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Determination of the hydrogen peroxide concentration in rotenone induced dopaminergic cells using cyclic voltammetry and amplex red

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DETERMINATION OF THE HYDROGEN PEROXIDE CONCENTRATION IN ROTENONE
INDUCED DOPAMINERGIC CELLS USING CYCLIC VOLTAMMETRY AND AMPLEX[®]
RED

by

KISHAN M. PATEL

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Molecular Biology and Microbiology
in the Burnett School of Biomedical Sciences
and in The Burnett Honors College
at the University of Central Florida
Orlando, Florida

Spring Term, 2012

Thesis Chair: Yoon-Seong Kim, Ph.D.

Abstract

Parkinson's disease (PD) is a neurodegenerative condition that affects millions of people worldwide. The exact etiology of PD is unknown. However, it is well established that environmental factors contribute to the onset of PD. In particular, chemicals such as the insecticide Rotenone have been shown to increase the death of dopaminergic (DA) neurons by increasing levels of reactive oxygen species (ROS). ROS such as hydrogen peroxide (H_2O_2) have been shown to be elevated above basal levels in PD patients.

Currently, to measure H_2O_2 concentrations, a commercially available (Amplex[®] Red) fluorescent assay is used. However, the assay has limitations: it is not completely specific to hydrogen peroxide and can only measure extracellular ROS concentrations. This research focuses on testing an electrochemical sensor that uses cyclic voltammetry to quantitatively determine concentrations of H_2O_2 released from a cell culture. The sensor was first tested in normal cell culture conditions. Next, chemical interference was reduced and the sensor was optimized for accuracy by altering protein concentrations in the media. Finally, Rotenone was added to a cell culture to induce H_2O_2 production. Near real-time measurements of H_2O_2 were taken using the sensor and comparisons made to the fluorescent assay method.

Overall, we are trying to determine if the electrochemical sensor can selectively and quantitatively measure H_2O_2 released from cells. Being able to track the production, migration and concentration of H_2O_2 in a cell can help researchers better understand its mechanism of action in cell death and oxidative damage, thus getting closer to finding a cure for PD.

Acknowledgments

To Dr. Yoon-Seong Kim, I would like to emphasize the deepest gratitude for giving me the opportunity to work in your lab, for your understanding, patience, invaluable advice and expecting only the best from me. In addition, I would like to thank Ana and all other lab members for their teachings, encouragement and tremendous help throughout.

Furthermore, I would like to thank Dr. Hyoung Jin Cho and his team for their dedication towards the completion of my project. Finally, to Dr. Self I would like to express many thanks for your lively personality, encouragement and for taking on this project.

Table of Contents

LIST OF FIGURES	V
ABBREVIATIONS	VI
INTRODUCTION.....	1
ROTENONE AND REACTIVE OXYGEN SPECIES	4
DETECTION OF REACTIVE OXYGEN SPECIES	5
OBJECTIVES, HYPOTHESIS AND SIGNIFICANCE	10
METHODS	11
FIRST SPECIFIC AIM.	11
SECOND SPECIFIC AIM.	12
THIRD SPECIFIC AIM.....	12
RESULTS AND DISCUSSION	14
INVERSE RELATIONSHIP OF H₂O₂ AND CV.....	14
CV IN MEDIA CONTAINING PROTEIN	15
CV DETERMINATION OF EXTRACELLULAR H₂O₂ IN N27 CELL CULTURE.....	19
AR DETERMINATION OF EXTRACELLULAR H₂O₂ IN N27 CELL CULTURE.....	27
CONCLUSIONS	32
REFERENCES.....	34

List of Figures

Figure 1 – Transverse cross-section of the brain showing Substantia nigra.	3
Figure 2 – Chemical Structure of Rotenone, an isoflavonoid compound.....	4
Figure 3 – Schematic representation of Amplex [®] Red conversion into Resorufin	6
Figure 4 – Electric Potential (V) varied with Time.....	7
Figure 5 – Cyclic Voltammogram Showing both Reductive and Oxidative Peaks	8
Figure 6 – Cyclic Voltammetry Setup	9
Figure 7 – CV of H ₂ O ₂ Concentration in RPMI	15
Figure 8 – CV with dialysis electrode in RPMI containing 10% FBS	16
Figure 9 – CV of Plain Gold Electrode in 10% FBS over 25 minutes	17
Figure 10 – CV of PBS alone and with 50µM of RT added	20
Figure 11 – Increase in Peak Height over 30 minutes of RT treatment in cell culture. ...	20
Figure 12 – Near real-time Effect of RT Addition at 30 minute mark	21
Figure 13 – RT Peak Reduction over Time	22
Figure 14 – Increase in Peak Height from the 42 – 60 minute mark	22
Figure 15 – Linear Rate of Increase of CV Curves at a fixed Potential (V).....	24
Figure 16 – CV of Untreated Cell Culture Used as a Control	24
Figure 17 – CV showing decrease in curves of RT with time.	25
Figure 18 – CV Calibration Curve of H ₂ O ₂	26
Figure 19 – Hydrogen Peroxide Calibration Curve using Amplex Red	27
Figure 20 – H ₂ O ₂ Detection using AR in Cell Cultures with and without RT	29

Abbreviations

AR	Amplex [®] Red
CV	Cyclic Voltammetry
DA	Dopaminergic
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
H ₂ O ₂	Hydrogen peroxide
N27	Immortalized Rat DA Cell Line 1RB3AN27
PBS	Phosphate Buffered Saline
PD	Parkinson's disease
ROS	Reactive Oxygen Species
RT	Rotenone

Introduction

Neurodegenerative diseases such as Parkinson's disease (PD) have been extensively studied by institutions and scientists worldwide in the hope to better understand their etiology and pathology. A clear and complete understanding of these diseases at the molecular level will enable scientists to generate an effective treatment or cure. An estimated ten million people live with Parkinson's disease worldwide.¹ Parkinson's disease was named in honor of an English doctor, James Parkinson, who published his research documenting symptoms of the disease.² The disease occurs due to the death of dopaminergic (DA) cells in the Substantia Nigra pars compacta region of the brain, which can easily be identified by the loss of the dark brown pigmentation of neuromelanine found in dopaminergic cells (Figure 1).

These DA cells produce dopamine, which is a neurotransmitter involved in voluntary movement, memory, cognition, and behavior. Resting tremor is a symptom exhibited by at least 70% of PD sufferers; relaxed muscles move in an oscillating fashion normally affecting one side of the body such as the hand or foot and spreads with mild severity to the other side of the body as the disease progresses.¹ Stiffness of muscles due to an altered muscle tone and bradykinesia (slow movement) will also be manifested in people with PD.

These obvious muscle impediments lead to postural instability and many PD patients fall down while walking. There is a loss of coordination and it is difficult to initiate movement therefore sufferers tend to feel "frozen" and can experience depression as a direct effect of PD or

as a side effect due to the decline in the quality of life. A host of other symptoms are possible such as trouble swallowing, speech irregularity and fatigue.

The etiology or cause of Parkinson's disease is not completely clear but it is widely accepted that genetic and environmental factors play a part. PD is a sporadic disease, which means that it is not genetically passed down family lines. In fact it is estimated that only 5-10% of PD patients show positive familial history.⁴ This must signify that there are external factors that contribute to the expression and progression of PD. Credibility was given to this statement when symptoms characteristic of Parkinson's were seen in users of a recreational opioid drug called MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), which when synthesized can have the impurity MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine).

MPTP is metabolized into MPP⁺, which is toxic to neuronal cells, by glial cells that are found in the brain. This toxic chemical leads to DA cell death in the Substantia Nigra, conveying PD-like symptoms to those individuals exposed to it.⁵ There are also other chemicals which are known to induce PD by DA cell degeneration, chemicals such as the very widely used herbicide Paraquat and the neurotoxin 6-hydroxydopamine (6-OHDA). Paraquat is known as a viologen because it is redox active and changes colors as it is reduced and oxidized. Since Paraquat is easily reduced and oxidized, it can produce high concentrations of hydrogen peroxide and other reactive oxygen species through a redox cycling mechanism.⁶

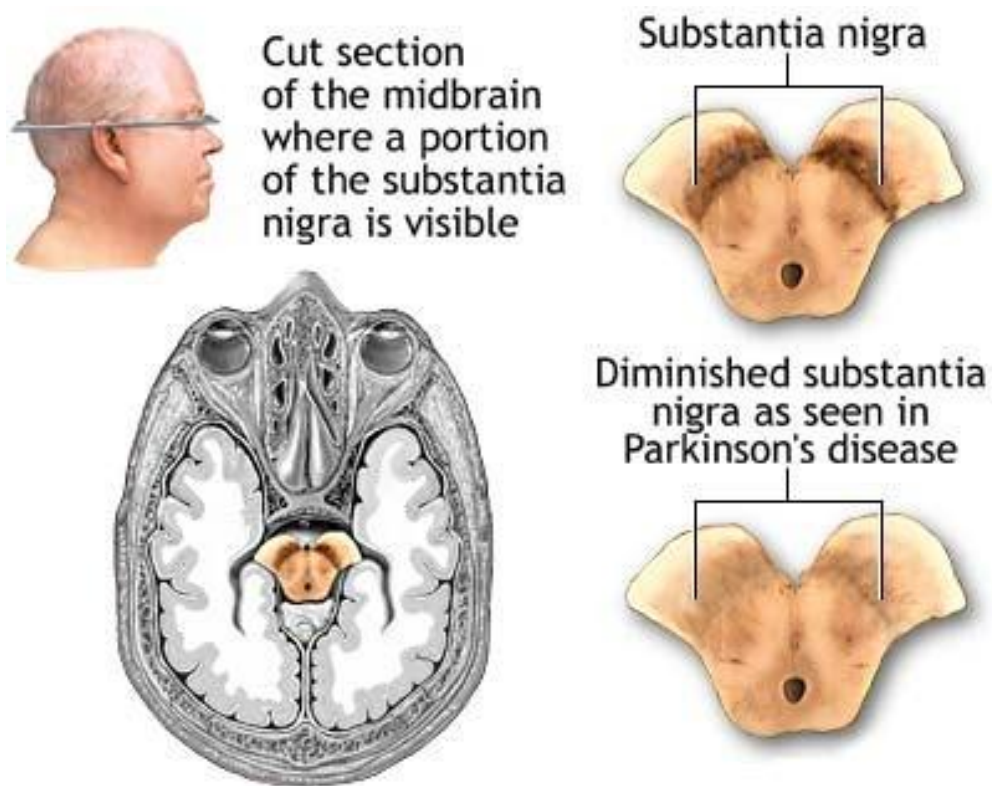


Figure 1 –Transverse cross-section of the brain showing Substantia nigra.

Normal Substantia Nigra (top right) compared to that of a PD affected individual (bottom right).

The reduced neuromelanine in the PD individual is indicative of dopaminergic cell death.

Image used under license from Springer Science, *Handbook of Neurochemistry and Molecular Neurobiology*.³

Rotenone and Reactive Oxygen Species

Rotenone (RT) is a naturally occurring organic compound, usually used as an insecticide and piscicide, which has been shown to reproduce PD characteristics in animal models.⁷

Rotenone is lipophilic in nature and is highly toxic to fish and birds. Due to its lipophilic properties, RT can penetrate the lipid bi-layer of cells and enter the mitochondria. On the other hand, RT is not as toxic to mammals because it is poorly absorbed through the digestive system. Inhalation or direct injection of RT into the bloodstream can be much more dangerous. Pure Rotenone is not very soluble in water due to its hydrophobicity. In addition, it is also sensitive to light and temperature.⁸

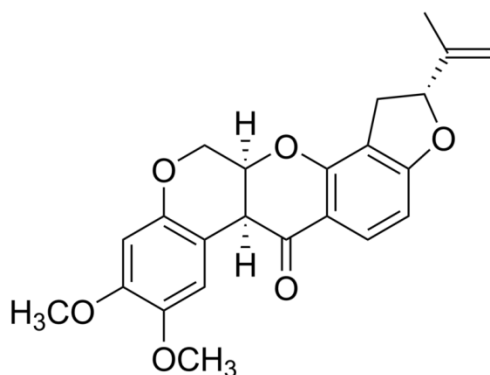


Figure 2 – Chemical Structure of Rotenone, an isoflavonoid compound

Rotenone reduces the activity of complex I in the mitochondrial electron transport chain (ETC) of cells thus reducing the level of adenosine triphosphate (ATP), the molecular unit of energy. However, the depletion of ATP is not the reason why DA cells die: Rotenone causes an increase in oxidative damage through reactive oxygen species (ROS).⁷ Reactive oxygen species such as hydrogen peroxide, nitric oxide derivatives, superoxide and a medley of other oxygen

containing species can cause damage to cells. ROS are produced as a byproduct of oxygen metabolism primarily in the mitochondria and are physiologically necessary in immunological functions as well as cell signaling.⁹

Normally functioning mitochondria produce ROS such as superoxide ($O_2\cdot^-$) because the ETC is made up of single electron transfers up to the final electron acceptor.¹⁰ Even though ROS are produced under normal conditions, the basal level of ROS causes little damage to the cells and surrounding tissue because of high antioxidant activity from enzymes such as superoxide dismutase (SOD) and small molecules like glutathione.¹¹ Oxidative damage occurs when there is an imbalance; ROS levels reach excessive limits. Prolonged exposure to stressful conditions such as the chemicals mentioned previously or through genetic mutations which either up-regulate the production of ROS or down-regulate its decomposition by enzymes lead to oxidative damage and cell death.

Detection of Reactive Oxygen Species

Due to the short lived but reactive nature of all radical species, it is very difficult to detect ROS. Therefore, end products in reactions involving ROS are normally measured. Hydrogen peroxide (H_2O_2) is a reactive oxygen species, but is not a radical. It has been used extensively in the detection of ROS. Currently, there are a number of fluorometric and colorimetric methods used to qualitatively measure ROS. One method is by the fluorescent detection of 2', 7'-dichlorofluorescein which is produced by the oxidation of 2',7'-dichlorodihydrofluorescein

diacetate (DCFDA). This method can actually measure intracellular ROS, however, it is not specific to one species and the oxidized fluorescent product can diffuse out of the cell.¹² A more specific ROS detection method is with Amplex[®] Red (10-acetyl-3, 7-dihydroxyphenoxazine) made by Invitrogen/Molecular Probes (Eugene, OR). Amplex[®] Red (AR) has been described as one of the most reliable methods to detect ROS and has extensively been used in many laboratories worldwide.¹³ It is a chemical which in the presence of horseradish peroxidase is converted into Resorufin in a 1:1 stoichiometric ratio with hydrogen peroxide. AR can be measured using both absorbance, for high concentrations (i.e. > 75 μ M H₂O₂) or the more sensitive fluorescence method for concentrations down to 0.01nM of hydrogen peroxide.¹⁴

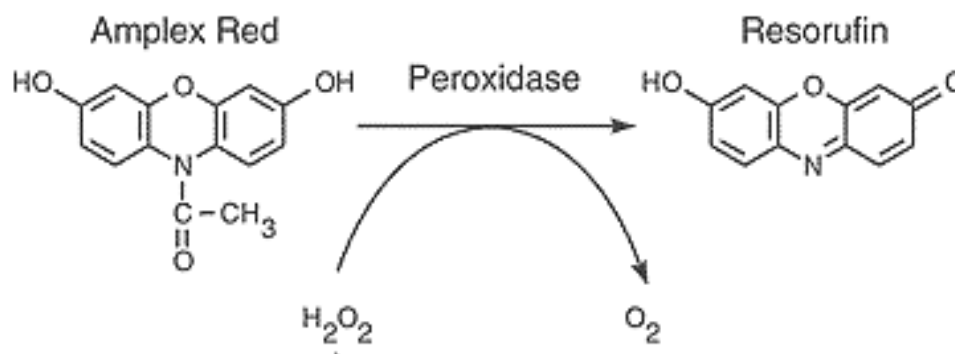


Figure 3 – Schematic representation of Amplex[®] Red conversion into Resorufin

Horseradish peroxidase (HPR) enzyme uses hydrogen peroxide as an oxidizing agent to convert AR into the fluorescent Resorufin. Image modified from Life Technologies, *Molecular Probes Handbook*¹⁵

In addition to detection of ROS by fluorescence and chemiluminescence, recently there has been great interest in using electrochemical methods due to the possibility of continuous *in vivo* measurement.⁹ Some drawbacks to the current fluorescent methods are that they are not

highly selective for one species compared to another, and real-time *in vivo* measurement is nearly impossible. The H2DCF assay, for example, requires 15-45 minutes of incubation time before image capture from confocal microscopy.¹⁶ In contrast, electrochemical sensors have the potential to be used *in vivo* by using advanced nanofabrication techniques to create electrodes less than 1 μm in diameter.¹⁷

In the experiments conducted in this thesis, cyclic voltammetry (CV) is the electrochemical detection method used.¹⁸ CV can be described as a scanning method in which the electric potential (V) is increased with time. As V is increased and subsequently decreased on the reverse scan, the current (I) is measured. The amplitude of the current is a result of redox reactions taking place at the surface of the working electrode.

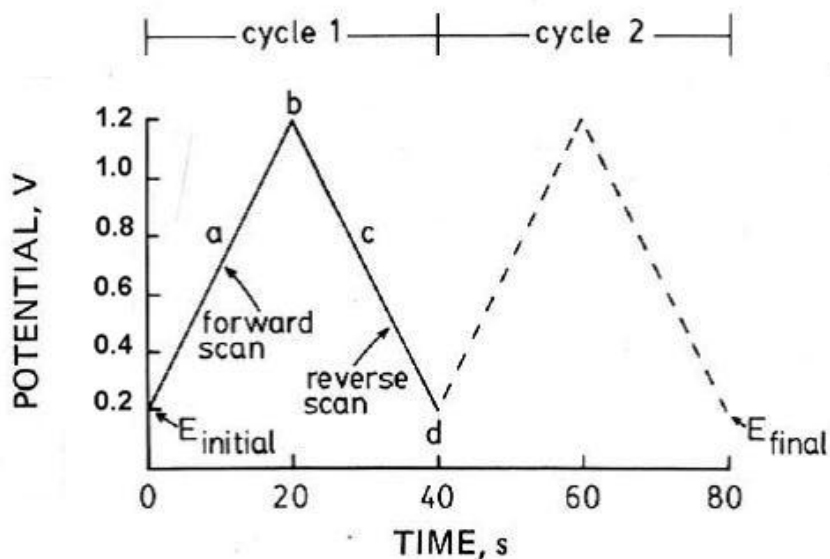


Figure 4 – Electric Potential (V) varied with Time

The voltage is altered, forward and then backwards at a particular rate. This rate of change is called Scan rate and is measured in V/sec. Image adapted from “*Cyclic Voltammetry*,” *Journal of Chemical Education*¹⁸

The three-electrode system used in our CV setup contains a working electrode made of gold (Figure 6). This is where the analyte is oxidized or reduced. A reference electrode provides a constant voltage while an auxiliary electrode balances the current produced at the working electrode. Quantification of an analyte is through a calibration curve and comparison of peak changes. Hydrogen peroxide is thought to have a redox potential (V) between 0.80V – 0.90V.¹⁹ A reaction is said to be reversible with fast electron transfer if the peak anodic current and peak cathodic current are equal.¹⁸

Equation 1: $(I_{pa} / I_{pc}) = 1$

Anodic peak current / Cathodic peak current = 1

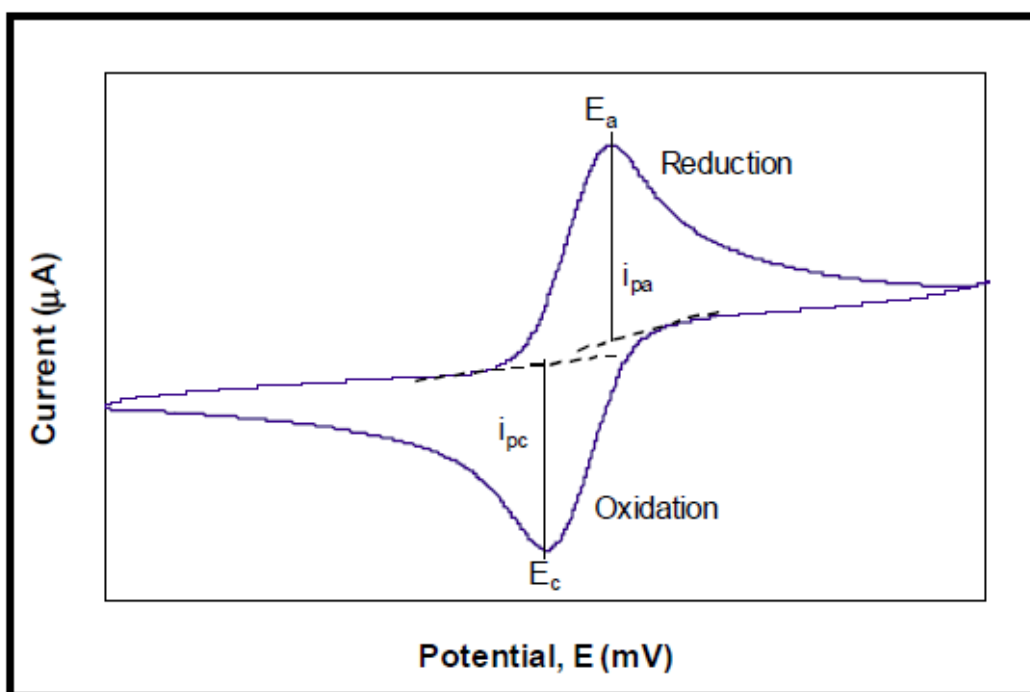


Figure 5 – Cyclic Voltammogram Showing both Reductive and Oxidative Peaks

As the potential changes in the forward scan there is an increase in current, shown as the reductive peak on the anode. In the reverse scan the oxidative peak is shown. The analyte is being oxidized while the anodic surface is reduced. This reduction causes an increase in current measured. Image from a Master’s thesis by Kadanthode R.²⁰

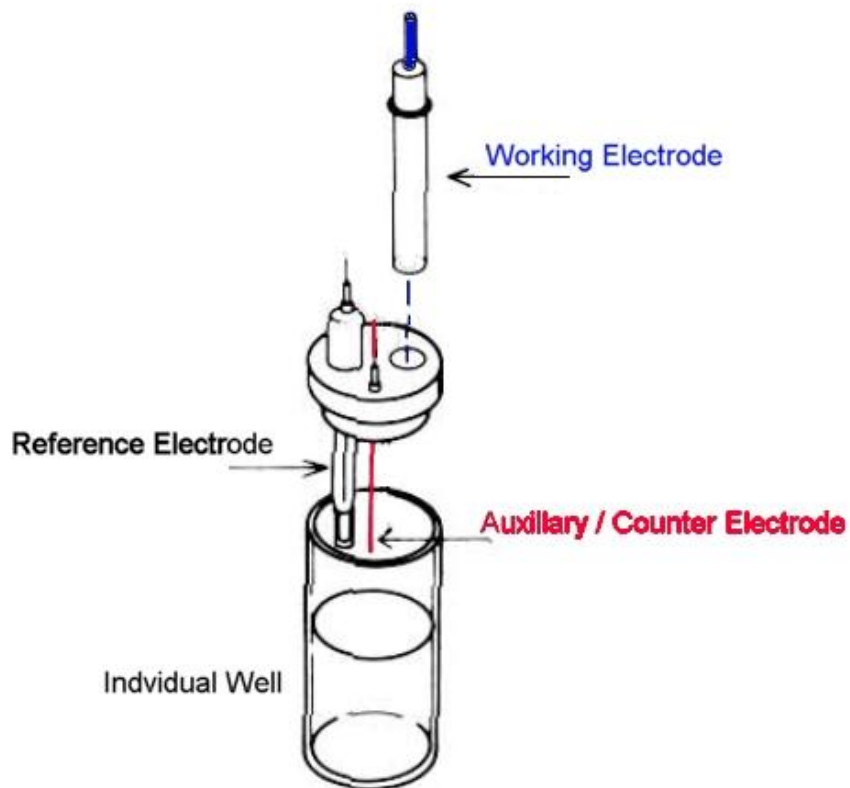


Figure 6 – Cyclic Voltammetry Setup

Three electrode setup: Reference electrode, Working electrode and Auxiliary electrodes are connected to a potentiostat and computer to record the Current (Amperes) vs. Scan rate (V/sec)
Image adapted from "Cyclic Voltammetry," *Journal of Chemical Education*.¹⁸

Objectives, Hypothesis and Significance

In our research, we propose to quantitatively determine the concentration of hydrogen peroxide in N27 cells. An electrochemical sensor developed by Professor Hyoung-Jin Cho in the Department of Mechanical, Materials & Aerospace Engineering will be used under collaboration to measure hydrogen peroxide levels released by the neuronal cells. The objective of this research is to determine if the sensor can be used in a biological environment and identify its limitations. If the sensor functions reliably and accurately then there is the possibility for future experiments to be conducted on a smaller scale and possibly inside a cell.

The first specific aim is to determine if the electrode with a dialysis membrane can detect hydrogen peroxide in a biological environment identical to that found in cell cultures.

The second specific aim is to determine the optimum conditions for measurement using the electrode. From previous research conducted by the collaborating lab, it is already known that the electrode has difficulty working efficiently in protein solutions. Therefore, a concentration of 10% and lower of protein will be tested to determine the optimum conditions, and recognize limitations of the sensor.

Finally the third specific aim is to determine if the sensor has any advantages in terms of near real-time detection of hydrogen peroxide compared to that of Amplex[®] Red in the DA cell culture.

It is hypothesized that the electrode-based system will give accurate and consistent results compared to the current methods of ROS measurement because it interacts directly with the ROS rather than measuring a byproduct of a chemical reaction. It is expected that any H₂O₂ released from the cells will cause a quantifiable change in the voltammogram. An alternate hypothesis is that the electrochemical sensor may not provide results drastically different compared to the Amplex® Red method or the sensor may not be selective enough due to background interaction with other chemicals. The significance of this research will determine whether this electrochemical sensor can be used as an alternative method for quantitative real-time hydrogen peroxide measurement *in vitro* and possibly *in vivo*.

Methods

First Specific Aim.

Determine whether the electrode works in conditions identical to those in which the cells grow. The gold working electrode fabricated with a dialysis membrane was placed into one well of a 6-well tissue culture plate. The well was filled with 2.0ml of RPMI 1640 growth medium. CV scans were run at a scan rate of 100mV/s from – 0.5V to + 1.5V using a PalmSens potentiostat and recorded using PSTrace software from PalmSens. This scan rate was identified as the most effective from previous research done by the collaborating lab with hydrogen peroxide. The CV setup includes a Ag/AgCl reference electrode, a platinum auxiliary electrode

and a membrane containing, gold working electrode. Every measurement curve was composed of three CV cycles to reduce any background noise and obtain consistent voltammograms. Varying volumes of hydrogen peroxide (300 μ M) were added to the RPMI solution to create concentrations ranging from 14 – 100 μ M.

Second Specific Aim.

Determine at which protein concentration the electrochemical sensor performs the best. A total working volume of 2.0ml RPMI 1640 media is added to four wells of a 6-well plate. Fetal Bovine Serum (FBS) is added to the media in 10%, 8% 6% and 0% concentrations. Then in each well a fixed concentration of hydrogen peroxide was added before CV measurement was conducted. The solution was mixed thoroughly for 10 seconds, and electrodes were cleaned before each test. The concentration of H₂O₂ in each well was measured almost every 4 minutes for just less than 30 minutes. This experiment is to test at which FBS (protein) concentration the electrode works most accurately and consistently.

Third Specific Aim.

Detect near real-time concentrations of hydrogen peroxide released from DA cells using CV as well as the Amplex[®] Red fluorescent detection method. The cells used were the immortalized rat mesencephalic dopaminergic neuronal cell line 1RB3AN27, more commonly known as N27. ²¹ N27 cells were grown in RPMI 1640 supplemented with 10% FBS and kept at a constant humidified temperature of 37 °C and 5% CO₂. Cells were plated in a 12-well tissue culture plate with each well containing 200,000 cells.

Before measurements were conducted, the media was removed and cells washed in PBS (0.1M pH 7.4). The media was replaced with 1.0ml of PBS. Cells were then treated with 50 μ M of RT and measurements taken every 2 minutes using the CV method and every 10 minutes using AR. At the 30 minute mark, an additional 50 μ M of RT was added. Measurements were taken for 60 minutes. As a control, CV and AR measurements were also taken for cells that were not treated with RT.

The AR kit was from Invitrogen/Molecular Probes (Eugene, OR) and the manufacturer protocol was followed.¹⁴ Fluorescence was measured in 96-well microplates using a Gemini EM microplate reader (Sunnyvale, CA) with an excitation of 530nm and emission at 590nm. Every 10 minutes 50 μ L of media was removed from the cell culture, combined with the AR assay and incubated in the dark for 30 minutes before being read. Three measurements at 10 minute intervals were taken from one well and the other three taken from another well under identical conditions. This was done so that as media is removed for measurement the total volume does not change significantly enough to alter the concentration of any species. Plain media not from cells was also run as a control. CV was conducted using a plain 99.99% gold electrode with a diameter of 25 μ meters; the setup and scan rate were the same as mentioned previously.

Results and Discussion

Inverse relationship of H₂O₂ and CV

The electrochemical sensor was preliminarily tested in DMEM and subsequently tested in RPMI 1640 growth media with increasing concentrations of hydrogen peroxide added. In both phenol red containing media, the voltammogram obtained showed a decreasing anodic peak as the concentration of hydrogen peroxide increased (Figure 7). This inverse relationship between the reduction/anodic peak and H₂O₂ is not intuitive and was unexpected. The fact that there is an anodic peak but no visible cathodic peak indicates that the reaction is irreversible ([Equation 1](#)).

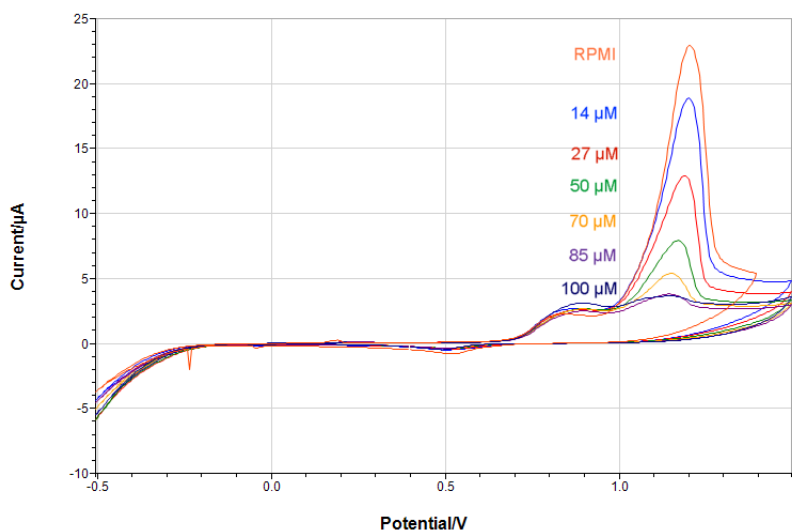
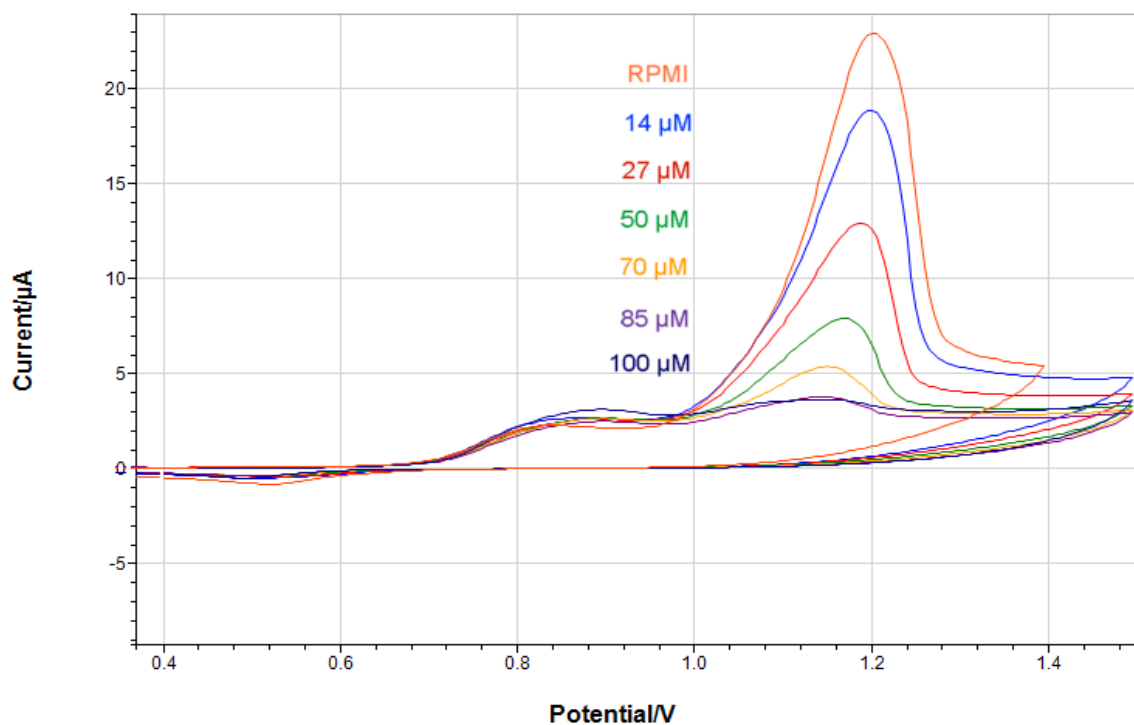


Figure 7 – CV of H₂O₂ Concentration in RPMI

Above image shows full CV range from -0.5V to +1.5 V

Exact image is shown below; but it is enlarged and focuses on peak currents.

All proceeding graphs have been enlarged and some curves may be removed for clarity unless stated otherwise



CV in Media Containing Protein

From previous research conducted, it is known that the electrode surface is prone to protein aggregation. This prevents any redox measurement to take place as more protein attaches to the surface of the electrode. To combat this, an electrode with dialysis membrane is used as our working electrode in our CV setup. Our findings clearly demonstrate that the dialysis membrane is efficient in preventing protein from attaching to the surface of the electrode. The electrode was tested in 10% FBS (Figure 8) and subsequently in 8% FBS (data not shown). In contrast to the plain gold electrode (without a dialysis membrane, Figure 9) the level of detection stays very consistent with time, as expected.

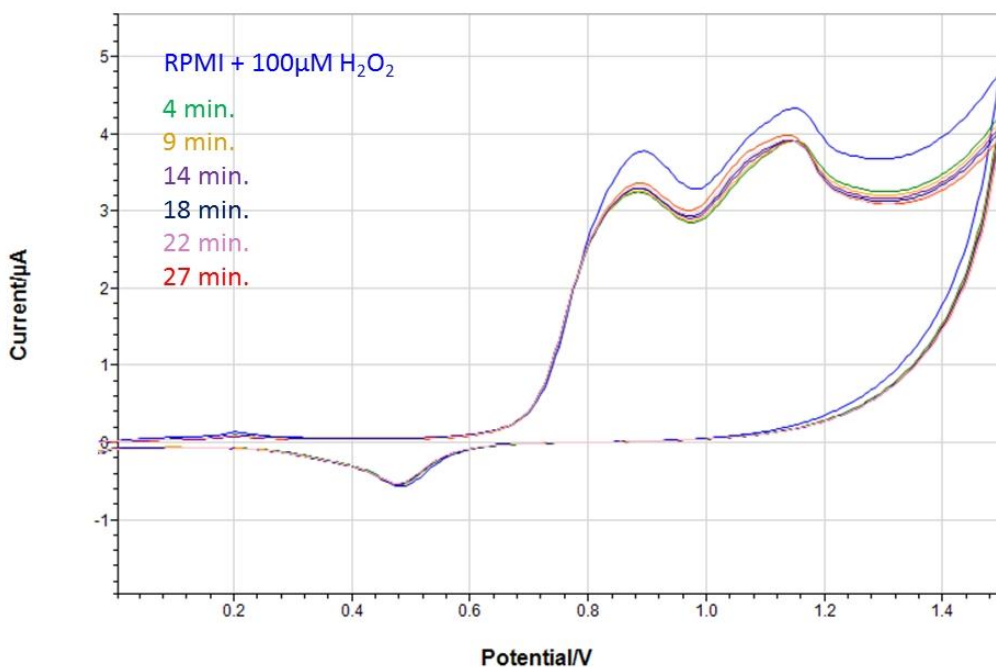


Figure 8 – CV with dialysis electrode in RPMI containing 10% FBS

There is minimal reduction in sensitivity due to protein, and as time increases to 27 minutes the detection level is fairly consistent.

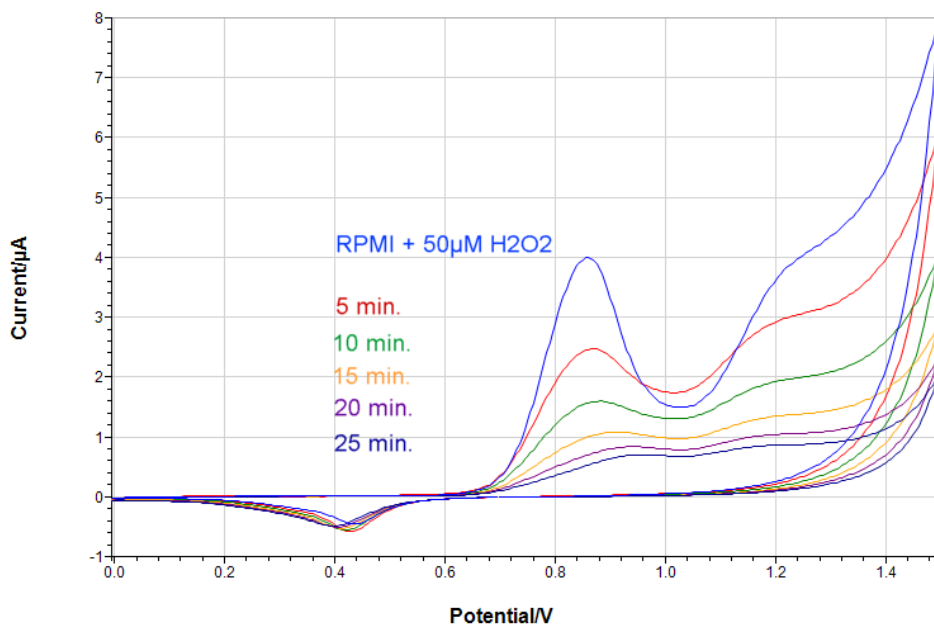


Figure 9 – CV of Plain Gold Electrode in 10% FBS over 25 minutes

The anodic peak decreases considerably as more FBS protein attaches to the plain gold electrode surface.

Even though the electrode with a dialysis membrane works well in protein, especially in 8% FBS there are still some drawbacks. When we measure H₂O₂ that is released from a cell culture we expect the concentration to be very low. For detection to occur, the hydrogen peroxide has to diffuse out of the cell into the surrounding media. Since H₂O₂ is a ROS, it will decompose relatively quickly due to temperature, light and even antioxidants in the cell. These factors make it difficult to detect *in vitro*. It is important that the electrochemical sensor is sensitive enough to reliably measure ROS. One of the most significant drawbacks of the dialysis membrane is that it reduces sensitivity by a factor of 10 compared to without a membrane: the lowest detectable H₂O₂ concentration was just under 10µM.

Due to the fact that CV in an unspecific scanning method, there are a numerous factors that can interfere with detection of ROS, and hydrogen peroxide in particular. For this reason, the initial protocol was changed and we decided to use a plain gold electrode for H₂O₂ detection in cell cultures. Furthermore, it was suspected that phenol red and amino acids or other chemicals in RPMI could affect the detection. To reduce any false peaks, the cells that were initially grown in RPMI were washed and placed in PBS with no protein. N27 cells are known to survive in minimum (near zero) protein for a duration long enough to conduct the experiment fairly.

The electrodes are an essential part to obtain quality data using CV measurement. The electrodes were assumed to be in proper working order and cleaned by the operator using nitric acid and deionized water. An initial test using the electrochemical sensor and RT was conducted to determine if there would be any interference. The voltammogram obtained (not shown) did not noticeably change, but it is important to note that the measured current was very low. Any change that was observed could have been due to background noise, vibration, or non-homologous portions of media. Thus, at this stage we could not conclusively say that RT affected the sensor.

CV Determination of Extracellular H₂O₂ in N27 Cell Culture

For CV measurements to be conducted in the cell culture containing RT, new electrodes that are more reliable were fabricated. The plain gold electrode was 25µm in diameter and quality tested by the fabricator before use. A baseline CV was taken of the cells in PBS. Immediately after RT was added and mixed in the media CV measurements were taken at 2-minute intervals. It must be noted that each measurement with 3 cycles takes 1.5 minutes. As shown in Figure 10, the RT elevated the graph.

Thereafter, the voltammogram of the cell culture with RT consistently decreased with time for 8 minutes. Each time RT is added the peak increases to a maximum height followed by a steady decrease up to a certain point. This can be clearly seen in Figure 13. This decrease is consistent with literature, where it has been shown in one previous study of RT using CV that the curve decreases with time. In addition, even when the electrode was cleaned the peak did not recover its initial height.²⁰ For this reason, we do not think that the surface of the electrode is saturated with RT and preventing further detection. Rather it is hypothesized that RT is chemically altered in the electrolytic conditions because it is not very stable and can be degraded by strong light and high temperatures.⁸ The decrease stopped 8 and 12 minutes after each addition respectively; it is thought that by this point all the RT has been chemically modified under the electrolytic conditions.

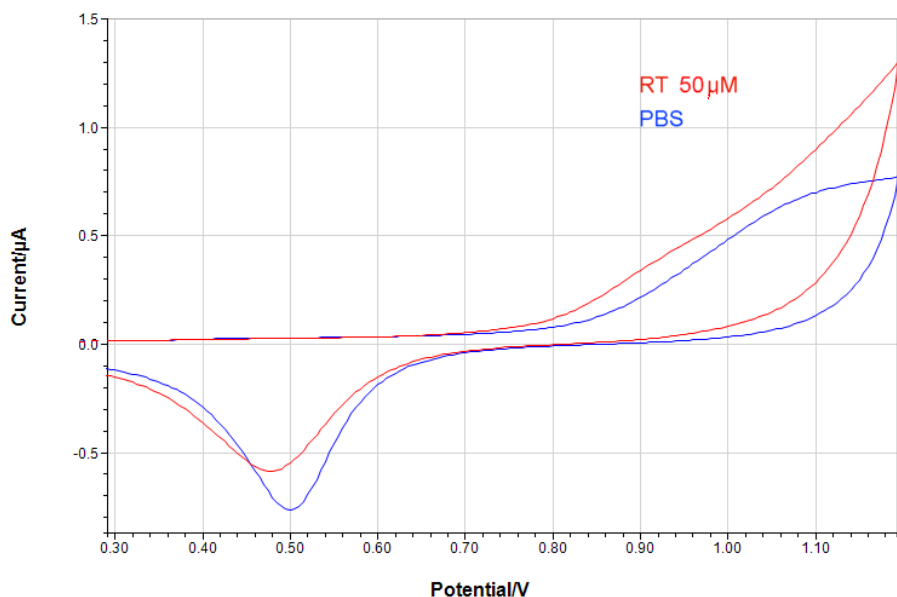


Figure 10 – CV of PBS alone and with 50µM of RT added

After the curve has stabilized and is not decreasing due to the effects of RT, the curve begins increasing steadily. The increase in peak height of the curves (Figure 11) was thought to be caused by ROS released by the N27 cells. However, as discussed later the increase cannot be definitively identified as hydrogen peroxide or ROS.

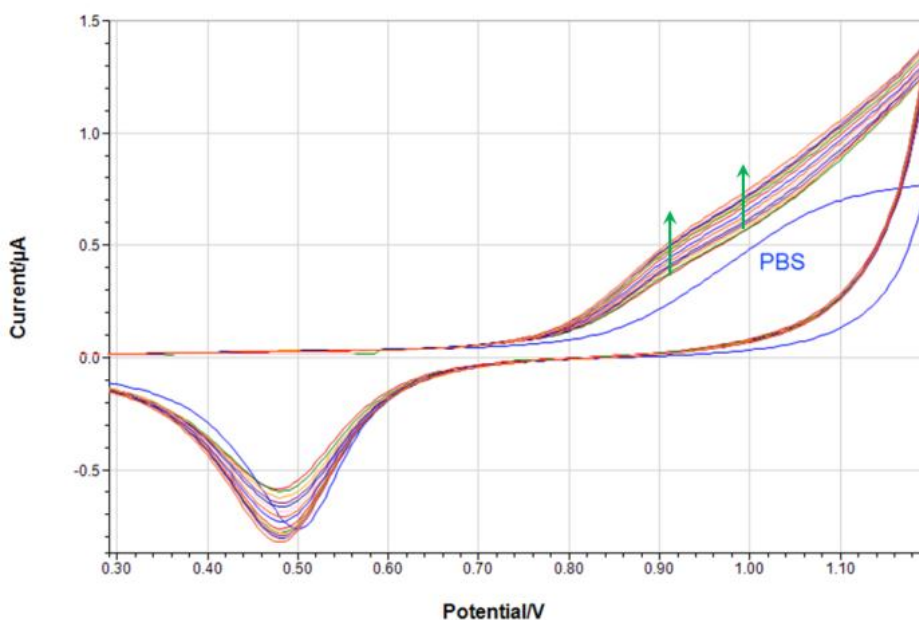


Figure 11 – Increase in Peak Height over 30 minutes of RT treatment in cell culture.

At the 30-minute mark, the RT concentration was doubled with the addition of 26 μ L of 2mM RT and as shown previously there is an immediately detected increase in the curve (Figure 12). Just as before, and as clearly shown in Figure 13, the peak of RT decreases with time and remains constant after the 42-minute mark (12 minutes after second addition of RT).

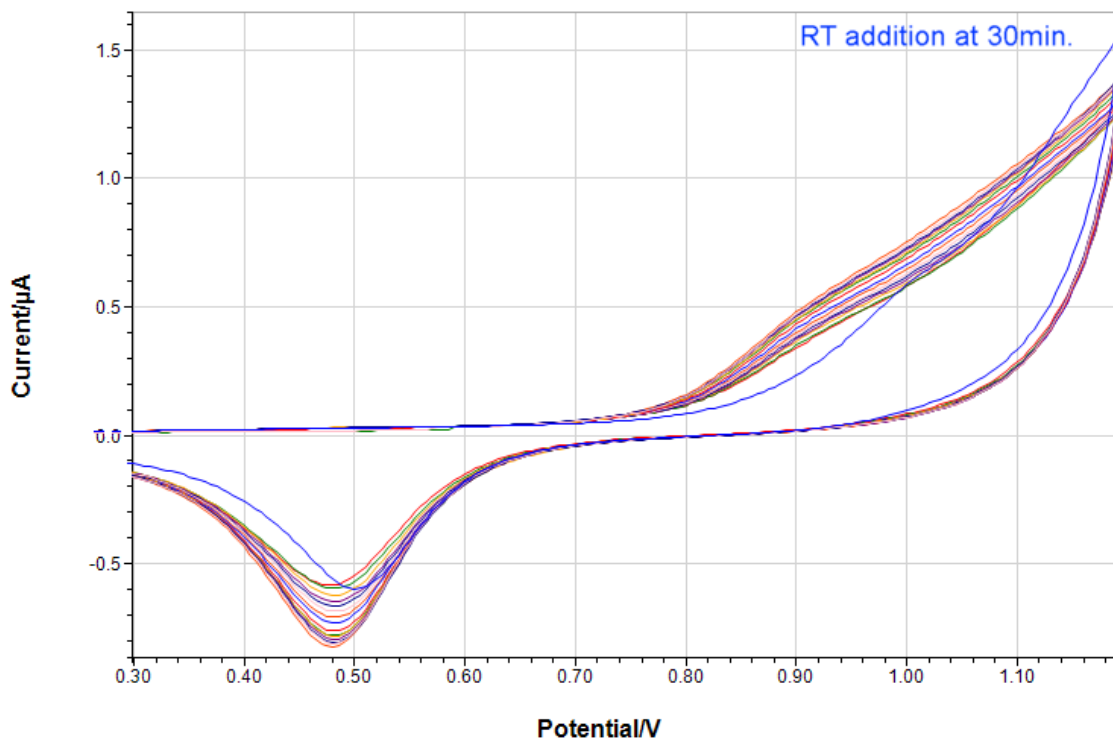


Figure 12 – Near real-time Effect of RT Addition at 30 minute mark
Curves show CV measurement every 2 minutes up to the 30 minute mark in cell culture

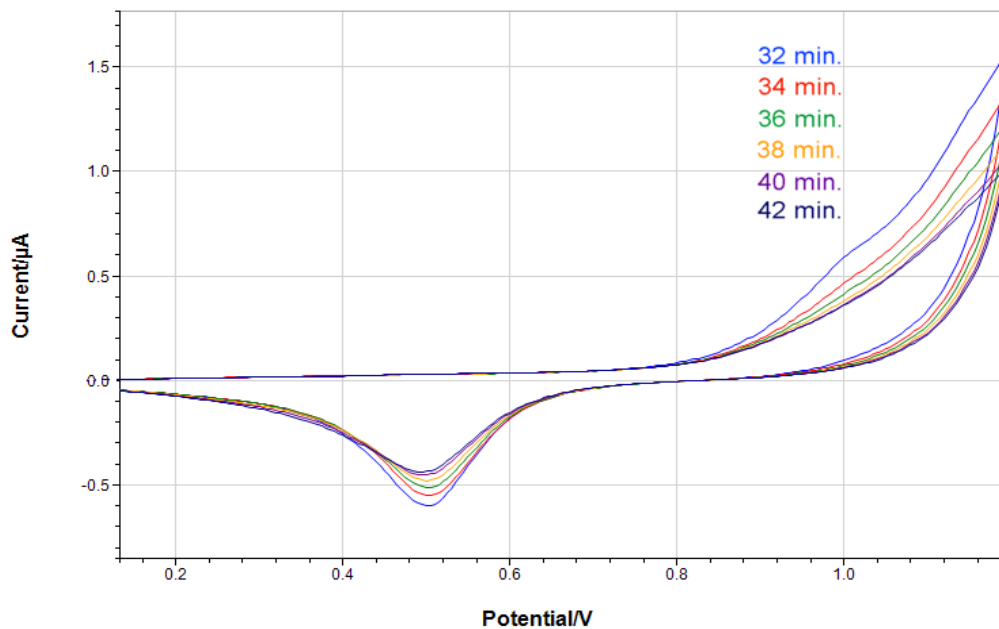
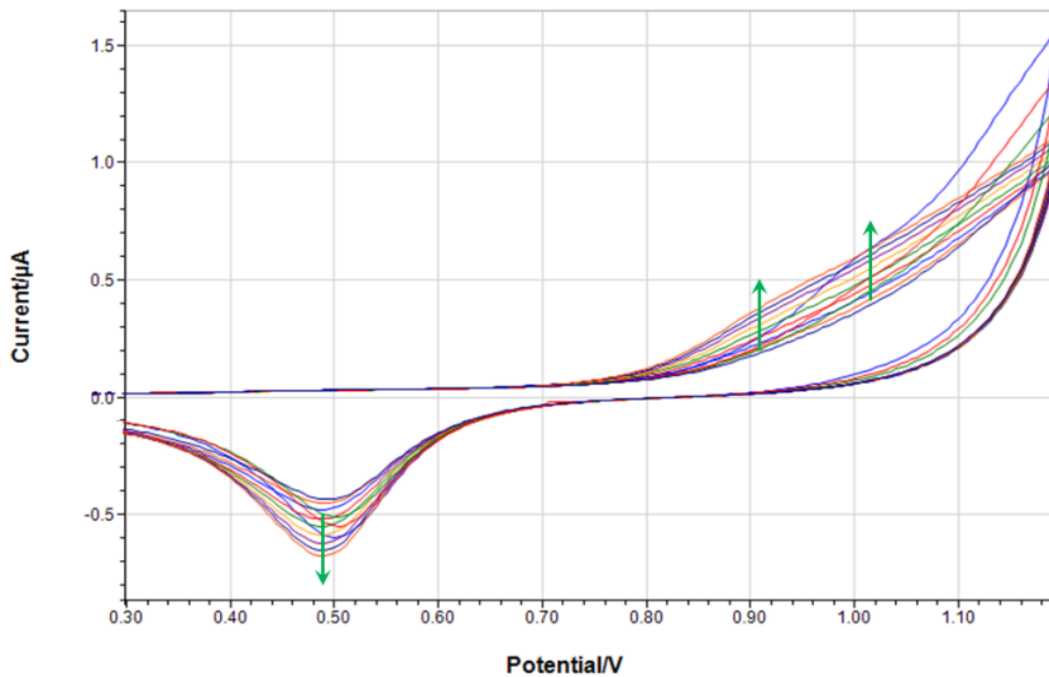


Figure 13 – RT Peak Reduction over Time

(Above) Peak continues to decrease with time for 10 minutes, after which no change is detected.

Figure 14 – Increase in Peak Height from the 42 – 60 minute mark



From the results obtained so far it is not possible to determine the cause of the increase in curves over the measured period. In Figure 15 we can see that the increase detected in the cell culture occurs at a steady and linear rate at a reduction potential of 0.9V/s. From previous experiments conducted by the collaborating lab, 0.9V/s is thought to be the range at which hydrogen peroxide is detected.

RT displays a broad reduction potential and thus interferes with detection. When we consider Figure 7 and Figure 17, it becomes clear that as H_2O_2 concentrations increase the curve decreases. Therefore, H_2O_2 cannot be responsible for any increased detected in the cell culture conditions. It can be speculated that the increase is due to some type of localized event: the RT is entering the cells and remaining trapped inside. This would cause the extracellular concentration of RT to decrease and cause any saturated RT on the sensor surface to vacate. Thus as the amount of RT on the sensor surface decreases the current detected becomes more positive. In other words, the increase could represent the indirect measurement of RT uptake by the N27 cells.

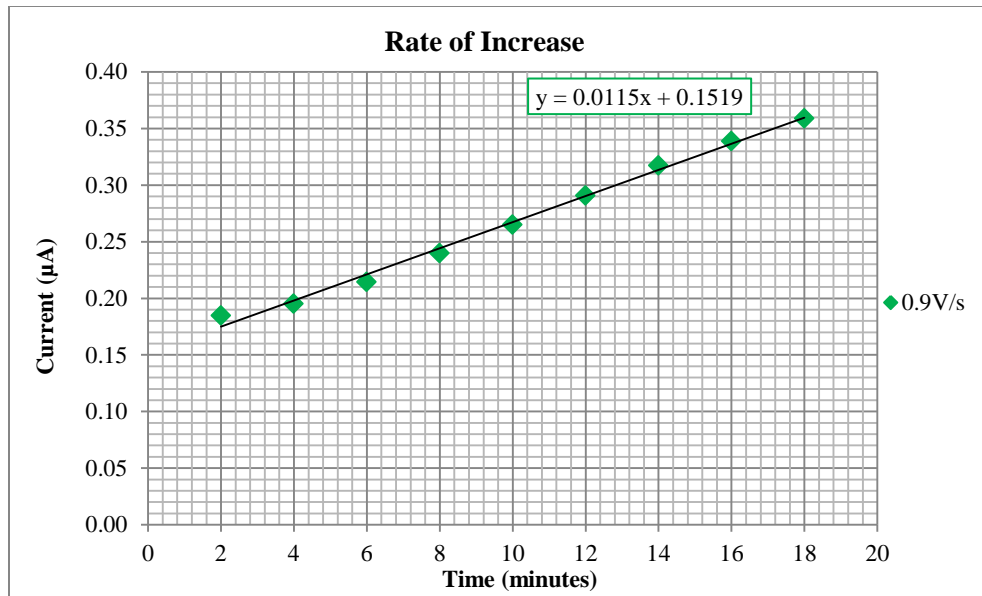


Figure 15 – Linear Rate of Increase of CV Curves at a fixed Potential (V)

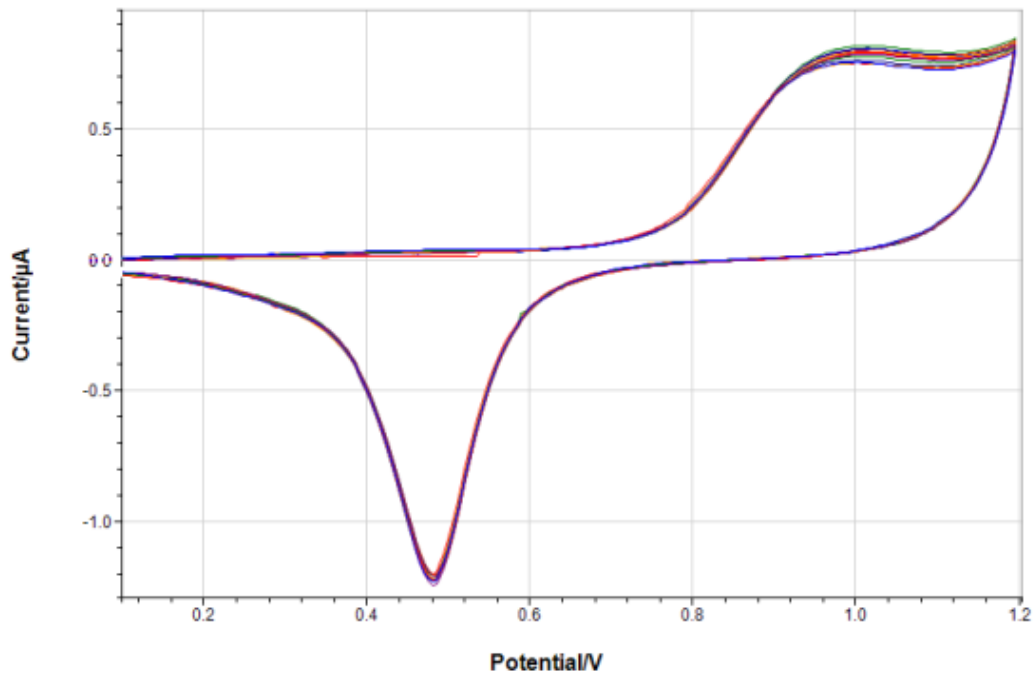


Figure 16 – CV of Untreated Cell Culture Used as a Control

CV measurements were taken every 2 minutes for the 60 minute period. No significant change can be detected

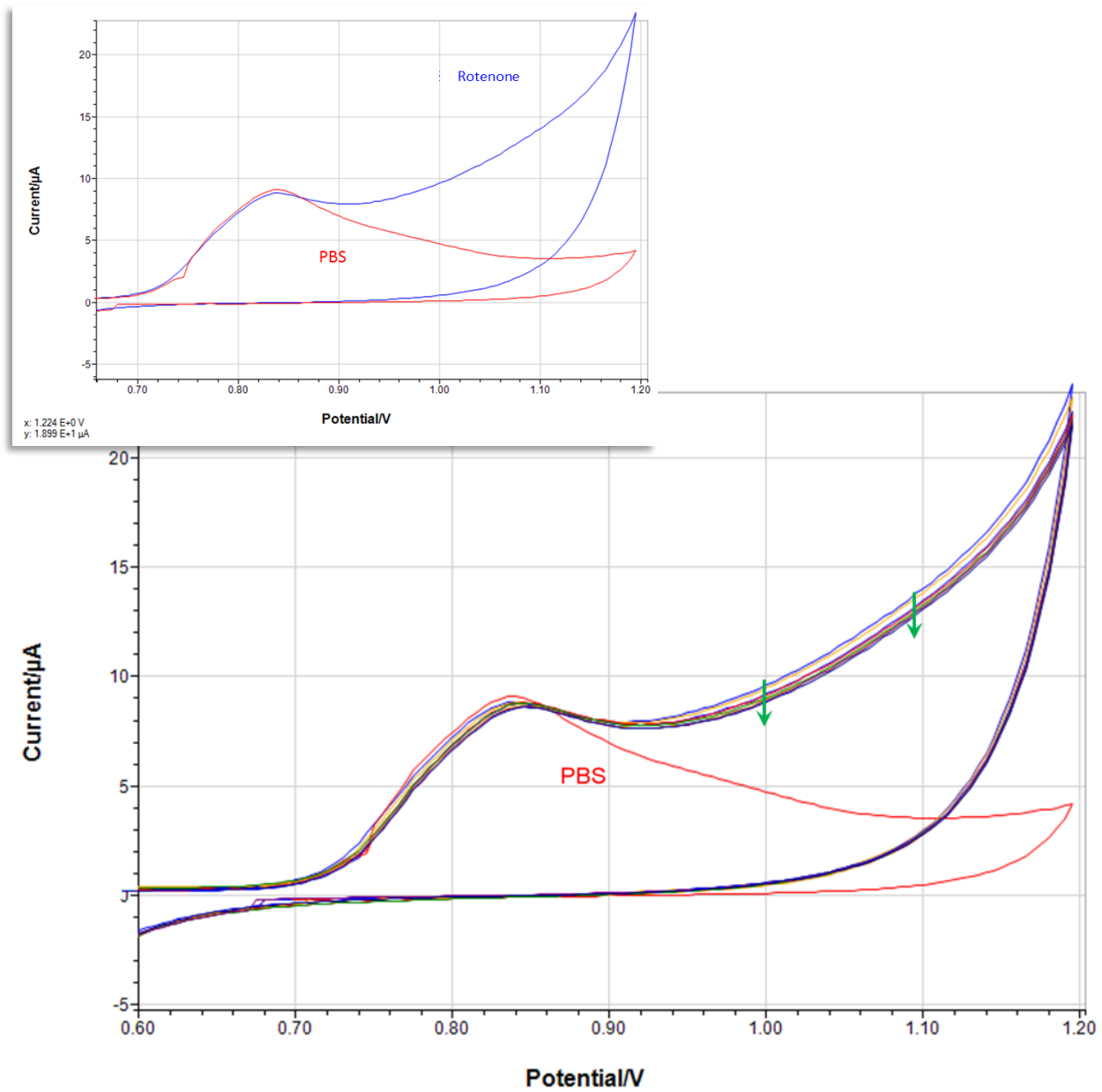


Figure 17 – CV showing decrease in curves of RT with time.

The curves continued to decrease for the full 20 minutes of measurement, possibly due to chemical modification of RT in the electrical environment. (No Cells Present in media)

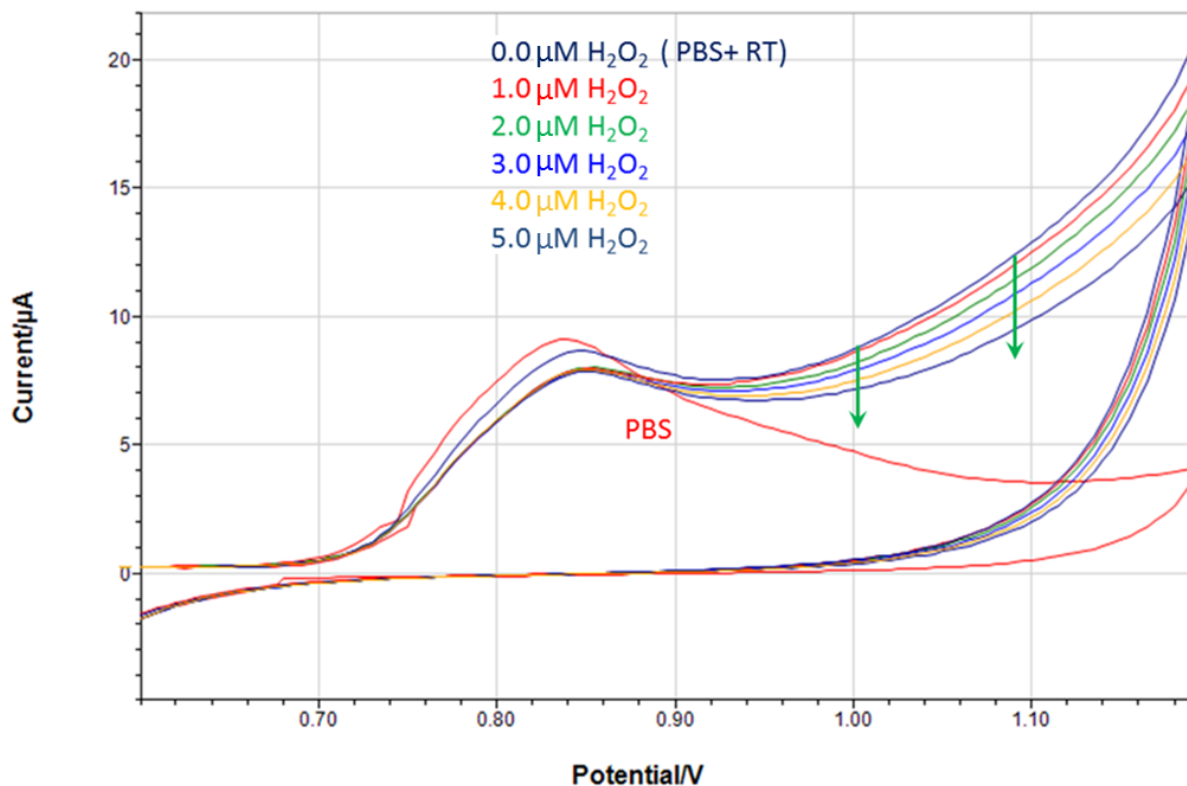


Figure 18 – CV Calibration Curve of H₂O₂

A number of well sizes were considered before selecting a 12-well plate in which to culture the N27 cells. Ideally, a large plate would hold many cells in a low volume of growth media, thus producing a higher H₂O₂ concentration. However, a limitation of the sensor is that, for a detectable change in current to be measured, the sensor must be submerged completely in the analyte for enough surface area to be in contact. Therefore, a well that was sufficiently deep but at the same time wide enough to hold many cells in a low volume was necessary.

AR Determination of Extracellular H₂O₂ in N27 Cell Culture

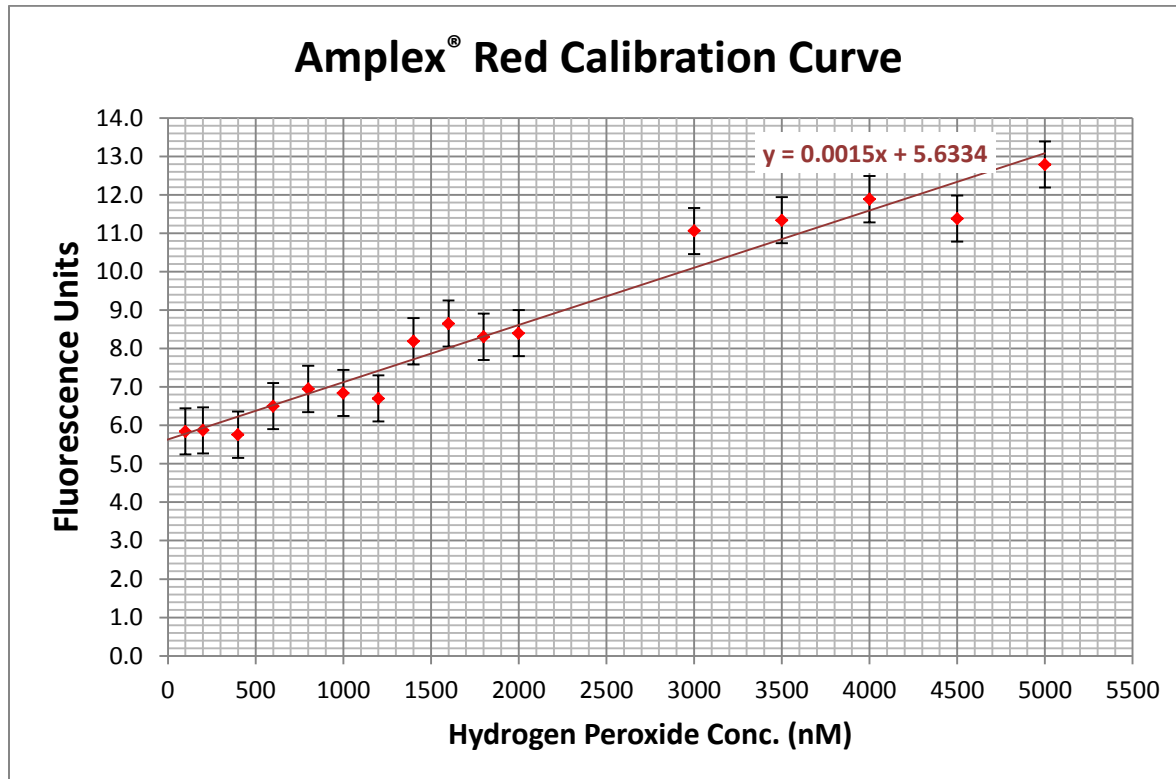


Figure 19 – Hydrogen Peroxide Calibration Curve using Amplex Red

H₂O₂ concentrations increasing every 200nM up to 2 μ M then increasing by 500nM until 5 μ M, Standard Error bars shown.

An AR calibration curve was made using triplicate results in one 96-well microplate. The fluorescence values were averaged and plotted. It must be noted however, that the first column of the microplate reader consistently gave systematic errors of the fluorescence. The value was always extremely high even when a negative control was placed in the first column. Therefore, data for 2.5 μ M of H₂O₂ is not included in Figure 19. When detecting H₂O₂ released from the cells using the AR method duplicate data was obtained. A control that was not treated with RT was also measured. As Figure 20 shows the control had a relatively constant hydrogen peroxide concentration of 500nM. This level can be expected because cells naturally produce ROS for physiological functions and as a by-product of metabolism. Furthermore, the levels are expected because the cells were placed in PBS with no FBS, in contrast to their more native RPMI 1640.

Consideration should be given to the fact that the experiment was only conducted once with a duplicate sample. It is also assumed that the cells cultures responded identically in their separate wells as RT was added and measurements taken. In Figure 20, the levels of H₂O₂ increase during the first 30 minutes and reach a maximum at just under 2.0 μ M.

The concentration of RT to use was determined by relating previous literature²² with the rate of H₂O₂ production that had been previously documented in our lab. Therefore, the measured concentration of H₂O₂ at 10 minutes was just as expected. However, after the 30-minute mark when more RT was added the H₂O₂ concentration that was detected using AR decreased. A possible reason for this decrease is that the N27 cells began to die due to the lengthy exposure and harsh conditions – no longer producing H₂O₂. However, the morphology of the cells was not

observed and a cell viability assay was not performed, therefore, there is insufficient evidence to conclusively support the statement.

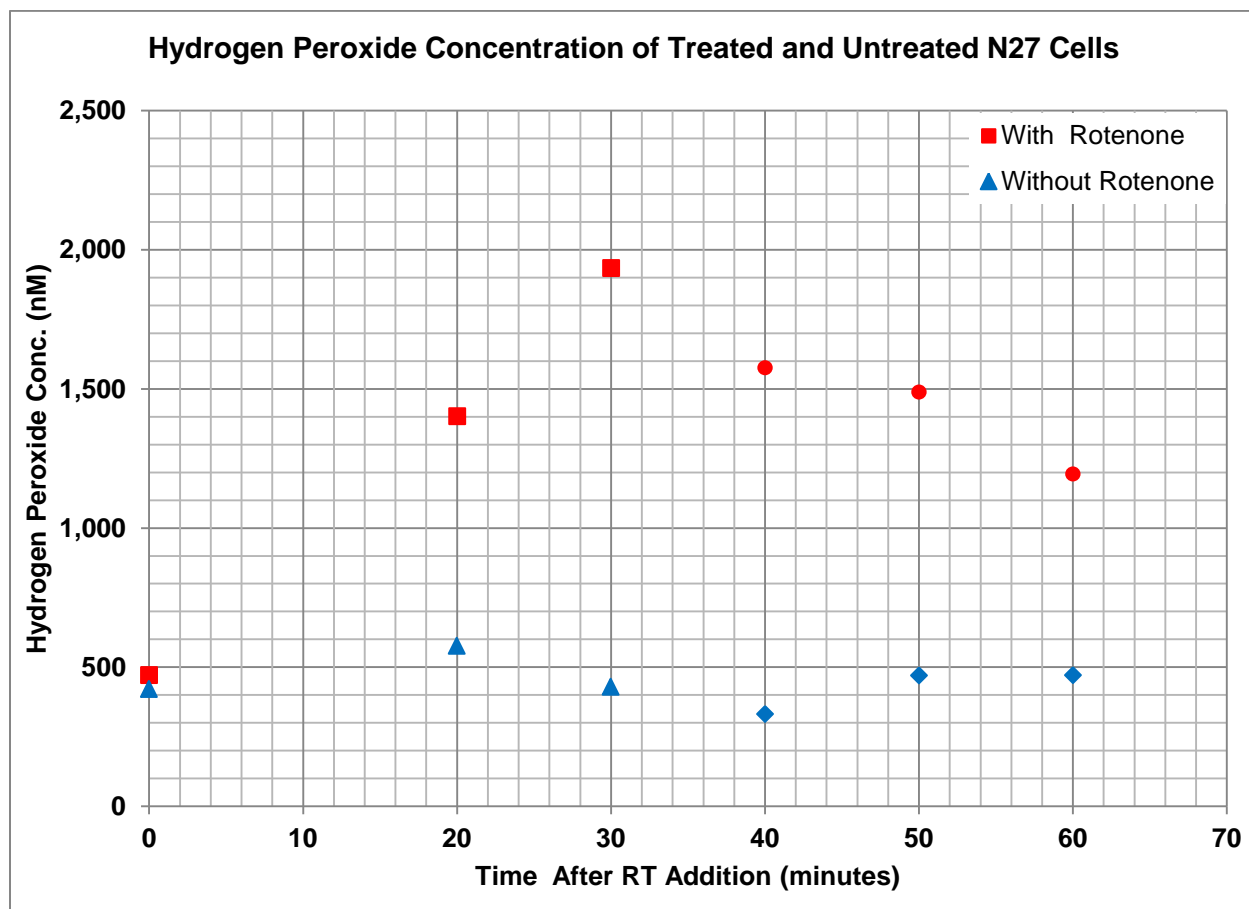


Figure 20 – H₂O₂ Detection using AR in Cell Cultures with and without RT

The different symbols after the 30-minute mark correspond to the second well from which AR measurements were taken. Conditions were kept constant and cells were from the same culture, therefore, it is assumed that the cells behave nearly identically.

As with any experiment, a larger sample size will reduce error and lead towards a true average. In the AR experiment not more than 2 independent trials were done therefore, as recommended in the Journal of Cell Biology²³, no error bars with Standard Error or confidence intervals are shown.

In the future, a larger number of wells would have to be measured to determine H₂O₂ concentrations definitively. The AR method is used as a benchmark in laboratories because it has few disadvantages and limitations. One limitation is that it cannot be used in the presence of DDT and thiols. Furthermore, excessively high concentrations are not fluorescent because the resorufin gets oxidized to resazurin.¹⁴ In comparison to the CV method of detection one of the major drawbacks is that AR needs incubation. The manufacturer recommends 30 minutes of incubation as mentioned in their protocol, but a product review found that 5 minutes of incubation was sufficient for detection.²⁴

Using the electrode, we were able to measure changes in the fluidic environment in near real-time. The setup can be modified with a faster scan rate, and less cycles to make reliable measurements nearly every 30 seconds. Further experimentation must be done to definitively identify what caused the increase in the curves shown in Figure 14 for example.

Oxygen will affect CV measurement and it is recommended that future tests are done in media that has had nitrogen bubbled through to remove any dissolved oxygen. In our experiment, our goal was to mimic biological environments and therefore oxygen was not removed. In addition, it would be very beneficial to use the enzyme catalase to rapidly decompose any hydrogen peroxide in the media and use CV to detect any changes. The use of catalase can be used as a negative control.

The experimental procedure was conducted at room temperature. The hydrogen peroxide was freshly made and stored in the dark and in a cool environment when not used. Nevertheless, it would be best if future experiments are conducted in a cooler environment to prevent decomposition and prolong H₂O₂ detection from cells.

Rotenone is not the most favorable chemical to use because as we have shown it is electro-active. Furthermore, studies have shown that RT is not specific to DA cells because it is lipophilic and can be difficult to replicate in animal models.²⁵ In future experiments the cells could be treated with RT for 5 minutes, the media should be replaced and then CV measurements made. This should provide more conclusive data since there should not be any chemical interference.

The cyclic voltammetry method gave variable results depending on the media and composition of electrode. The pH and chemicals in the media such as phenol red affect the position of the redox potential (V) of the analyte. The CV setup that was used occasionally gave different current (μA) values even though the electrode and other variables were kept constant. The operator is extremely important. Many experimenters do not understand the principles involved in CV, leading to unreliable data.¹⁸ Preliminary electrodes used were not very reliable and a number were broken by the operator. Therefore, new quality-tested electrodes were fabricated and maintained by the collaborating lab. An advantage of the CV method is that the cell culture or measurement environment is thought to be unaltered and undamaged. The media volume is left unchanged and no additives are necessary for measurement.

Conclusions

In regard to the specific aims:

The electrochemical sensor was able to function in RPMI 1640 growth media. An inverse relationship between hydrogen peroxide concentration and peak current was identified.

Furthermore, the dialysis membrane electrode was able to function effectively in protein containing media, with almost no sensitivity reduction compared to time in 8% FBS. However, the dialysis membrane electrode was not sensitive enough to detect hydrogen peroxide concentrations below 10 μ M.

The most significant drawback of the electrochemical sensor setup is that it is not specific to ROS, yet alone hydrogen peroxide. A variety of substances that have high and broad reduction potentials (V) generate an electric current (μ A) and interfere with measurement. In our case, the hydrogen peroxide peak could not be differentiated from that of the electro-active Rotenone because the peaks were overlapping.

For electrochemical detection in a cell culture, the more sensitive plain gold electrode was used. From the data obtained using the cyclic voltammetry setup, no conclusive statement can be made regarding the concentration of H₂O₂ released from cells. On the other hand, it is possible that the indirect detection of RT uptake by cells was measured and caused the increase in curves observed in Figure 11 and Figure 14.

Nevertheless, by using the Amplex[®] Red detection method and creating a calibration curve of known hydrogen peroxide concentrations we can report from our data, with considerations from the discussion in mind, that slightly less than 2.0 μ M of H₂O₂ was released from RT treated N27 dopaminergic cells in a period of 30 minutes.

The electrochemical sensor is sensitive, and has the unique ability for near real-time measurement in a dynamic setting. Nonetheless, further development must be done and the possible incorporation of an enzyme/catalyst on the surface of the working electrode⁹ could increase its specificity, thereby enhancing its potential use in a variety of environments.

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