gold electrodes
- Show nanoporous gold electrodes demonstrate Nernstian or near-Nernstian behavior using ascorbic acid as the analytic reagent
- Evaluate redox potential changes in specific pathologic conditions
- Evaluate redox potential changes in specific therapies to pathologic conditions

Production of Nanoporous Gold Redox Electrodes

Introduction:

This procedure was heavily based on the one already being performed in the Collinson lab.[50] Commercially available white gold leaf was immersed in concentrated nitric acid. This selectively de-alloyed the silver in the leaf, causing the remaining gold to develop a nanoporous framework with a much larger surface area.[51] The major variation from that previously described protocol was the addition of two layers of nanoporous gold on the gold substrate. An example of a double-layer nanoporous gold redox electrode is shown in Figure 1. In Figure 1, the gold plated portion of the electrode is exposed on the left for the potentiostat cable to connect to and the rest is covered with Teflon PFTE tape and parafilm with the exception of the nanoporous circle on the right. The second leaf addition increased the surface area of the electrode, which created more nanoporous framework for electron exchange at the electrode electrolyte interface. The second leaf is not visibly discernable to the naked eye compared to the single leaf method once the electrodes are completely taped.

Figure 1: Nanoporous gold redox electrode

Figure 2 shows a scanning electron microscope image of a planar gold redox electrode surface. Although the surface has some imperfections, it is relatively flat. Figure 3 shows a scanning electron microscope image of a nanoporous gold redox electrode. Due to the selective de-alloying of silver, the gold has formed pores that are between 5-50 nm in diameter. These nanopores not only give the electrode a much larger surface area for the analyte to interact with but selectively allow redox active molecules access to the deeper aspects of the nanoporous surface and exchange electrons with the electrode.

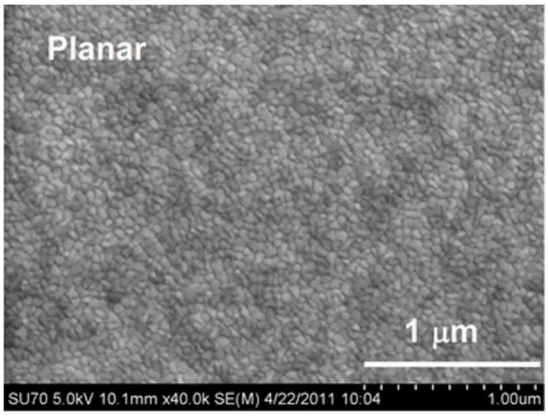

Figure 2: Scanning Electron Microscope image of a planar gold redox electrode[4]

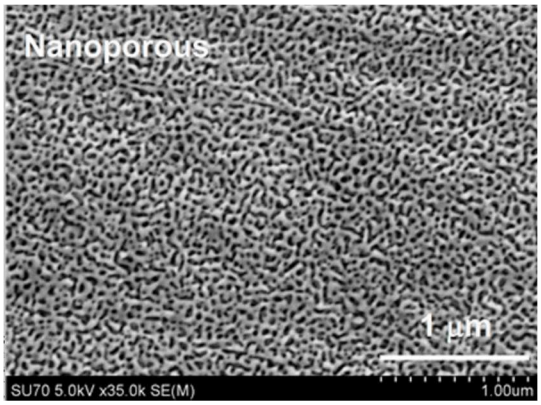

Figure 3: Scanning Electron Microscope image of a nanoporous gold redox electrode[4]

Procedure:

Manetti 49% Au:Ag white gold leaf (purchased at FineArtStore.com) was placed on a glass plate with a sheet of paper underneath and taped on all four sides. 1 cm square sections were cut and submerged in concentrated nitric acid (CAS No. 7697-37-2; Sigma Aldrich St. Louis, MO) for 12 minutes then placed in deionized water bath.

Titanium-adhesed gold-plated evaporated metal films purchased from EMF Corporation were cut with a glass cutter into approximately 0.75cm wide sections (x2.5cm) and cleaned in a sonicator with deionized water for approximately 10 minutes.

The de-alloyed gold leaf section in deionized water was picked up on the end of the gold-plated slide section and blown with medical air for several seconds until the leaf completely dried so the gold would stick to the gold-plated slide. Once the de-alloyed leaf had completely dried, another de-alloyed leaf section was picked up directly on top of the first section and blow dried to create a second nanoporous gold layer. If electrodes were not going to be immediately used, they were stored in a covered petri dish under a 20W UV light container to minimize electrode dirtiness and possible contamination.

Just before use, a 1/8" diameter hole punch was used to make a hole in medical grade PTFE tape (CS Hyde Co) which was then taped so that an exact, known geometric area of nanoporous gold was exposed. The tape was pressed firmly on the electrode and the base of the tape was wrapped in parafilm to ensure there was no exposed metal to the solution the electrodes were used in. Electrodes were stored prior to the taping step in a covered petri dish in UV light to minimize electrode dirtiness and possible contamination if they were not going to be immediately used.

Electrode Quality Analysis Protocol

Introduction:

Metal redox electrodes work best when they are clean and have a large surface area.[46] The goal of these experiments is to show that nanoporous gold redox electrodes are clean, have a high surface area, and have a nanoporous framework in order to effectively allow electron exchange of redox active species.

Cyclic voltammetry is an electroanalytical technique measuring the current across an electrode while a potential is applied in a linear fashion against time to electrode and then an equal, reverse potential is applied. This technique can be used to identify reaction reversibility, identify redox intermediates, or evaluate electrode performance.[52] Cyclic voltammetry uses a three electrode system, a working electrode, where the reaction occurs; a reference electrode, where the potential change is being measured against; and a counter electrode, where the current flows toward in order to prevent ohmic drop at the reference electrode.[53] A potential is applied to the working electrode linearly against time, then a reverse potential is applied to the working electrode vs. time. The potential added is plotted as the independent variable and the current is the dependent variable. If the potential is cycled from a more electronegative potential to a more electropositive potential then back to the more electronegative potential, it first displays oxidation with an anodic peak, then reduction with a cathodic peak. Based on information obtained from the hysteresis plot in cyclic voltammograms in specific solutions, information about the electrode quality was obtained.

Potassium ferricyanide is a commonly used salt used in electrochemistry due to its known electron transfer properties. Because potassium ferricyanide oxidation and reduction is a reversible reaction, cyclic voltammograms of electrodes in dilute ferricyanide solution show

similar anodic and cathodic peaks with a peak separation of approximately 59.2 mV.[54] If an electrode had a dissimilar peak currents or peaks that were spread too far apart, then it was considered dirty or excluded from analysis.[55]

The electroactive surface area of a gold electrode can be calculated based on the charge required to reduce a gold oxide layer off of its surface.[51, 56, 57] The gold oxide layer formed on the surface by applying an oxidizing potential in dilute sulfuric acid. The electrode surface was immediately reduced and a cathodic peak was obtained, the area of which is proportional to the electrode surface area. The actual area was calculated by integrating the cathodic peak, dividing that area by the scan rate then dividing by a conversion factor of 390 $\mu C/cm^2$, a figure specific to gold, found in literature review.[51,56,57] The electroactive surface area was divided by the known geometric surface area in order to give a roughness factor.[58] If the roughness factor were 50, it means the electrode is 50x larger than its planar equivalent. Larger surface area means more nanoporous framework for continuing electron exchange between electrode and electrolyte in the presence of biofouling protein molecules.

Additionally, blood has a distinct cyclic voltammogram due to the different redox components in it. After OCP measurements were made, a voltammogram was run in blood to make sure there wasn't major electrode dysfunction, noted by substantial qualitative differences in the hysteresis curve.

Procedure

Separate cyclic voltammogram experiments were performed in blood, 1 mM potassium ferricyanide (CAS No. 13746-66-2; Sigma Aldrich St. Louis, MO) in 0.1 M KCl (CAS No. 7447-40-7; Sigma Aldrich St. Louis, MO), or 0.5 M sulfuric acid (CAS No. 7664-93-9; Sigma Aldrich St. Louis, MO). A software program was written to run simultaneous cyclic voltammograms on 4 working electrodes. A specific description of the program layout is included in the appendix for all electrochemistry procedures. A platinum wire was used as the counter electrode and a silver chloride coated silver wire was used as a reference electrode.[59] The reference electrode was visually inspected prior to each experiment for obvious deterioration in silver chloride coating and regular OCPs were run against commercially available fritted Silver Silver Chloride reference electrode purchased from Basi Scientific or in Zobell's solution, a redox standard solution that was made in the Collinson lab. All of the cyclic voltammograms shown are the second consecutive run and all were run at 100 mV/s. For potassium ferricyanide solutions, the potential was cycled from 0.5 V→0 V→0.5 V. For blood, the potential was cycled from 0 V→0.7 V→0 V. For sulfuric acid, the potential was cycled from 0.2 V→1.7 V→0.2 V.

As convention, each CV experiment was run twice and second run data was used in analysis. This began due to the current running through the electrode cleaning off any possible trace dirt on the electrode and was maintained throughout all cyclic voltammetry experiments.

All electrochemistry was performed on a Metrohm Autolab "PGSTAT128N Multi BA" and their proprietary software, Nova 2.0 was used. Details of program written for multi-electrode analysis in Appendix. All peak analysis was done with Nova 2.0. All graphs were created using SigmaPlot 13.0 and all statistical analyses were done with JMP Pro 12.

Statistical Analysis:

Surface area roughness factors was calculated for nanoporous gold electrodes and planar gold electrodes then nanoporous gold electrodes before and after use. Normality of data was assessed with normal quantile plot and variance was assessed with Brown Forsythe test.[60] The null hypothesis that there was no difference in surface area between the groups was tested. Based on the results of the Brown Forsythe test, the equal variance t-test was used. The null was to be rejected in favor of the alternative if the observed p-value is <0.05, otherwise the null would have failed to have been rejected.

For the surface area analysis of electrodes before and after use, a repeated measures t-test was used. Normality was assessed with normal quantile plot. The null hypothesis that there was no difference in surface area after electrode use in blood was tested. The null was to have been rejected in favor of the alternative if the observed p-value was <0.05, otherwise the null would have failed to have been rejected.

Results

Figure 4 shows a ferricyanide cyclic voltammogram, illustrating similar cathodic and anodic peaks with peak splitting, ΔE_p= 73 mV. For reversible electrode reactions at normal temperature, ΔE_p should be relatively close to 59.2mV. This is characteristic of a reversible kinetics of a single electron redox reaction.

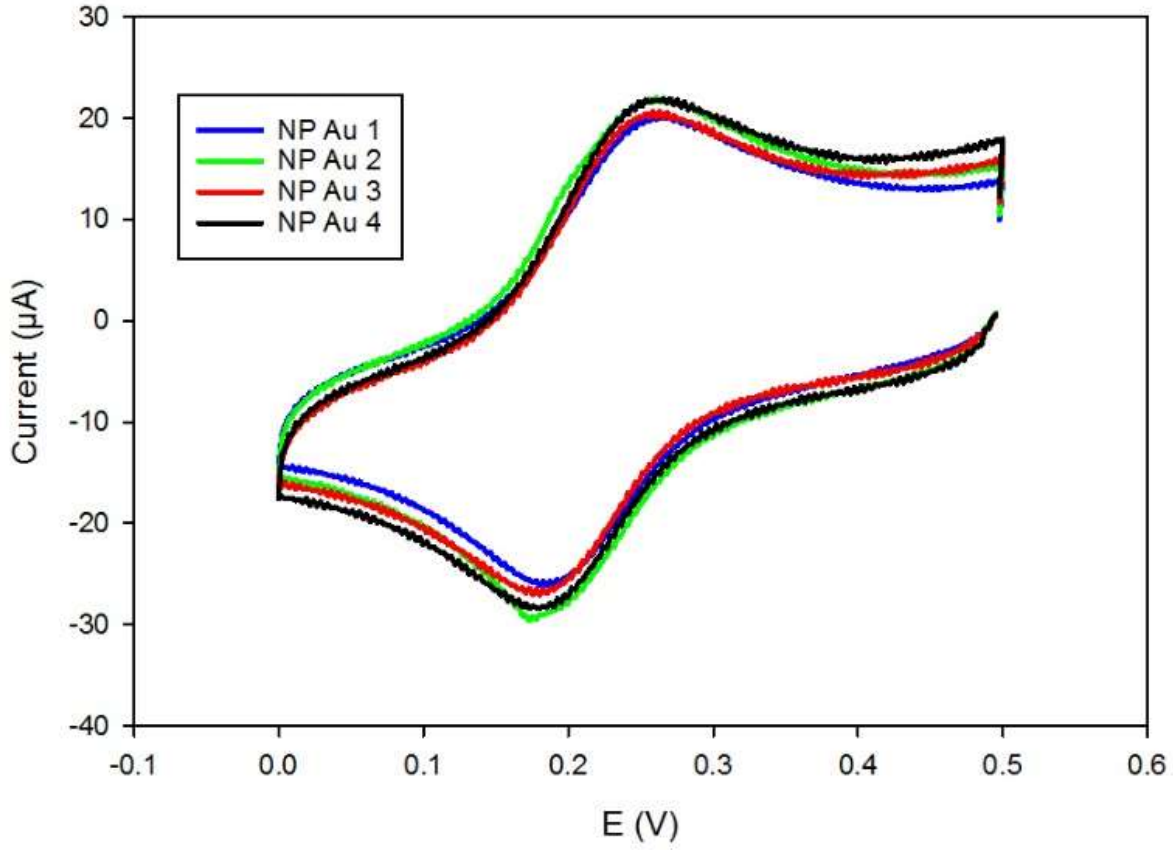

Figure 4: Cyclic voltammogram in 1 mM potassium ferricyanide at 4 nanoporous gold redox electrodes, Ag/AgCl wire reference electrode, and Pt wire counter electrode 0 V→0.5 V→0 V at 100 mV/s

The double layer nanoporous gold electrodes have a much larger electroactive surface area and roughness factor than the planar gold electrodes based on the directly correlated reduction peaks seen in Figures 5-7 at approximately 0.6 V. Figure 5 shows the cyclic voltammogram of 4 nanoporous gold electrodes in 0.5M sulfuric acid. Figure 6 shows the same procedure run with planar gold electrodes used as the support for the nanoporous gold leaf. Figure 7 shows Figures 5 and 6 overlaid to illustrate the large cathodic peak differences between the nanoporous and planar gold electrodes.

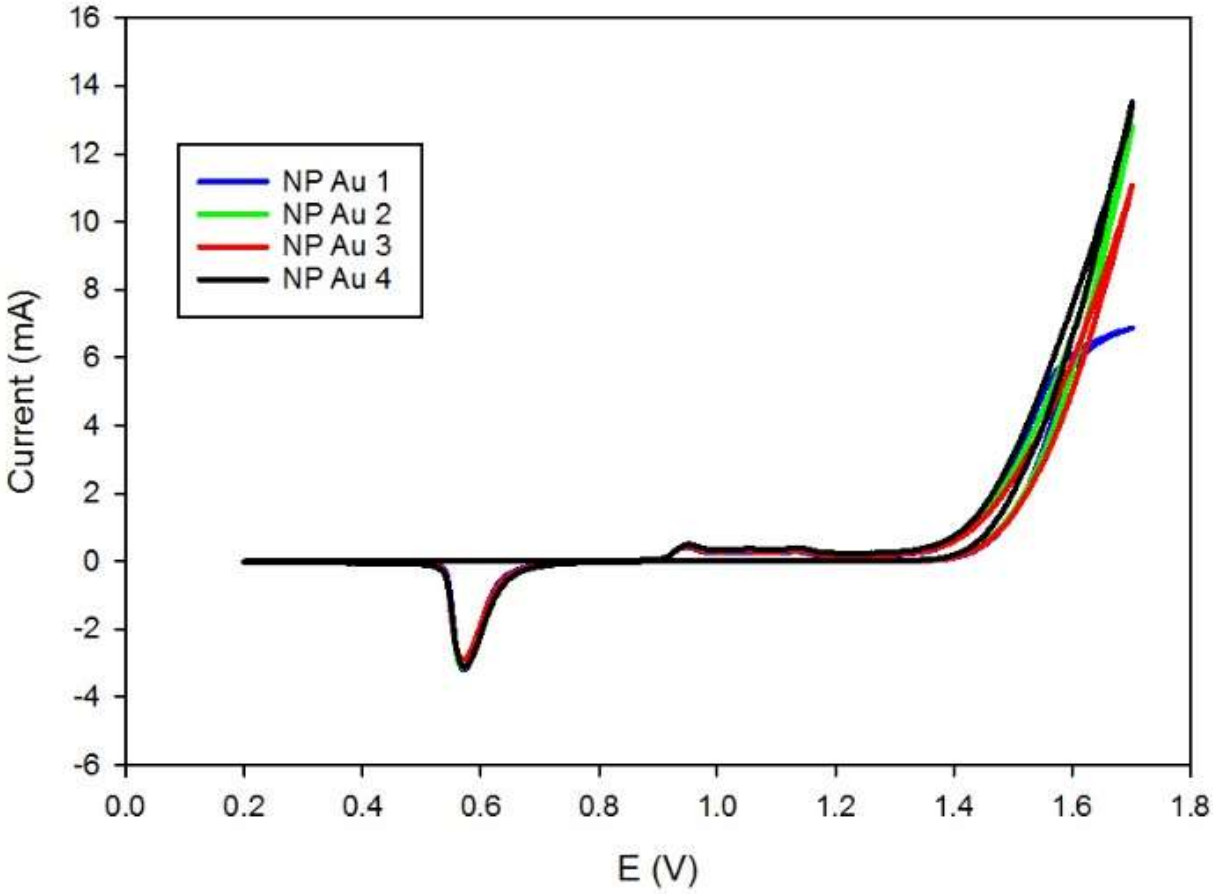

Figure 5: Cyclic voltammogram in 0.5 M sulfuric acid at 4 nanoporous gold redox electrodes, Ag/AgCl wire reference electrode, and Pt wire counter electrode 0.2 V→1.7 V→0.2 V at 100 mV/s

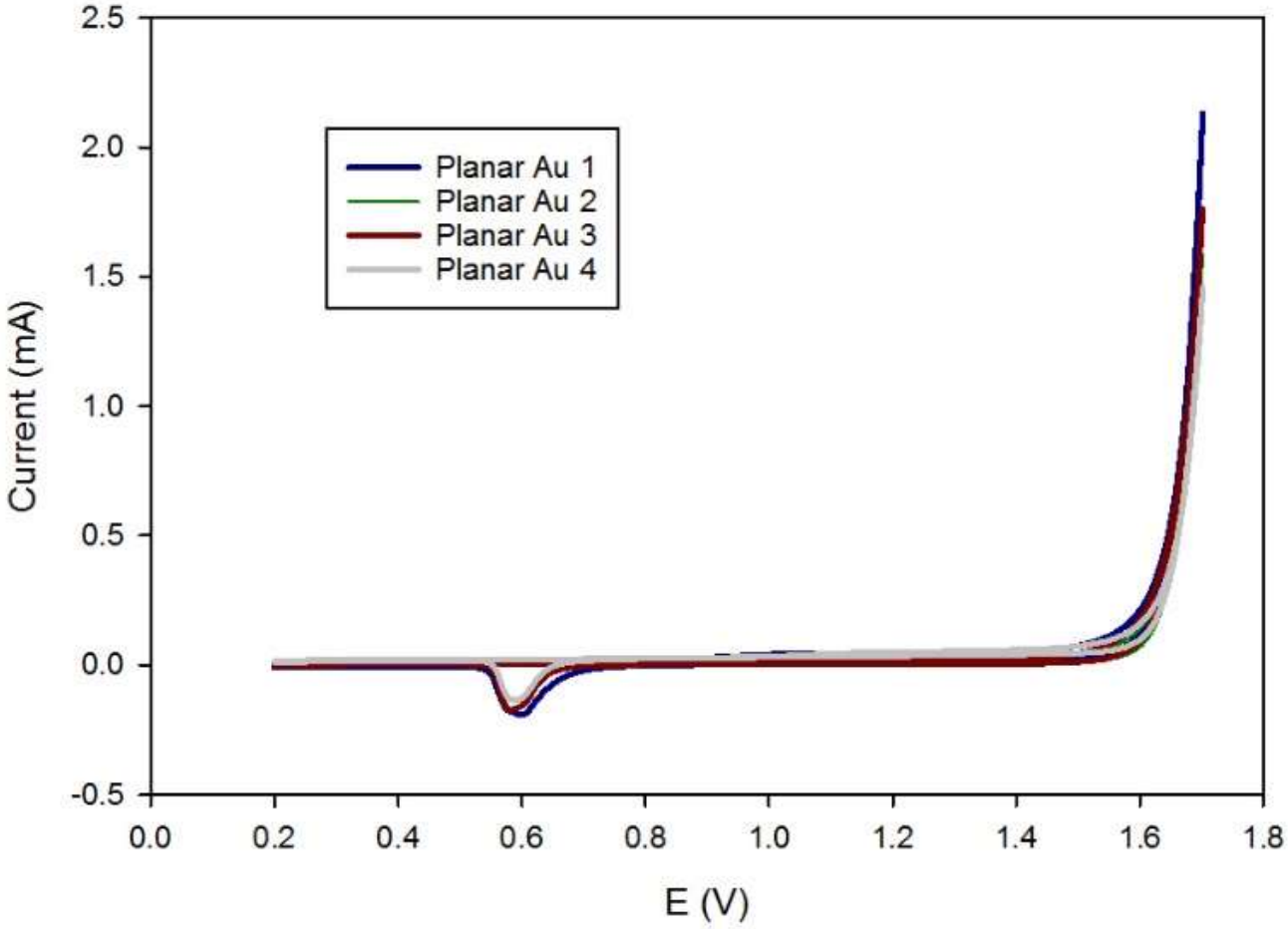

Figure 6: Cyclic voltammogram in 0.5 M sulfuric acid at 4 planar gold redox electrodes, Ag/AgCl wire reference electrode, and Pt wire counter electrode 0.2 V→1.7 V→0.2 V at 100 mV/s

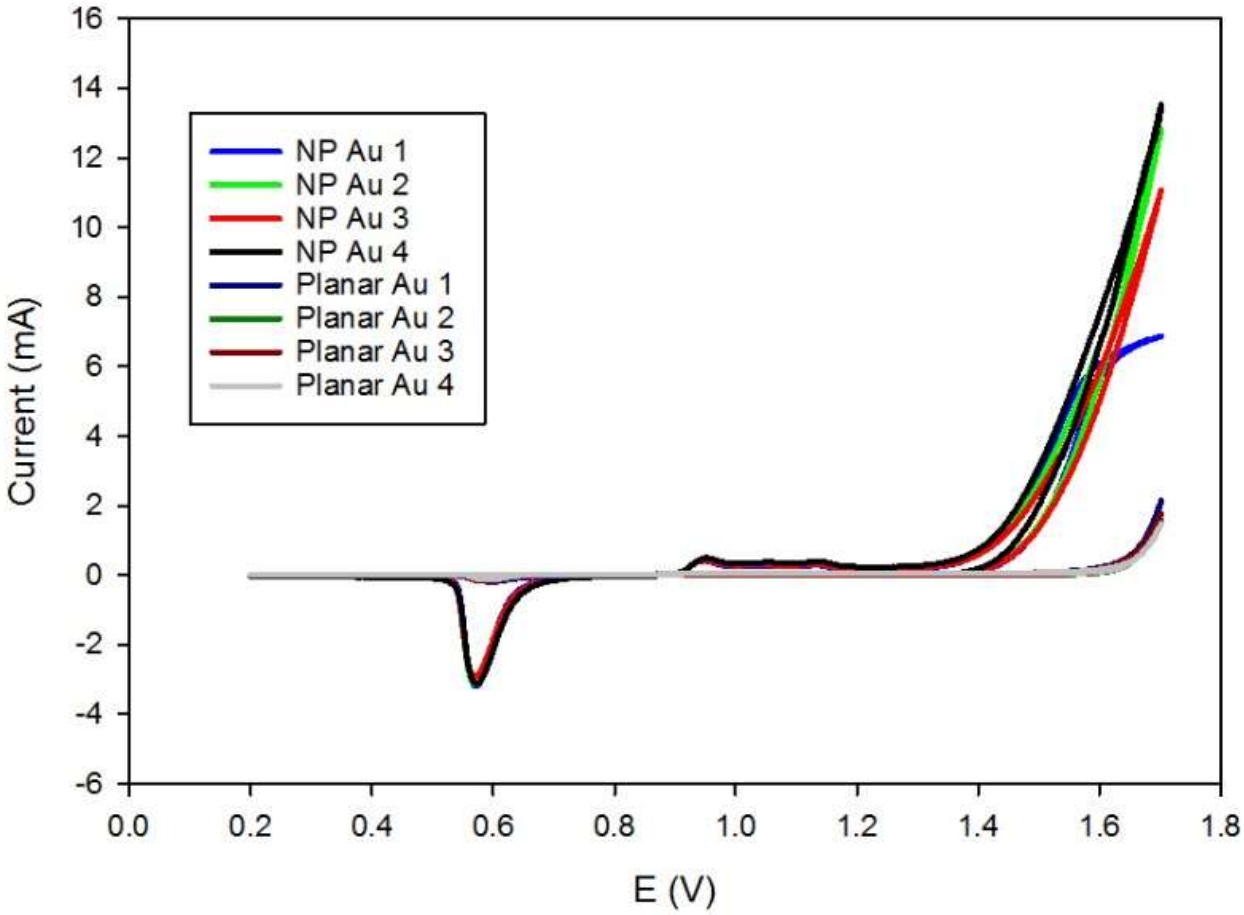

Figure 7: Overlay of figures 5 and 6

Surface area roughness factors was compared between the double leaf nanoporous gold and planar gold using a two sample t-test. Data appeared normal on quantile plot. Brown Forsythe test was done, giving a p-value>0.05, therefore equal variance was assumed. The equal variance t-test results (t_6=-43.5346, p-value<0.0001) indicate a significant difference between double layer nanoporous gold roughness factor and planar gold roughness factor. Summary Statistics are shown in Table 1.

Table 1: Summary statistics of two-sample t-test comparing roughness factors (unit less) of nanoporous and planar gold redox electrodes

	Mean	SD	95% CI	n
DL NP	63.40	2.597	59.27, 67.54	4
Planar	3.80	0.869	2.41, 5.18	4
	Mean	SE	95% CI	
Difference	-59.607	1.369	-56.26, -62.96	

Surface area analysis was done comparing the electrode roughness factor before and after electrode use using a paired t-test. Normality was assumed based on quantile plot. The paired t-test (t_3=3.83, p-value=0.016) indicate a difference between unused nanoporous electrode and used nanoporous electrode surface area. Summary statistics are shown in Table 2. While there is a statistically significant difference after use, the used nanoporous gold electrodes are still much larger than the planar ones summarized in Table 1.

Table 2: Summary statistics of two-sample t-test comparing roughness factors (unit less) of nanoporous gold redox electrodes before and after use in blood

	n	Mean	SD	95% CI
Before	4	63.40	2.597	59.27, 67.54
After	4	52.98	6.836	42.1, 63.86
Difference	4	10.42	5.444	1.76, 19.08

Ascorbic Acid Standard Additions Protocol

Introduction:

In these experiments, blood redox potential was measured in human blood donated as waste from the VCU apheresis clinic from polycythemia patients. Polycythemia is a disease where the body produces excess hemoglobin and requires regular apheresis. It is unclear how polycythemia specifically affects redox potential, but it was hypothesized that the blood will still change redox in a similar fashion to normal blood in response to the addition of an antioxidant.[61] The source of blood was chosen based on the logistic ease of obtaining it as it would otherwise have been disposed of. Blood was de-identified and was considered waste, therefore was IRB exempt. No information, including hematocrit, was known about the samples donated.

200 µL aliquots of 0.19M ascorbic acid were added to 15 mL blood during potentiometry and changes were measured and plotted. Data was plotted against the common logarithm of ascorbic acid concentration and hypothesized to conform to the Nernst Equation (Equation 2). Plotting potential as the dependent variable and common logarithm of ascorbic acid concentration as the independent variable, a linear relationship with the slope approximating $\frac{RT}{zF}$ was expected. The slope value would approximate 59 mV at room temperature with z=1, as the number of electrons transferred per unit oxidant. However, ascorbic acid transfers two electrons, therefore, at room temperature, the slope is expected to approximate 29.5 mV. The goal of this experiment was to show that nanoporous gold electrodes change with addition of antioxidant and demonstrate "Nernstian" behavior in blood, as a normal redox electrode would in an electrolyte solution that does not contain protein molecules.

Procedure:

Blood was heparinized to 3u/mL by apheresis nurses at time of blood draw. Blood was refrigerated, then allowed to warm to room temperature prior to use. All blood analysis occurred within 24 hours of blood draw. 15 mL of room temperature blood was put in a small container with spin bar. The setup is shown in Figure 8.

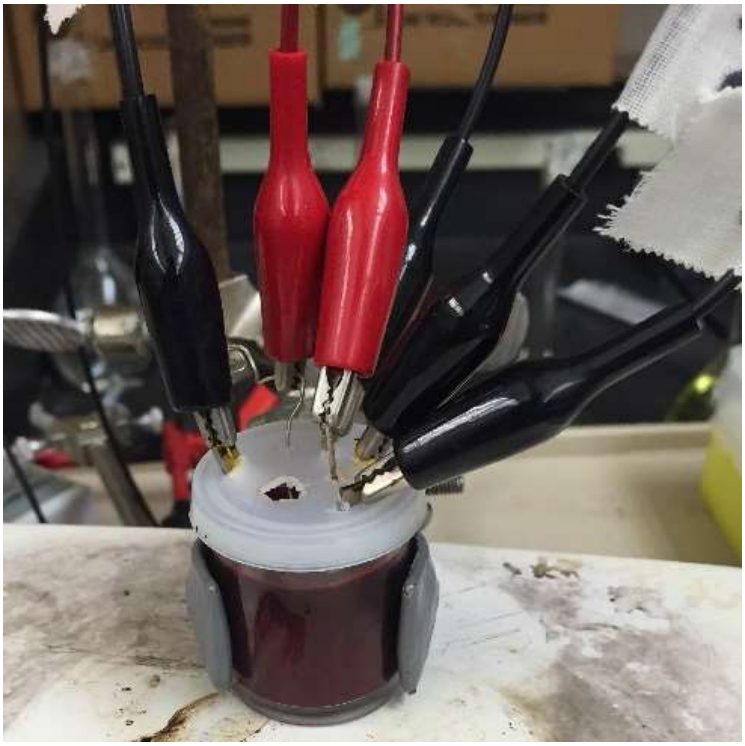

Figure 8: Potentiometric measure of blood redox potential with 4 nanoporous gold working electrodes and Ag/AgCl wire reference electrode. Pt wire counter electrode is also in solution for post-test analysis

Potentiometric OCP software described in the appendix was set to run for 300 s. At the end of each run, a small volume dose of ascorbic acid was given and the OCP was restarted. Each dose was 0.19 M 200 µL doses. Ascorbic acid purchased from Sigma Aldrich was mixed in deionized water immediately prior to the beginning of the experiment. This was repeated multiple times and the data points were plotted against the common logarithm of ascorbic acid concentration in blood, based on the ascorbic acid added.

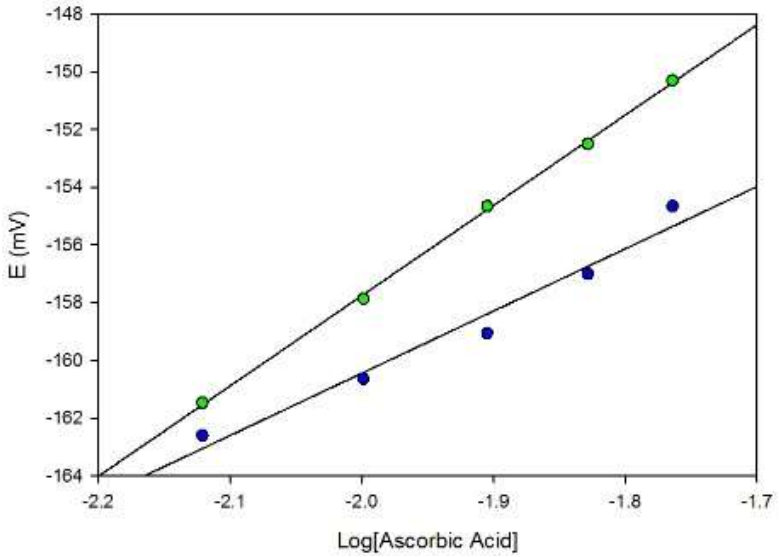

Figure 9: Natural logarithm of ascorbic acid concentration (M) vs. open circuit potential (mV) in human blood

Results:

Figure 9 shows the redox response of two nanoporous gold electrodes to additions of ascorbic acid; each point represents the signal measured after 300 s and the electrodes are separated by color. Ascorbic acid is a potent antioxidant, so it was hypothesized that addition of it to blood would make the blood's redox potential more electronegative. By adding known aliquots to the solution, a standard addition curve was created, plotting the redox potential as the dependent variable and the natural logarithm of the total known ascorbic acid concentration as the dependent variable. The blood redox potential responded linearly to the natural logarithm of the known ascorbic acid concentration. With addition of a single electron exchanging molecule such as ascorbic acid, the slope of the line was predicted to be 59.2 mV based on the Nernst Equation (Equation 2) at normal temperatures. If ascorbic acid donates two electrons to blood, then the slope of the standard addition curve would be half of that value, or 29.6 mV. The two electrodes in Figure 9 are measuring the same sample at the same time and variations may be due to position in solution related to the stir bar or electrode quality.

Redox potential measured by nanoporous gold electrodes appeared to have a linear relationship with the common logarithm of the ascorbic acid concentration. R^2, slope and Y-axis intercepts are shown in Table 3.

Table 3: Linear regression summary for standard addition Potentiometry experiment illustrated in Figure 9.

	Blue	Green
Intercept	-117.33	-95.20
Slope	21.56	31.28
R^2	0.9671	0.9992

Human Cardiac Bypass Sampling

Introduction:

Open cardiac surgery is when the heart is stopped for the surgeon to work on it. This requires extracorporeal circulation of the blood through a heart lung machine, or bypass pump. The blood is circulated through large cannulas into an extracorporeal membrane where it can be oxygenated. This is done on a short term basis for a surgeon to perform a procedure on the heart or aorta, but may be done for longer periods of time if the patient is in cardiopulmonary compromise.[62] This patient population is usually considered to be very sick and often has severe inflammatory response and poor outcomes in regard to mortality and morbidity.[63] It has been shown that running blood across a non-organic surface leads to decreases in ascorbic acid concentration[64] and increases in inflammatory markers.[65]

Procedure:

At the time of this experiment, patients undergoing cardiac surgery that required the placement of ECMO or extra corporeal membrane oxygenator were being enrolled in a coagulation study. The protocol for lab draws in this study had the technician draw saline out of a venous line, draw 3-4 mL of just blood, then draw the samples. The 3-4 mL of blood was considered waste blood and would otherwise have been discarded. Because the blood was de-identified and considered waste, this experiment was considered IRB exempt.

Blood was obtained and the open circuit potential was measured for 600 s while gently stirring. Due to small volume of blood per sample, a small electrochemical cell that could only hold three working electrodes was used and one of those was a planar gold electrode used as a control. All blood was tested within an hour of being drawn, however there were several missed time points due to blood draw technician forgetting to not dispose of waste samples. After the blood samples were run through OCP software, electrodes were gently rinsed in DI water and a ferricyanide CV was obtained to determine if the electrodes were working properly. An electrode's OCP reading was excluded if it had a large amount of obvious noise on the CV or if the CVs did not qualitatively or quantitatively match up to the standard described above. For each patient, there were four time points, the first being just before going onto the ECMO machine, the second mid-procedure, the third just before coming off of ECMO, and the fourth being an hour after being off of ECMO. During the four days that data was collected, there was some misunderstanding about the anticoagulation used in the blood draw and sample delivery. Patient 1 had all blood drawn into citrated tubes, but only had 3 of the 4 time points delivered. Patient 2 had blood drawn into citrated tubes then transferred into a heparinized syringe and had all 4 of the time points delivered. Patients 3 and 4 had blood drawn into heparinized syringes,

but patient 3 only had 3 of the 4 time points delivered and patient 4 only had 1 of the 4 time points delivered.

Statistical Analysis:

Due to the extremely limited sample size and anticoagulant variation, comparison between time points was not feasible. Data was compared to previously collected blood redox potential data of healthy volunteers measured with similar electrodes and heparinized blood.[66]

Distribution of data were analyzed using normal quantile plots and variances for each group were compared using the Brown-Forsythe test. Data were summarized with means, standard deviations, and 95% confidence intervals.

Data collected from cardiac bypass patients were compared to healthy volunteer redox potential with a two-sample T-test. Equal variance was assumed based on the Brown Forsythe test, therefore, the equal variance t-test was used. Observed differences, standard errors, adjusted confidence intervals and adjusted p-values were reported. The null hypothesis that there is no difference between groups was tested against the alternative. The null was to have been rejected in favor of the alternative if the observed p-value <0.05, otherwise the null would have failed to have been rejected.

Results:

Figures 10-13 are of the same two nanoporous electrodes and planar electrode with numbering convention maintained. Electrodes were rinsed in deionized water between procedures in different fluid (ferricyanide or blood). Figure 10 shows a ferricyanide cyclic voltammogram using two nanoporous gold electrodes and one planar gold electrodes. Prior to testing in blood, electrodes were tested by obtaining ferricyanide cyclic voltammograms to identify any obvious electrode dysfunction. As evidenced by peak splitting, the planar gold electrode in Figure 10 may be dirty even though it was made with a comparable technique to the nanoporous gold electrodes.

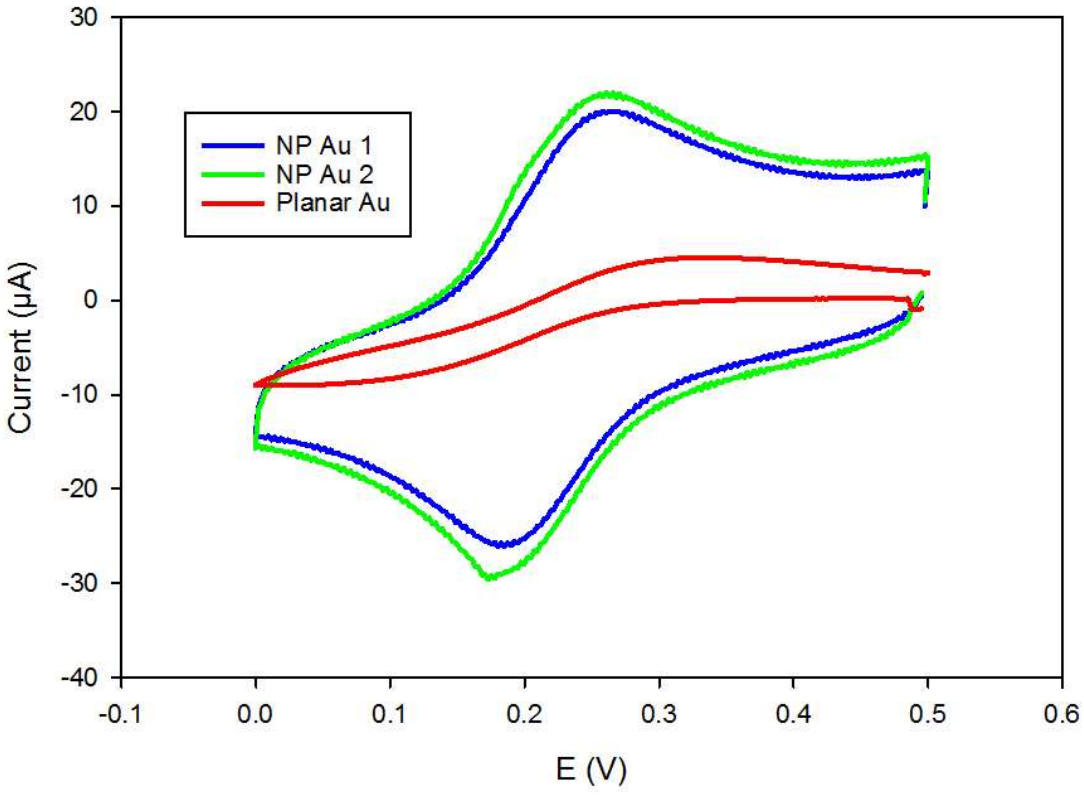

Figure 10: Cyclic voltammogram in 1 mM potassium ferricyanide with 2 nanoporous gold redox electrodes, 1 planar gold redox electrode, Ag/AgCl wire reference electrode, and Pt wire counter electrode 0 V→0.5 V→0 V at 100 mV/s. Just prior to use in cardiac bypass experiment.

Once the electrode pre-test was complete and electrodes were rinsed in a deionized water bath, an open circuit potential experiment was run in blood with a Ag/AgCl wire reference electrode and a slow spin bar. Figure 11 shows an example of a potentiometry experiment done on blood with two nanoporous gold electrodes and a planar gold electrode in heparinized blood. It is evident that the two nanoporous electrodes have very similar signals while the planar gold signal is drastically different. This is hypothesized to be due to the mechanism of nanoporous gold and its ability to continue electron exchange and proper electrode function in the presence of biofouling protein molecules that are found in blood.

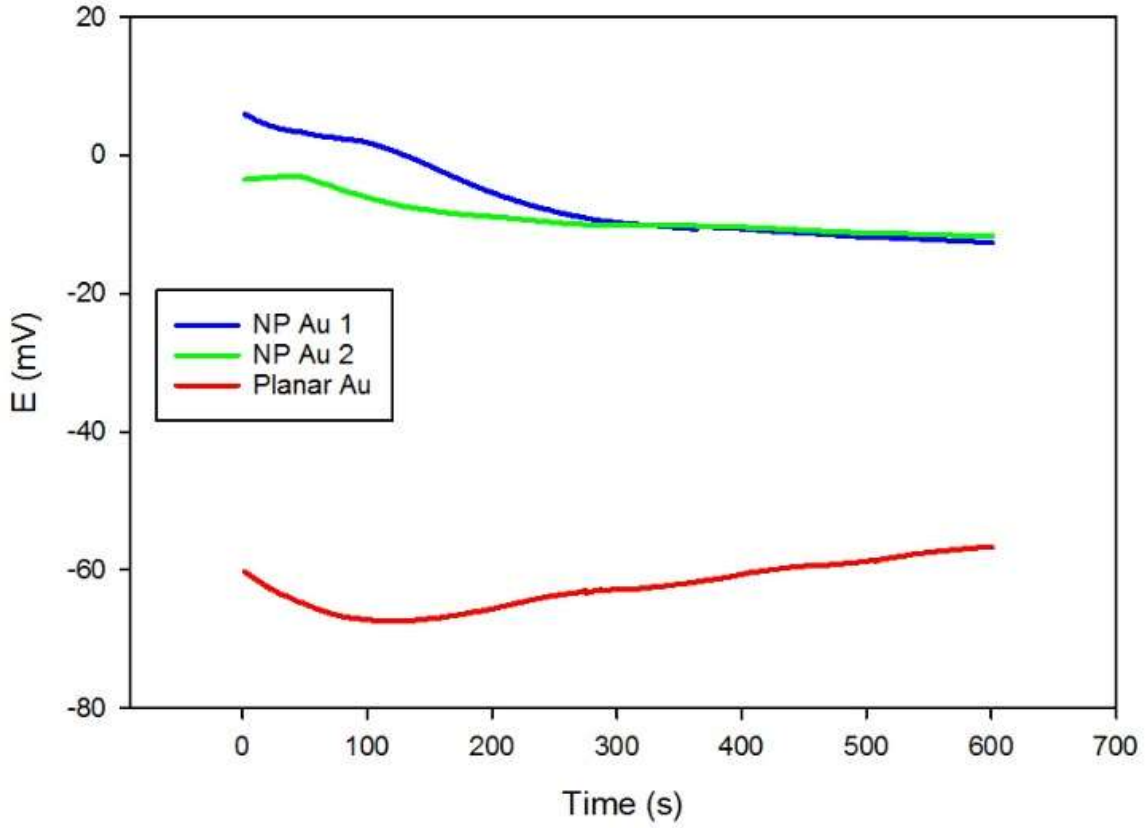

Figure 11: Open circuit potential of human blood sample of patient undergoing cardiac bypass. Sample was drawn in heparinized syringe.

After the redox potential was measured for the sample, a platinum wire counter electrode was added and a cyclic voltammogram was run from 0V→0.7V→0V at 100mV/s. This was done to help identify any major artifact that may have occurred during the previous experiment. An example of a blood cyclic voltammogram is shown in Figure 12. The anodic, or oxidation, peak noted at 0.4V in the nanoporous eleectrodes may be representative of the oxidation of ascorbic acid and uric acid in blood.

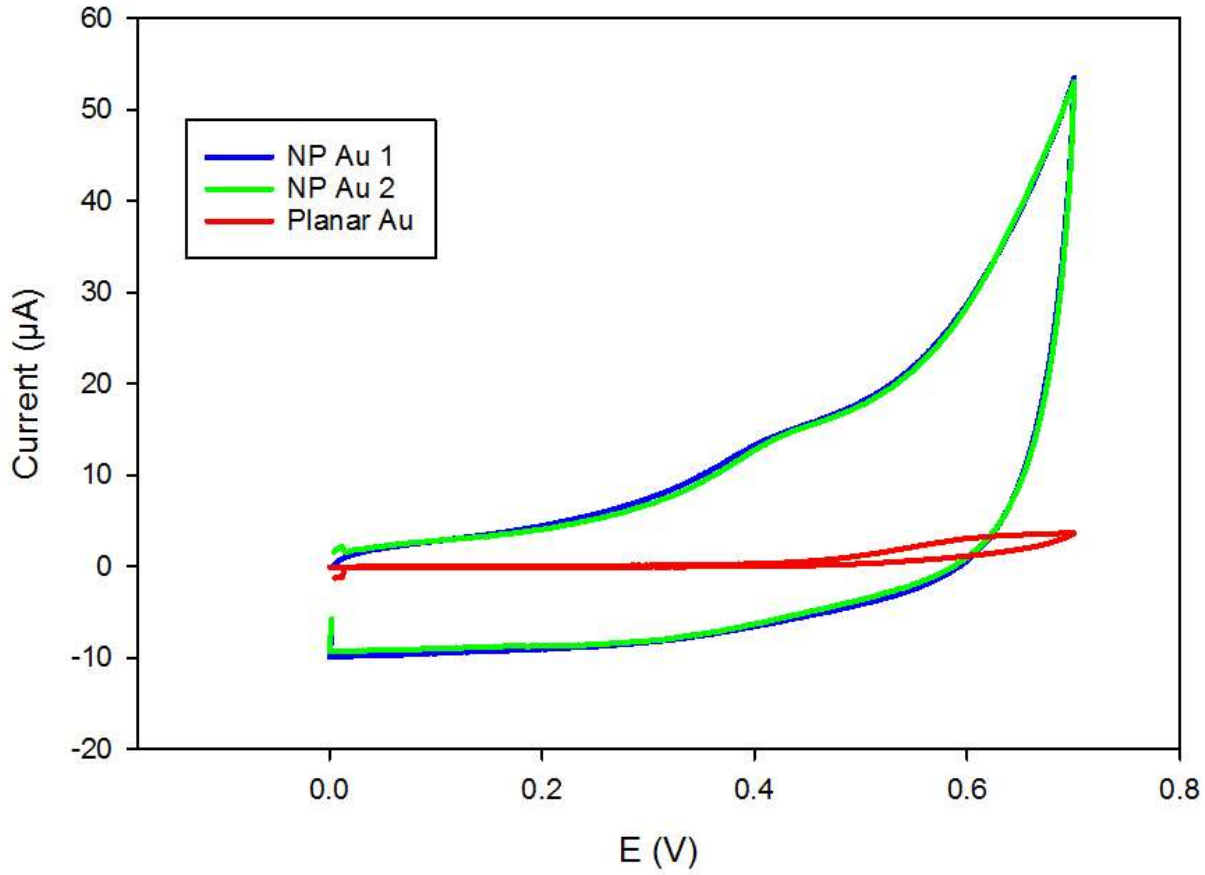

Figure 12: Cyclic voltammogram of blood, run immediately after Figures 11 and 12 were run 0 V→0.6 V→0 V at 100 mV/s. Sample was drawn in heparinized syringe.

Once the blood analysis was completed, the electrodes were submerged in deionized water and agitated until all gross blood was not visible. Once they appeared clean, a repeat ferricyanide cyclic voltammogram was done in order to identify any drastic changes in electrode characteristics. The post analysis cyclic voltammogram for the previous figures is shown in Figure 13. In this voltammogram, minimal changes from figure 10 should be noted in the two nanoporous gold electrodes while the planar gold peaks are barely identifiable let alone demonstrating Nernstian behavior. The inclusion of the planar gold control in these experiments further illustrates the superiority of nanoporous gold over planar for making electrochemical measurements in blood.

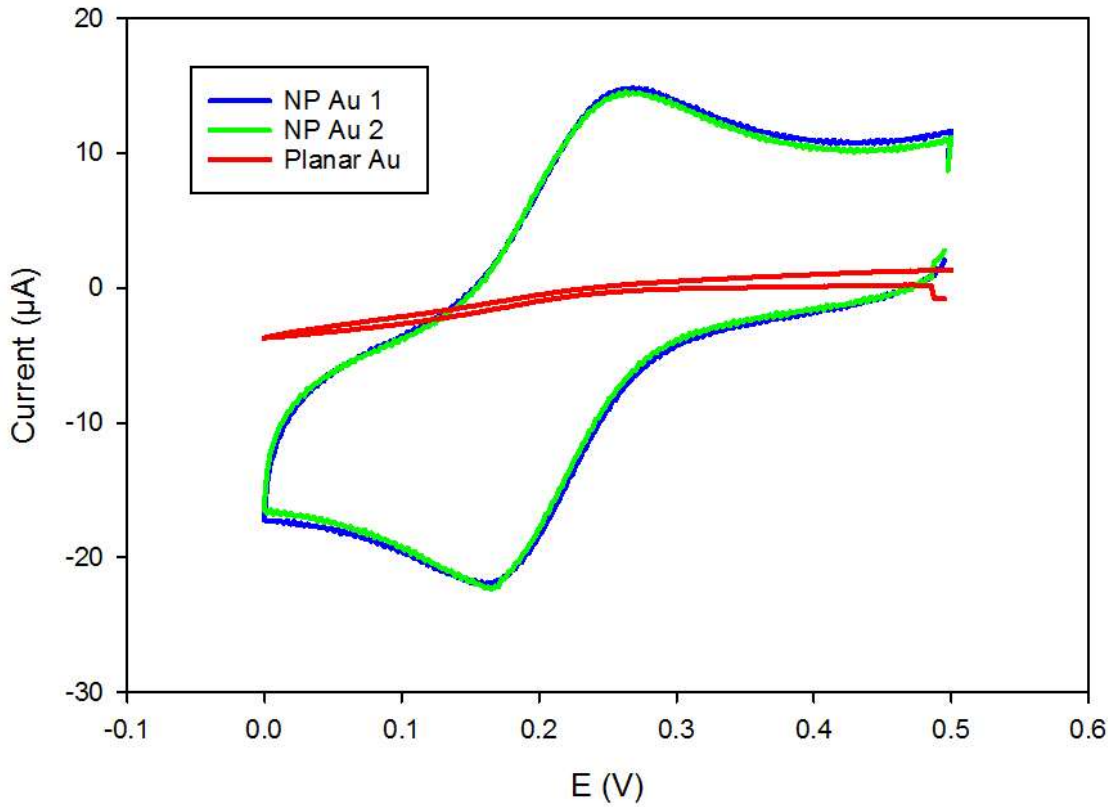

Figure 13: Cyclic voltammogram in 1 mM potassium ferricyanide with the same electrodes used in Figures 10-12 just after use in human blood of cardiac bypass patient sample 0 V→0.5 V→0 V at 100 mV/s.

Figure 14 illustrates the four cardiac bypass patient's average redox potentials plotted with the 49 healthy volunteers' redox potentials measured with nanoporous gold electrodes. Each individual was plotted; the healthy volunteers had one measurement taken per person and the cardiac bypass patients had multiple measurements per person, so the data shown is the individual average for each of the cardiac bypass patients. The healthy volunteers' redox potential appears to illustrate a bell curve, although slightly right skewed. The average redox potentials of the cardiac bypass patients all fall outside of that bell curve.

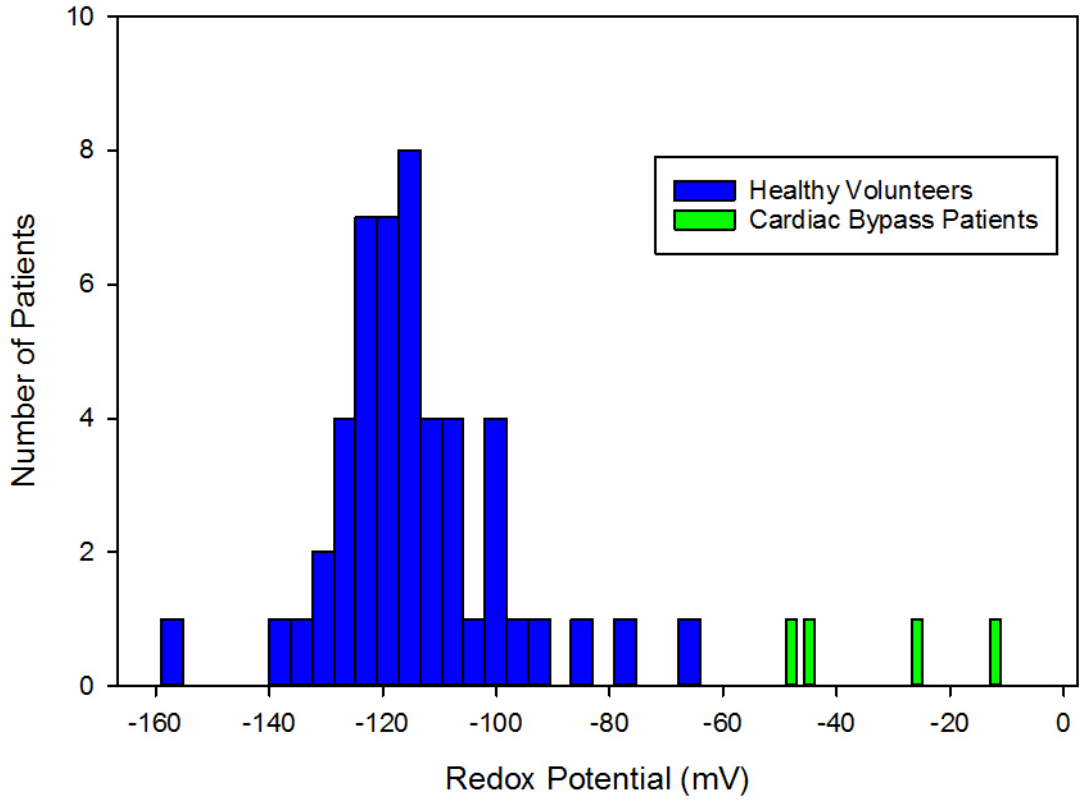

Figure 14: Redox Potential Distribution of 49 healthy volunteers and 4 patients undergoing cardiopulmonary bypass. All healthy volunteer samples were heparinized. All cardiopulmonary bypass patient samples were either heparinized or citrated.

For the comparison of patients undergoing cardiac bypass and healthy volunteers, the Brown Forsythe test had a p-value>0.05, therefore, equal variance was assumed. The results from the equal variance t-test (t_{51}=-10.146, p-value<0.0001) indicate that there is a significant difference in redox potential between patients undergoing cardiac bypass and healthy volunteers. Summary statistics are reported in Table 4.

Table 4: Summary Statistics for two sample t-test comparing redox potential of patients undergoing cardiac bypass and healthy volunteers[66]

	Mean (mV)	SD	95% CI	Sample Size
CPB	-32.68	16.849	-59.49, -5.867	4
Healthy	-114.57	15.435	-119.01, -110.14	49
	Mean	SE	95% CI	
Difference	-81.9	8.072	-65.69, -98.1	

Potentiometry in swine hemorrhagic shock experimental model

Introduction:

Hemorrhagic shock is a specific kind of shock involving blood loss through blunt and/or penetrating trauma. Even though it is a specific kind of trauma, the body still undergoes all of the associated problems with systemic hypoperfusion such as systemic inflammatory response syndrome, ischemia-reperfusion injury, and multiple organ dysfunction syndrome. The goal of this experiment was to assess how blood redox potential changes over the course of hemorrhagic shock and resuscitation in a swine experimental model.

Experimental Design:

General anesthesia was induced; the pig was intubated and sedation was maintained with isoflurane. The femoral veins and arteries were cannulated for continuous mean arterial pressure monitoring, central venous pressure monitoring with a Swan Ganz pulmonary artery catheter, blood samples and hemorrhage. Once the procedures were completed and the animal was appropriately anesthetized based on arterial blood gas readings, the injury part of the experiment was started. The Pringle surgical technique was used to cut blood flow off to the liver for 15 minutes, then released for 5 minutes. This hepatic artery constriction was done three consecutive times. During the liver ischemia/Pringle portion, the animal was shot with a spring-loaded gun to cause a femur fracture. After the hour of liver injury, the pig was hemorrhaged through one of the femoral catheters. Hemorrhage was based around maintaining a “hypotensive” mean arterial pressure for an hour. After the hemorrhage, the pig was resuscitated with 500 mL hetastarch solution, to mimic standard of care field resuscitation, low concentration IV ascorbic acid; or high concentration IV ascorbic acid and monitored for 4 hours.

Blood Draw:

Time points were chosen based around other sample time points (ABG, plasma ascorbic acid concentration, CBC, coagulation panel) with the exception of the added "Post Pringle" time point where the only other data collected was ABG analysis. Time points were baseline (BL) which occurred just prior to liver ischemia portion, post Pringle (PP) which occurred immediately after the liver injury before hemorrhage, end hemorrhage (T0) was at the end of hemorrhage just prior to resuscitation, T15 was 15 minutes after resuscitation, T2 was 2 hours after resuscitation, and T4 was 4 hours after resuscitation. Blood was drawn into three Lithium Heparin commercially available blood tubes and produced approximately 5-6 mL per sample. Samples were run at bedside immediately after being drawn.

Electrochemical Analysis:

For each sample, 4 nanoporous gold redox electrodes were used simultaneously to measure open circuit potential for 600 seconds against a AgCl coated Ag wire reference electrode. A small stir bar was placed in solution and was spun at low speed throughout all electrochemistry. Immediately after open circuit potential was run, a cyclic voltammogram was performed in the blood solution with a Pt wire as a counter electrode. The cyclic voltammogram was cycled from 0 V→0.7 V→0 V at 100 mV/s and was run twice. All data reported is from second run of cyclic voltammogram. The cyclic voltammetry experiment was done to identify electrodes that were not working correctly. If the CV had very obvious artifact or did not reach a current peak greater than 75 μA, then it was deemed not to be working correctly and OCP for that electrode was excluded from the analysis.

Statistical Analysis:

Distribution of data were analyzed using normal quantile plots and variances for each group were compared using the Brown-Forsythe test. Data were summarized with means, standard deviations, and 95% confidence intervals.

Three separate analyses were performed, separately looking at the control resuscitation, low dose ascorbic acid resuscitation, and high dose ascorbic acid resuscitation in order to identify if there was a difference between any time points.

For the two ascorbic acid analyses, an analysis of variance (ANOVA) was used to test the null hypothesis that there was no change in redox potential between time points (against the alternative that at least two of the means differ) at the 0.05 significance level. If the resulting p-value from the ANOVA model was <0.05, then the null hypothesis was rejected and multiple comparisons between group levels would have been made using the Tukey-HSD method and observed differences, standard errors, adjusted confidence intervals, and adjusted p-values would have been reported; otherwise, we would have failed to reject the null hypothesis.

For the control analysis, a univariate mixed model analysis of variance (ANOVA) was used, accounting for between-subject fixed effects. If the resulting p-value from the model had been <0.05, the null would have been rejected and a series of paired t-tests would have been performed, using the Bonferroni method and Greenhouse-Geisser adjustment for sphericity violation.[67] Observed differences, standard errors, adjusted confidence intervals and adjusted p-values would have been reported; otherwise, we would have failed to reject the null hypothesis.

Below, Figure 15 shows an example of an open circuit potential measurement, using 4 nanoporous gold electrodes, in the swine hemorrhagic shock model. All four electrodes show the redox potential normalizing and reaching an asymptote at the redox potential over the course of 600s. At approximately 100s, a sharp spike can be seen; this is hypothesized to be artifact noise due to motion.

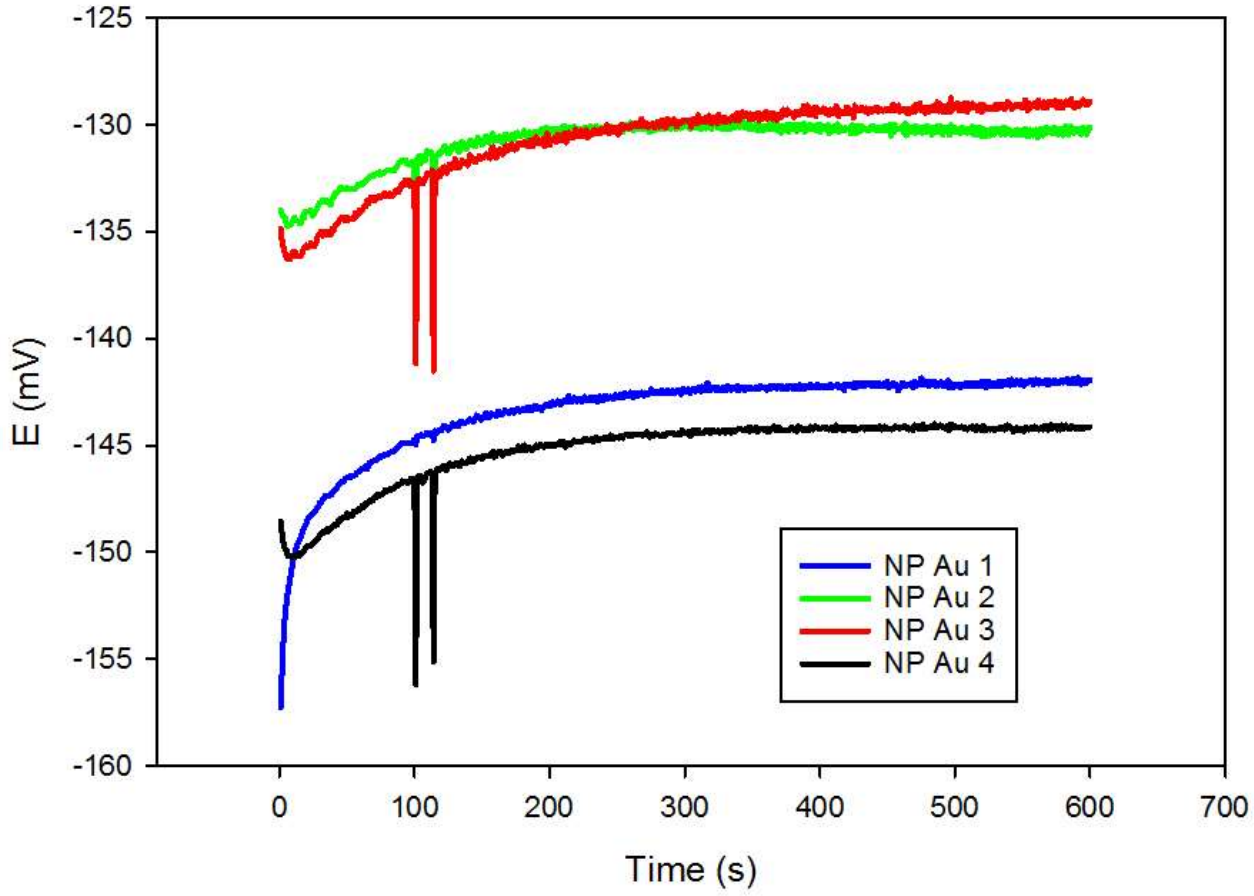

Figure 15: Open circuit potential of swine blood during hemorrhagic shock experimental model. Sample was drawn in heparinized syringe.

Figure 16 shows a cyclic voltammogram post analysis of the same electrodes used in Figure 15. This experiment was done to identify any qualitative differences in electrodes and objectively rule out electrodes that were not functioning properly.

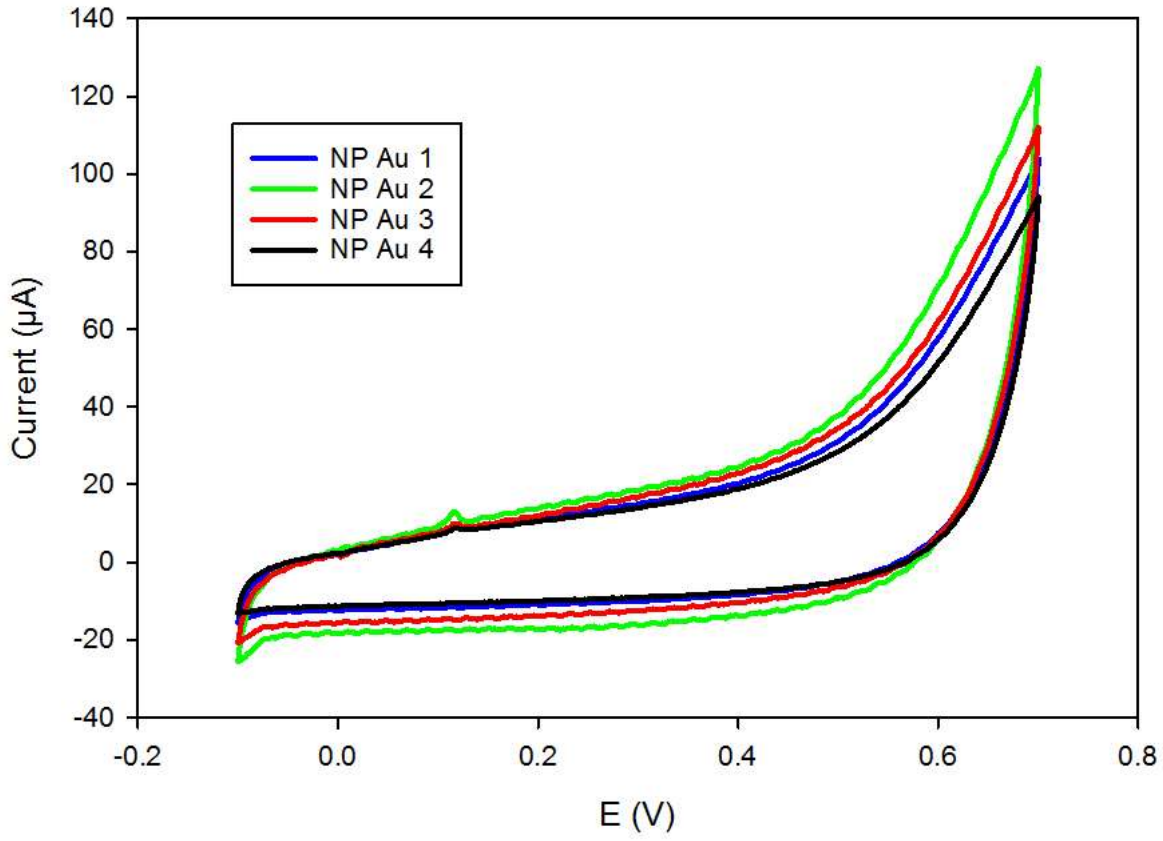

Figure 16: Cyclic voltammogram of swine blood during hemorrhagic shock experimental model. Run immediately after Figure 15. -0.1 V→0.6 V→-0.1 V at 100 mV/s. Sample was drawn in heparinized syringe.

Figures 17 and 18 show the average redox potential at each time point for each pig that data was collected from. Figure 17 shows all four of the animals that received the control resuscitation of hetastarch and Figure 18 shows the two animals that received ascorbic acid in their resuscitation phase. In Figure 18, the animal represented by the green line received a high dose of ascorbic acid while the animal represented by the blue line received a lower dose of ascorbic acid.

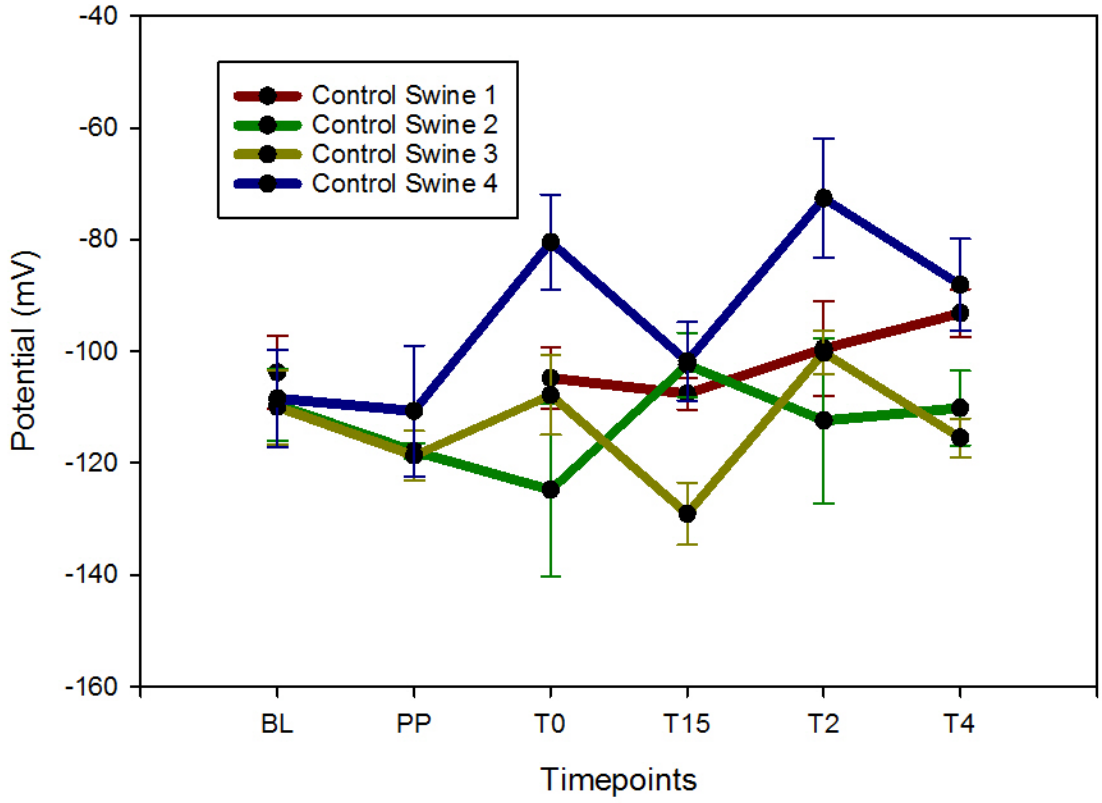

Figure 17: Data summary of swine hemorrhagic shock model showing mean redox potential of animals given control resuscitation at each time point with error bars representing standard deviation. All samples were drawn into heparinzed tubes and immediately run.

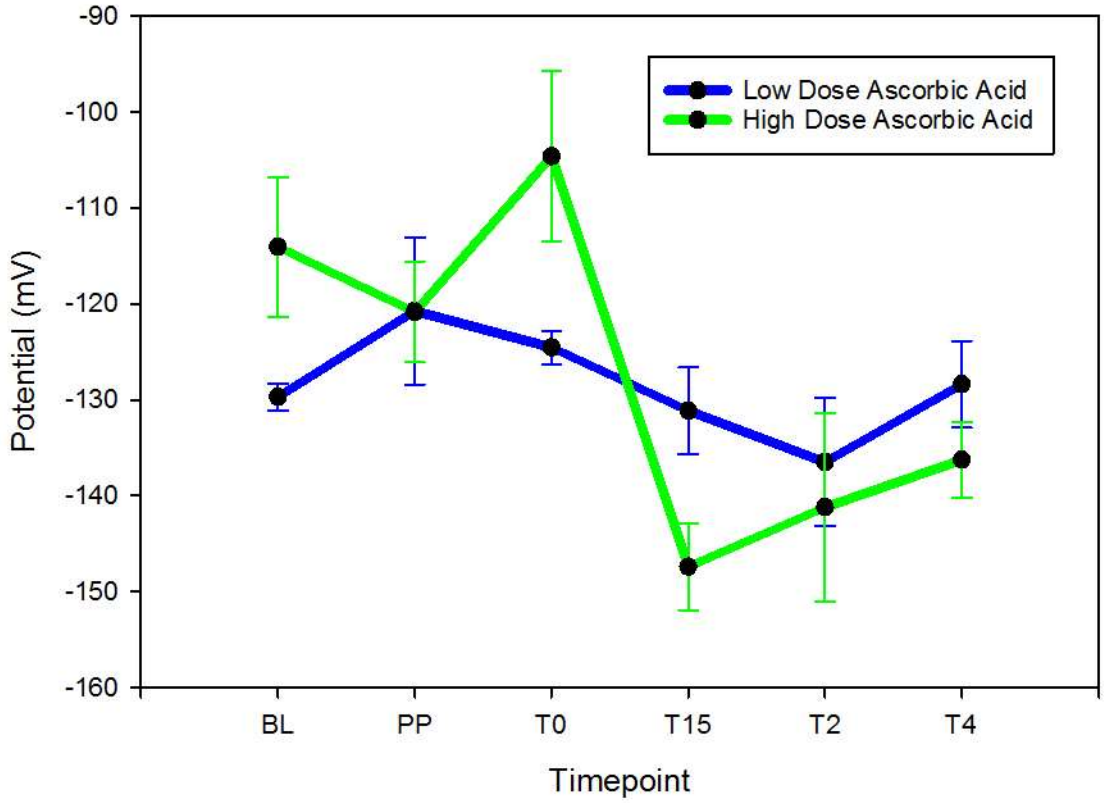

Figure 18: Data summary of swine hemorrhagic shock model showing mean redox potential of animals given ascorbic acid resuscitation treatment. Error bars at each time point represents standard deviation. All samples were drawn into heparinzed tubes and immediately run.

Table 5: Summary statistics for high dose ascorbic acid time points (in mV)

Timepoint	n	Mean	SD	95% CI
BL	4	-114.06	14.56	-137.23, -90.88
PP	4	-120.83	10.489	-137.52, 104.14
T0	3	-104.61	17.77	-148.76, -60.46
T15	4	-147.41	9.08	-161.87, -132.95
T2	4	-141.22	19.6	-172.4, -110.03
T4	4	-136.28	7.87	-148.8, -123.76

Table 6: Summary statistics for high dose ascorbic acid significant differences, adjusted with Tukey HSD (in mV)

Comparison	Difference	SE	Adjusted 95% CI	Adjusted p-value
BL-T15	33.35	9.677	2.4, 64.31	0.0307
T0-T15	42.8	10.452	9.38, 76.23	0.0083
T0-T2	36.61	10.452	3.17, 70.04	0.0275

Results

The data for the high dose ascorbic acid were reasonably normally distributed based on normal quantile plot and standard deviations did not indicate a violation of homogeneity of variance. The results from the ANOVA ($F_{5, 17}$=5.45, p-value=0.0036) indicate that there was a difference between time points. We therefore, reject the null hypothesis in favor of the alternative and post hoc analyses were done, which demonstrated three specific differences in redox potential between time points. The baseline redox potential was more positive than the 15-minute post resuscitation redox potential and the end hemorrhage time point was more positive than both the 15-minute post resuscitation and 2-hour post resuscitation time points. Summary statistics shown in Table 5 for overall ANOVA and in Table 6 for the Tukey HSD post hoc comparisons.

The data for the low dose ascorbic acid and the control therapy did not demonstrate a significant difference between time points, therefore the we failed to reject the null hypotheses

for those experiments. The low dose ascorbic acid pig did appear to have a more negative redox potential, however it was not a statistically significant change, so more data is required for further analysis.

Discussion and Future Directions:

Nanoporous gold electrodes measure redox potential in blood where planar gold electrodes demonstrate dysfunction. The increased electroactive surface area and nanoporous framework allows electron transfer between the electrode and solution to continue in the presence of biofouling molecules. Compared to the previously used nanoporous gold electrodes, the second layer of de-alloyed leaf increases the surface area dramatically.

Making the electrodes requires a great deal of dexterity and as with anything that is handmade, it is highly susceptible to reproducibility shortcomings. The technique of de-alloying gold leaf, placing it on a commercially available gold plated piece of glass, then isolating a known geometric area on it would not be impossible to manufacture with a small amount of startup capital. Removing the human element of electrode manufacture would likely greatly reduce the reproducibility issues.

Blood redox potential changes with addition of ascorbic acid, which will prove invaluable with the increasing attention to parenteral ascorbic acid as a therapy in sepsis and possibly other diseases. Ascorbic acid is likely not the only redox active chemical component that changes blood redox potential, however further testing is required.

Redox is profoundly higher in humans undergoing cardiopulmonary bypass than healthy volunteers. Due to limitations in blood sampling and variances in anticoagulants, which may affect redox, we were unable to discern any differences between different time points during the cardiac bypass procedure. Additionally, minor variances in technique may have skewed the redox data; all electrodes that were used on cardiac bypass samples underwent a pre-test of a ferricyanide cyclic voltammogram in order to make sure the electrodes were functioning normally. It is possible that ferricyanide may have remained in the porous framework despite rinsing in deionized water and altered the redox analysis of those samples. While completely blinded to any further patient information,

people requiring cardiac bypass are typically not healthy individuals. They are likely suffering from some inflammatory response due to being connected to non-biologic circuitry in addition to their disease state and are at high risk for cardiogenic or septic shock.[68] Future studies should include anticoagulant analysis, comparison to other inflammatory biomarkers such as C-reactive protein, procalcitonin, ferritin, and malondialdehyde, and a larger sample in order to determine any possible trending of redox changes during the timeline of a disease such as cardiogenic or septic shock.

Hemorrhagic shock in pigs did not appear to change redox potential in a significant way. This may be due to electrode reproducibility, which is a limitation of human electrode production, however there may be another process explaining the lack of change. Pigs, unlike humans, guinea pigs, bats and certain monkeys, have a functioning enzyme called L-gulonolactone oxidase that synthesizes ascorbic acid.[69] The loss of L-gulo function in humans is thought to be related to ancestral tropical diets that were abundant in citrus.[70] Based on the sensitivity of redox to ascorbic acid concentration, pigs may have been upregulating their endogeneous ascorbic acid production as a way to compensate for the redox insult provided during hemorrhage. Blood ascorbic acid concentration was measured at the time points that redox was measured, however, that data was unavailable at the time of this analysis. Pigs may not be an ideal model organism for redox studies due to this compensatory mechanism that humans do not have.[7] Pigs did have decreased redox potential when given the high dose ascorbic acid therapy.

This concept of redox compensation is analogous to pH compensation. Organisms have many different physiologic mechanisms to compensate for pH imbalances and keep pH within normal limits. Cells simply don't work as well outside of normal pH ranges; they similarly don't work outside of normal redox balance. Humans likely have other compensatory mechanisms, but the loss of L-gulo may mean redox imbalances are more prevalent in human disease processes such as circulatory shock than is realized.

Redox potential appears to be altered in cardiopulmonary bypass and it may be linked to inflammatory response and circulatory shock. Blood redox changes both in vitro and in vivo to parenteral ascorbic acid administration. Nanoporous gold electrodes provide a mechanism to effectively measure blood redox potential without electrode dysfunction. With improvement in electrode standardization, open source handheld potentiostats,[72] and the developments of nanoporous gold electrodes that only require a drop of blood,[73] redox measurement may develop into a clinically useful point of care test as a biomarker of SIRS and shock.

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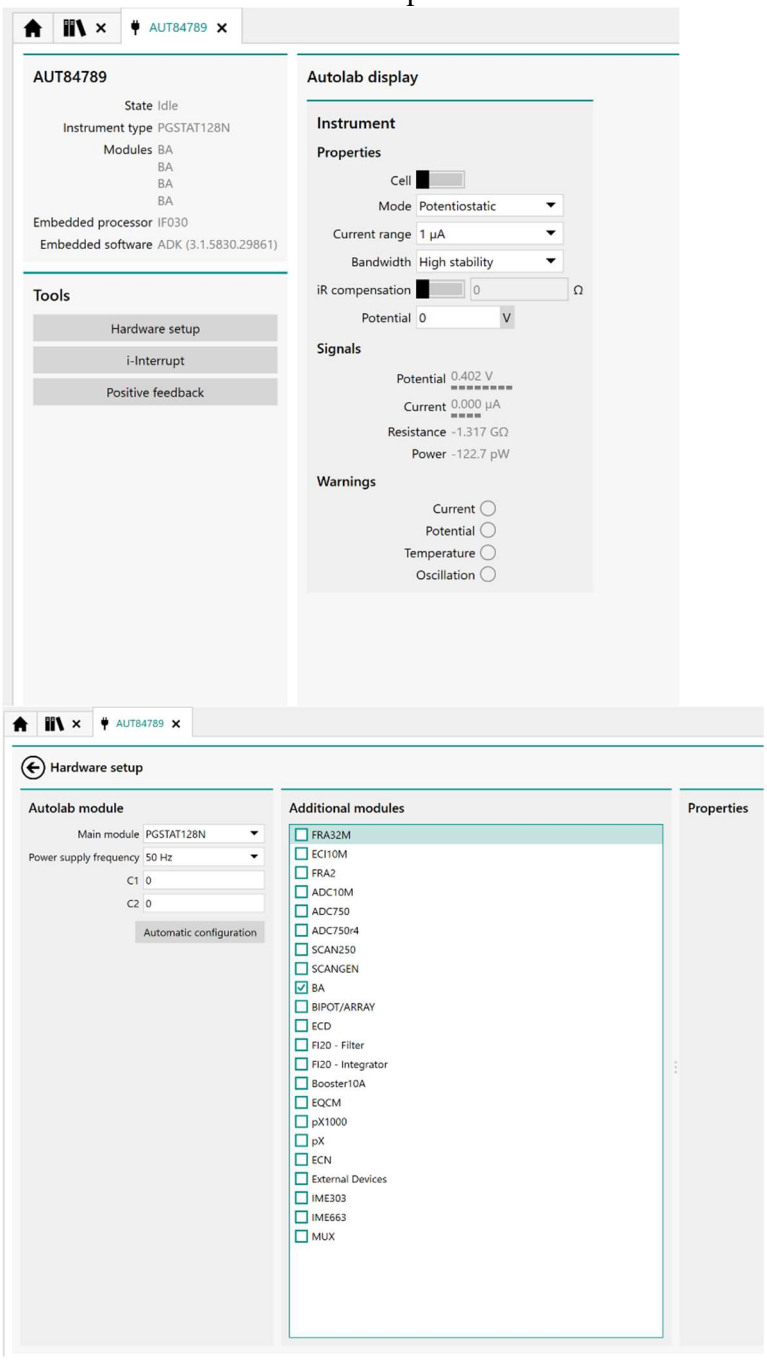
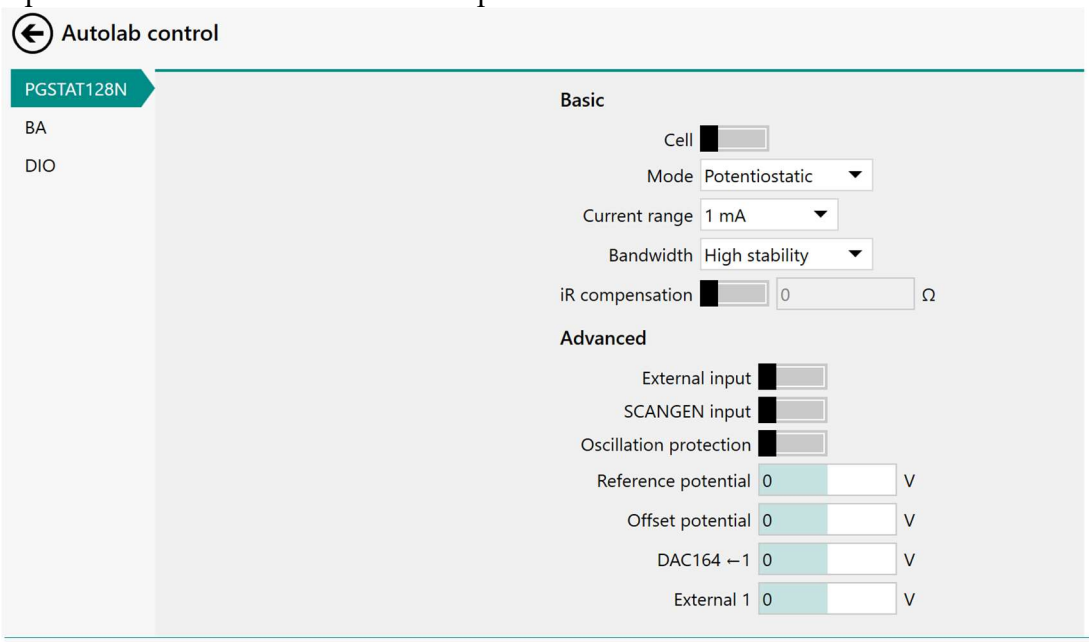
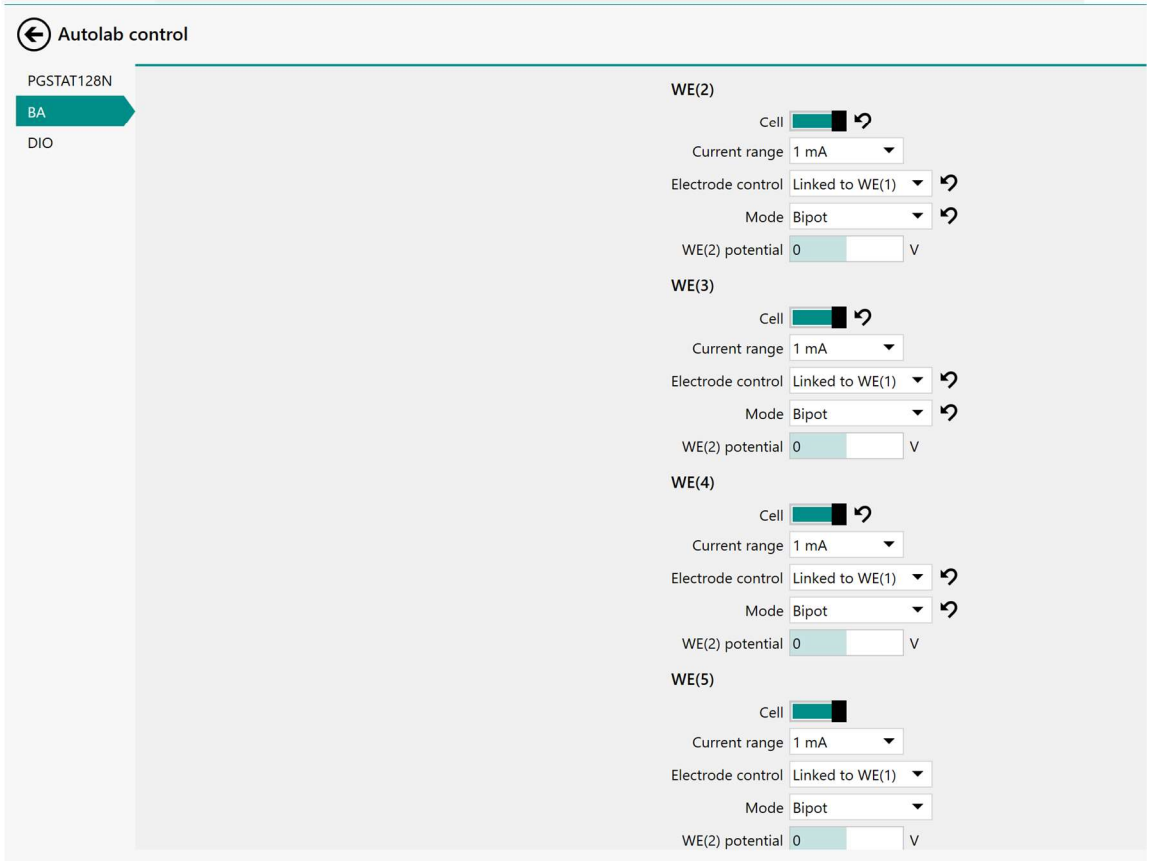
Appendix

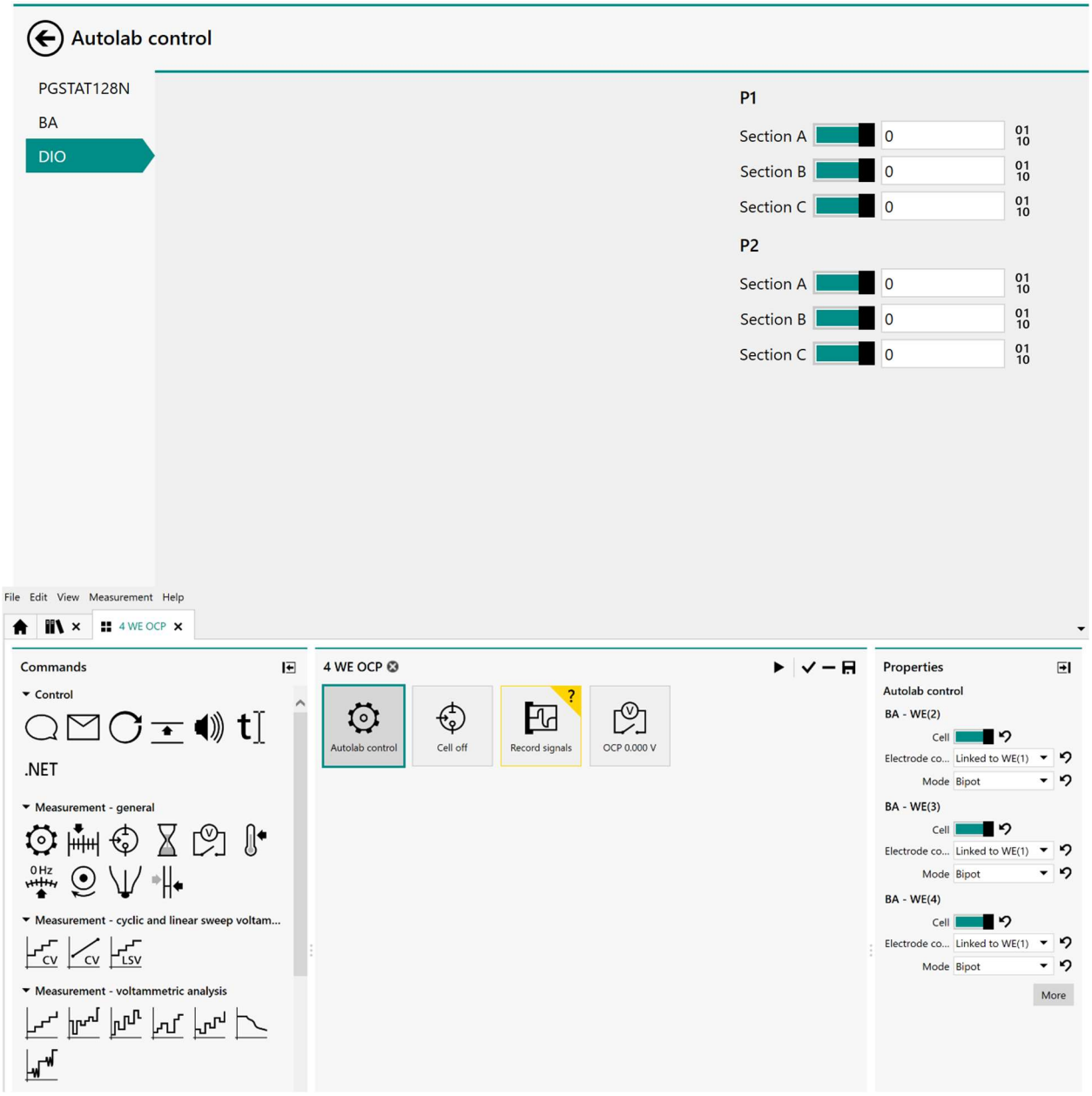
General Multi Potentiostat Setup



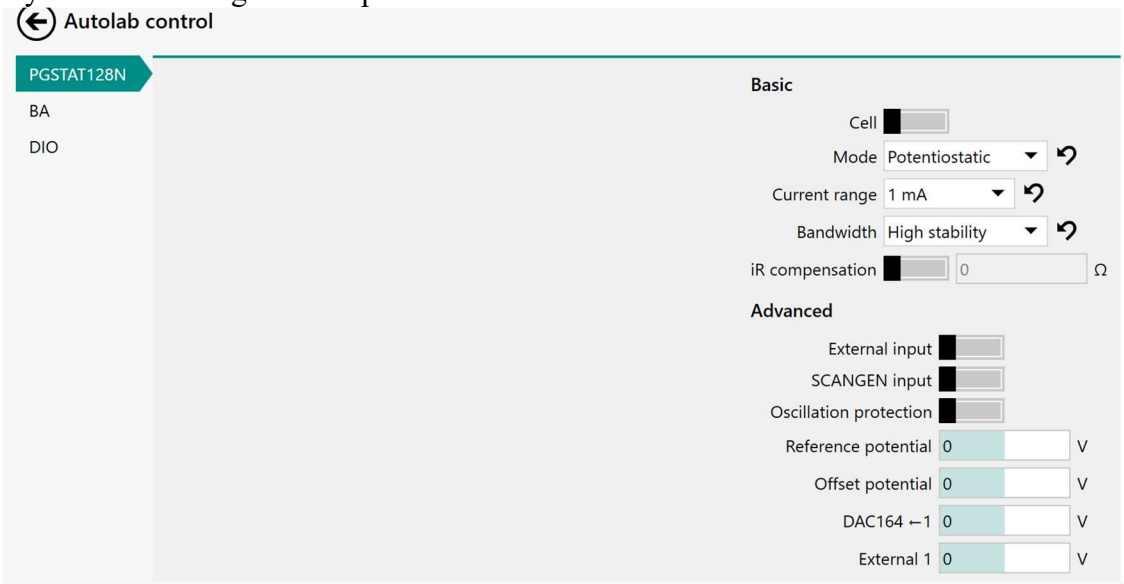
Open Circuit Potential Software Setup

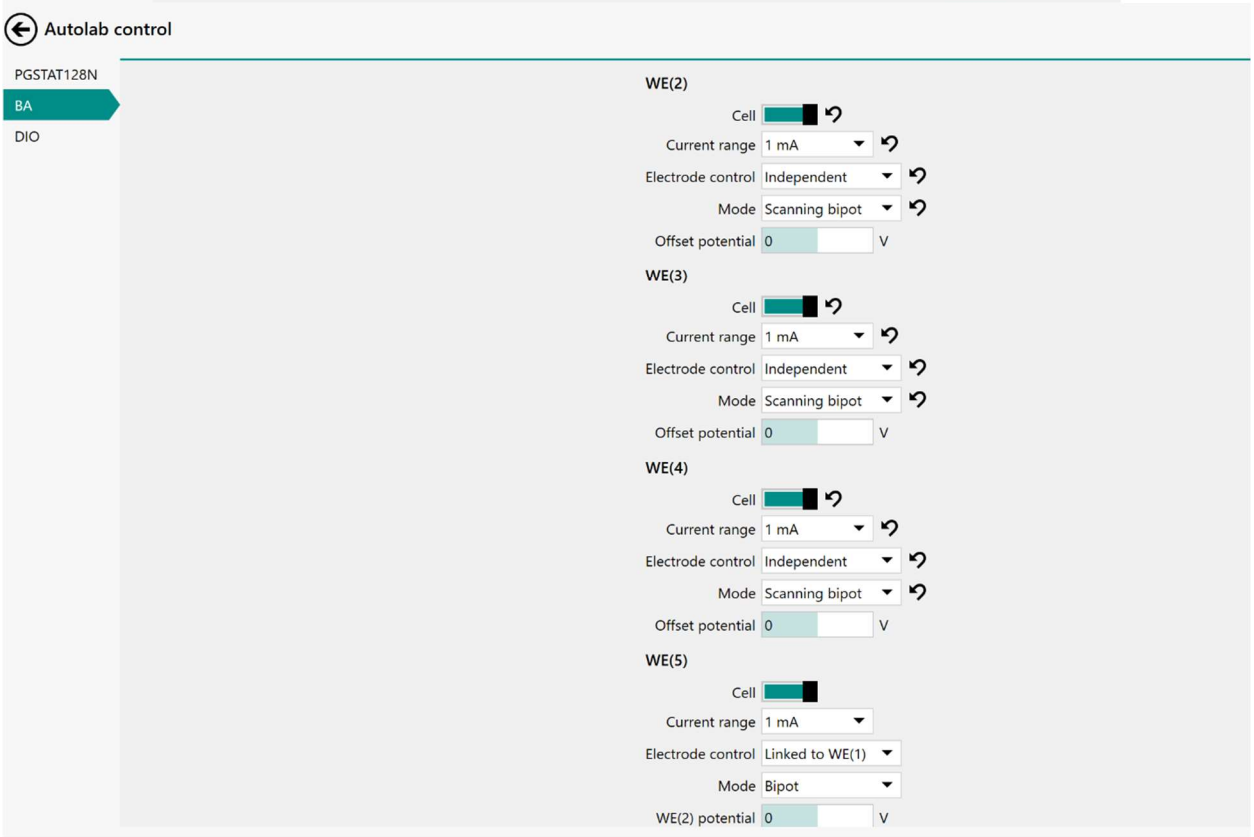


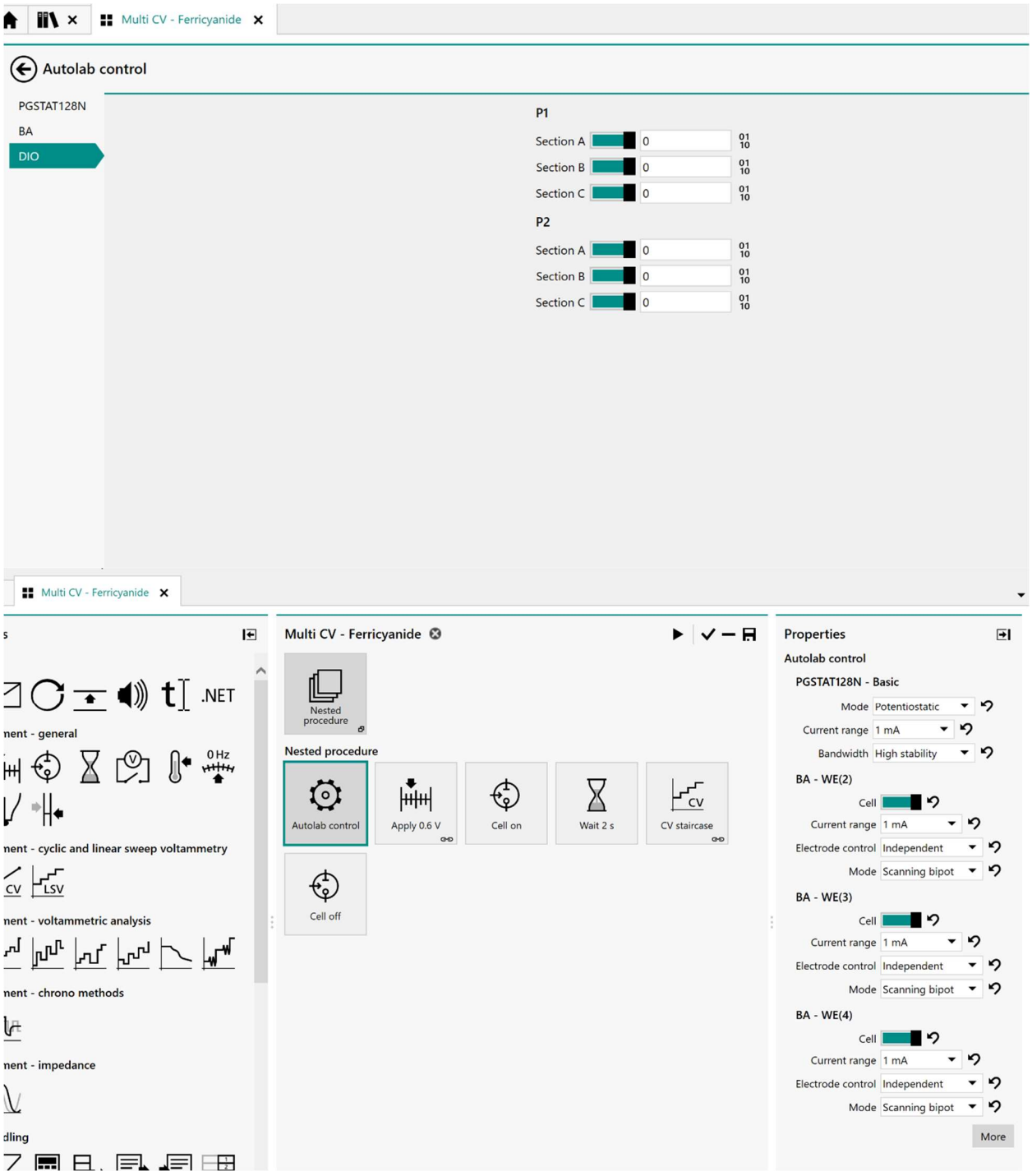


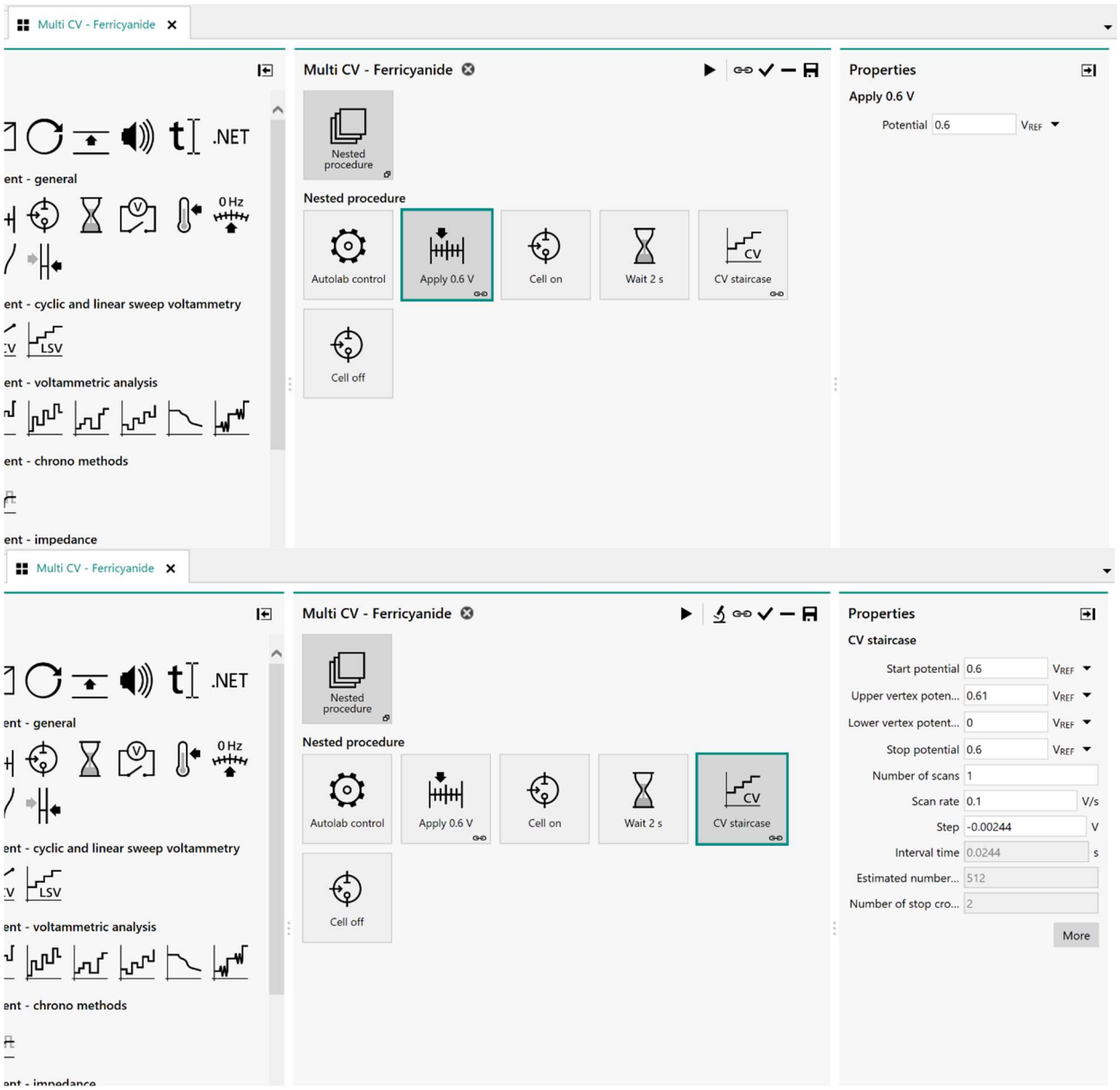

Autolab control
PGSTAT128N
BA
DIO
P1
Section A
0
Section B
0
Section C
0
P2
Section A
0
Section B
0
Section C
0
File Edit View Measurement Help
4 WE OCP
Commands
Control
.NET
Measurement - general
Measurement - cyclic and linear sweep voltam...
CV
CV
LSV
Measurement - voltammetric analysis
4 WE OCP
Autolab control
Cell off
Record signals
OCP 0.000 V
Properties
Autolab control
BA - WE(2)
Cell
Electrode co...
Linked to WE(1)
Mode
Bipot
BA - WE(3)
Cell
Electrode co...
Linked to WE(1)
Mode
Bipot
BA - WE(4)
Cell
Electrode co...
Linked to WE(1)
Mode
Bipot
More

Cyclic Voltammogram Setup






Multi CV - Ferricyanide
Autolab control
PGSTAT128N
BA
DIO
P1
Section A 0
Section B 0
Section C 0
P2
Section A 0
Section B 0
Section C 0
Multi CV - Ferricyanide
Nested procedure
Nested procedure
Autolab control
Apply 0.6 V
Cell on
Wait 2 s
CV staircase
Cell off
Properties
Autolab control
PGSTAT128N - Basic
Mode Potentiostatic
Current range 1 mA
Bandwidth High stability
BA - WE(2)
Cell
Current range 1 mA
Electrode control Independent
Mode Scanning bipot
BA - WE(3)
Cell
Current range 1 mA
Electrode control Independent
Mode Scanning bipot
BA - WE(4)
Cell
Current range 1 mA
Electrode control Independent
Mode Scanning bipot
More


Multi CV - Ferricyanide
.NET
ent - general
0 Hz
ent - cyclic and linear sweep voltammetry
CV
LSV
ent - voltammetric analysis
ent - chrono methods
ent - impedance
Multi CV - Ferricyanide
Nested procedure
Nested procedure
Autolab control
Apply 0.6 V
Cell on
Wait 2 s
CV staircase
Cell off
Properties
Apply 0.6 V
Potential 0.6 VREF
Multi CV - Ferricyanide
.NET
ent - general
0 Hz
ent - cyclic and linear sweep voltammetry
CV
LSV
ent - voltammetric analysis
ent - chrono methods
Multi CV - Ferricyanide
Nested procedure
Nested procedure
Autolab control
Apply 0.6 V
Cell on
Wait 2 s
CV staircase
Cell off
Properties
CV staircase
Start potential 0.6 VREF
Upper vertex poten... 0.61 VREF
Lower vertex potent... 0 VREF
Stop potential 0.6 VREF
Number of scans 1
Scan rate 0.1 V/s
Step -0.00244 V
Interval time 0.0244 s
Estimated number... 512
Number of stop cro... 2
More