ME550: Advances in BioSensors
Lab1: Fabrication and Sensing of Glucose Oxidase Biosensor
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1. Abstract

Glucose Oxidase Biosensors are important sensors used excessively in the medical industry for the clinical detection of Diabetes. Worldwide, Diabetes is one of the rapidly increasing Chronic and life-threatening diseases. For a diabetes patient, blood glucose level needs to be monitored at regular intervals so it can be controlled under a certain limit. So that negative impact can be minimized. Recently, some research has been done on implementing nanotechnology to improve the surface area of the sensor, improve the properties of the catalyst, and produce small-scale compact nanoscale sensors. [11] In this lab experiment, a carbon and platinum nanoparticle-based electrode glucose oxidase biosensor was developed to test glucose concentration in an unknown mystery solution. This type of sensor provides accurate results for the micromolar changes in glucose levels. In this experiment, Glucose oxidase enzymes were immobilized with cross-linking method on a preprinted electrode. The figure of merits, such as sensitivity, linear range, and response time, was determined for lab-developed biosensors. In this experiment, the discrete plateau for each addition of glucose in the solution was found. From the experiment result, Response Current vs Concentration linear curve was plotted. Later, experimental data were used to plot the Lineweaver-Burk plot as well as the Eadie-Hofstee plot to determine different kinetics variables. From the calibration curve concentration of unknown sample were determined. From both plots, different kinetic constant values of enzyme and performance data of the biosensor were evaluated and compared to data from the "BRENDA" database. The reason for deviation from the 'Brenda Database' has also been discussed. Later, another unknown concentration of glucose solution was tested with a commercial digital and lab-developed sensor. Data from the digital sensor was compared with lab developed sensor. This lab provides the opportunity to understand the working principle, functionality, types, and characteristics of biosensors and the kinetics of enzymes in a more detailed fashion.

2. Introduction

Diabetes is a common and rapidly growing disease affecting many people regardless of age (children to adults). It is a chronic and metabolic disease caused due to rise in sugar levels in the blood. The main reason for this increase in glucose is the low generation of insulin hormone in the body. Blood glucose level needs to be monitored and controlled at a patient-specific level based on their risk for future health complications due to the disease. Glucose Biosensors are extensively used to detect and manage diabetes, both Type I and Type II. The main motivation for this laboratory comes from the high demand and usage of glucose biosensors in the food and medical industries. According to statistics from the Centers for disease control and prevention (CDC), 37.3 million people have diabetes in the USA, accounting for 11.3% of the total US population. Around 85% of the biosensor market is populated by glucose biosensors [1,2]. The electrochemical detection method is one method that is highly used for glucose sensing. Glucose sensors are widely used to detect blood glucose concentration in the medical industry. The number of diabetic diagnoses is rapidly increasing worldwide. Early detection is necessary to analyze and manage glucose levels in the body. Most blood glucose sensors are categorized as Amperometric sensors, Optical

transduction sensors such as absorption sensors, light scattering, Raman spectroscopy, and implantable glucose sensors [2].

There are three general strategies for electrochemical sensing with a glucose biosensor. The first strategy is measuring oxygen consumption, the second is measuring the amount of hydrogen peroxide produced by the enzyme reaction, and the third is using a diffusible or immobilized mediator to transfer the electrons from GOx to the electrode [3]. Research is also carried out to produce cost-effective, non-enzymatic wearable glucose sensors such as smart patches, etc. Along with research in different strategies of using biosensors to determine glucose concentrations, there is also headway on using these strategies to determine glucose concentration in the body from different fluid mediums. An article from the Institute of Physical Chemistry in 2016 compiled research in this area to describe improvements in glucose biosensing by finding less invasive mediums to sense than blood. Teams are currently working on glucose biosensing using urine, saliva, sweat, tears, and breath. All of these mediums have a much lower concentration of glucose than blood, which provides a challenge for the research and technological advancements [6].

The central hypothesis of this laboratory is that if an electrochemical glucose biosensor is used to create a calibration curve based on response current generated with the variation of glucose concentrations, a Lineweaver Burke plot and Eadie-Hofstee plot can both be utilized to help identify the glucose concentration of an unknown solution. In this experiment, a detailed study of a glucose biosensor's response spectrum at different glucose concentration levels was conducted. This type of sensor effectively calculates the glucose percentage in different solution concentrations.

Initially, a biosensor is developed with the adsorption of glucose oxidase and glutaraldehyde on the sensor's microfluidic detection surface. The carbon with a platinum nanoparticle-based working electrode along with graphite counter electrode and AgCl reference electrode is used. All three electrodes are placed inside a small beaker with phosphate buffer saline solution.

Glucose Oxidase(GOx) is a popular, cheap, and stable enzyme used for glucose sensing. In the presence of glucose oxidase (GOx), glucose reacts to oxygen and produces hydrogen peroxide, which acts as a target molecule, while at the same time, GOx transform glucose to d-glucono-1,5-lactone. Increases in hydrogen peroxide concentration are directly linked to the addition of glucose levels in the solution. Due to capillary action, the hydrogen peroxide reached the working electrode through microfluidics and reacted with the electrode to release oxygen and electrons. These electrons are responsible for current flow. With the addition of more glucose, more electrons are generated, and the current flow increases. Hence, the increase in current flow is linked with glucose concentration in the solution. [11]

The main objective of this experiment is to showcase the difference in current response due to different concentrations in glucose levels in solution, to understand the working principle of biosensors, s, to evaluate different figures of merits (FoM) (such as sensitivity, the limit of detection, response time and linear range) of developed biosensor, and to evaluate different kinetics parameters of the enzyme (glucose oxidase). A mystery solution was also tested with a commercial and lab-developed biosensor.

From the experimental data, sensitivity, limit of detection, response time, and the linear dynamic range of bio-sensor are analyzed and reported in the discussion of this report. From the response vs concentration plot, the slope of the curve is directly related to the sensitivity of the sensor. From the literature [4], the sensitivity of a sensor is defined as a change in response current due to a unit change in the concentration of the analyte.

$$Sensitivity(SEN) = \frac{\Delta R}{\Delta a} \quad (1)$$

Limit of detection(LOD) is a measurement of limit up to which a reliable and quantifiable response can be detected from the sensor. It is defined in the literature [2] as

$$Limit\ of\ Detection(LOD) = \frac{3\ x\ Standard\ Deviation}{SEN} \quad (2)$$

Sensor dependency on a linear range of concentration is defined as Dynamic Range [4].
Response time is defined as the time to reach 95% of a stable signal [4].

Kinetic Theory

Kinetic factors of an element may be used to obtain values from the Lineweaver-Burk plot and Michaelis-Menton descriptors, as well as a few other methods, which can be used to help evaluate the data found in the lab. The Michaelis-Menton equation is listed in equation 3 below, where v is the velocity of the reaction, V_{max} is the maximum velocity of the enzyme found at the saturation point of the system, $[S]$ is the substrate concentration, and K_m is the Michaelis constant.[4]

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (3)$$

V_{max} and K_m can be found by rearranging the Michaelis-Menten equation. One such way to do so is described in equation 4 below, known as the Lineweaver-Burk form. This is set up in the fashion of a typical Another way to go about this could be by using the Eadie-Hofstee plot, as illustrated in equation 5 below. In the Lineweaver-Burk plot, the slope can be found by using the rise over run method, as it is a linear plot, whereas the slope in the Eadie-Hofstee plot is found to be K_m.[4,5]

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}\frac{1}{[S]} \quad (4)$$

$$V = V_{max} - K_m\frac{V}{[S]} \quad (5)$$

At the maximal rate of the reaction, the rate constant is found to be k_{cat}. This variable is a representation of the limiting rate of the reaction and is found in units of 1/sec. The catalytic rate variable can be used to identify the specificity constant. This can be found by dividing the catalytic rate, k_{cat}, by the Michaelis constant, K_m. This value measures how efficiently the enzyme can convert the substrate into a product. These values are important when determining the best method of collecting data, and how accurate specific methods are in this process.

3. Methods and Materials

System Functionalization

To initially prepare for this test, first place a working electrode in a petri dish and put 5 microliters of 6% glucose oxidase and 5 microliters of 0.25% glutaraldehyde into an empty area of the petri dish. Inject the 0.25% glutaraldehyde into the glucose oxidase. Mix the solution by keeping the tip of the Eppendorf pipette submerged in the droplet and retracting and ejecting into the droplet around 10 times being careful not to add air bubbles. Once mixed, 8 microliters of this solution can be ejected onto the working electrode without touching the tip of the pipette to the electrode. Ensure the solution covers the entire space on the working electrode. Label electrode petri dish and set in the fridge for 48 hour time period.

Sensor Calibration

Using a 10mL beaker, add 5mL phosphate buffer saline (pH 7.4), a platinum counter electrode, and an Ag/AgCl reference electrode. Set up on a magnetic stir plate/hot plate. Make electric connections to the electrodes and lower below the surface of the phosphate buffer saline. Ensure the buffers are submerged but that the clips are not. Turn on the stir rate to 120 rpm. Make sure the stir bar stays in the middle of the beaker. Use the CHI program. Select input potential of +0.6 V, sample interval of 0.5 seconds, quiet time of 20 seconds, and sensitivity of e-6. Click play to begin.

After around 500 seconds, begin glucose additions according to Table 1 below. Observe a response current and add glucose according to the table or once the signal has linearized. Once the solution is saturated, end the test and save the data. Evaluate.

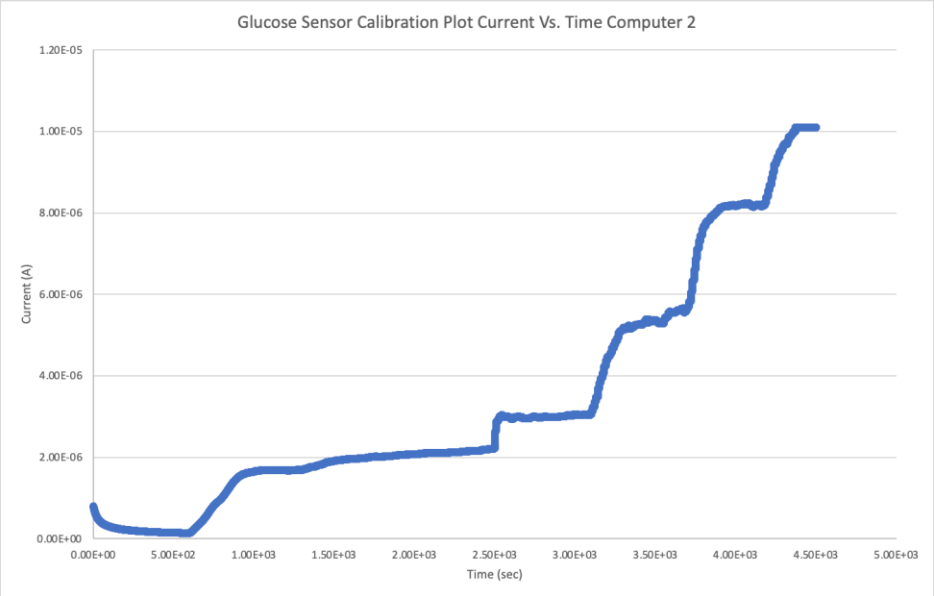
Table 1: Time, Concentration, Volume, and Current Response of Added Glucose

Time (sec)	Concentration Total (uM)	Concentration Added (uM)	Volume Added (uL)	Current Response (uA)
0	0	0	0	0.146
500	50	50	0.5	1.68
1200	100	50	0.5	2.10
2500	500	400	4	2.99
3000	1000	500	5	5.45
3600	1500	500	5	8.21
4000	2000	500	5	10.1

4. Results

Glucose was added to the solution at the time intervals listed in Table 1. Data was collected in intervals of 0.5 seconds and recorded in an excel file. Figure 1 shows the current response to the glucose additions with respect to time.

Figure 1: Glucose Sensor Calibration Plot - Current(A) vs. Time @ Computer 2



Mystery Solution

A commercially available glucose sensor was used to detect the glucose concentration of Solution C. It was found to be 312 mg/dl. The same solution was tested using the lab-developed glucose sensor and phosphate buffer saline.. Figure 2 shows the current response in uA with respect to the time in seconds after the addition of Mystery Solution C to the phosphate buffer solution.

Figure 2: Mystery Solution C - Current(A) vs. Time

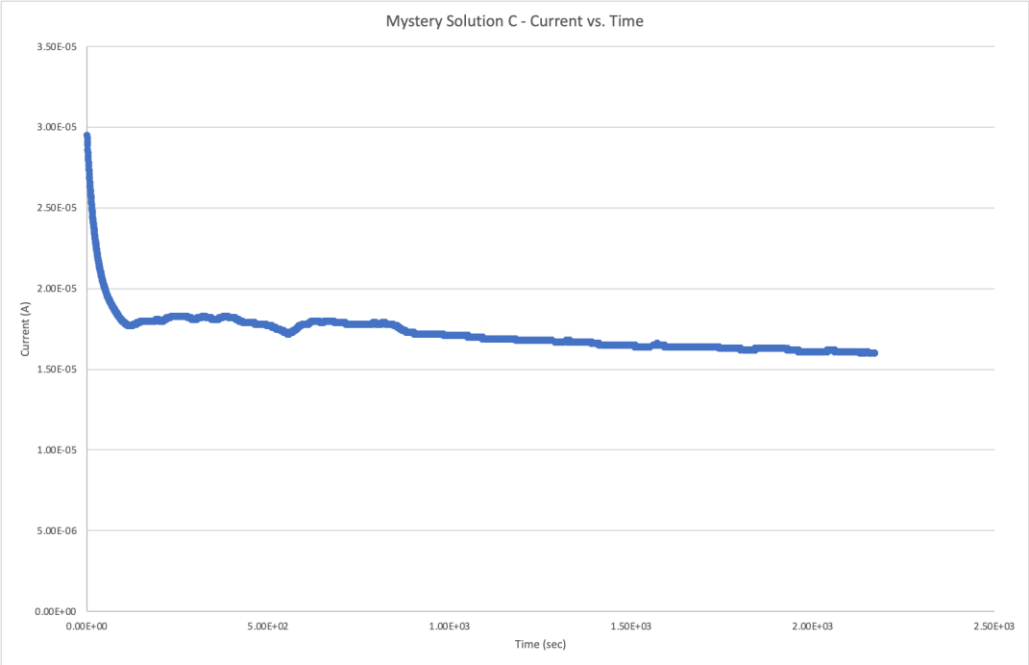


Figure 3: Current Response vs Total Concentration

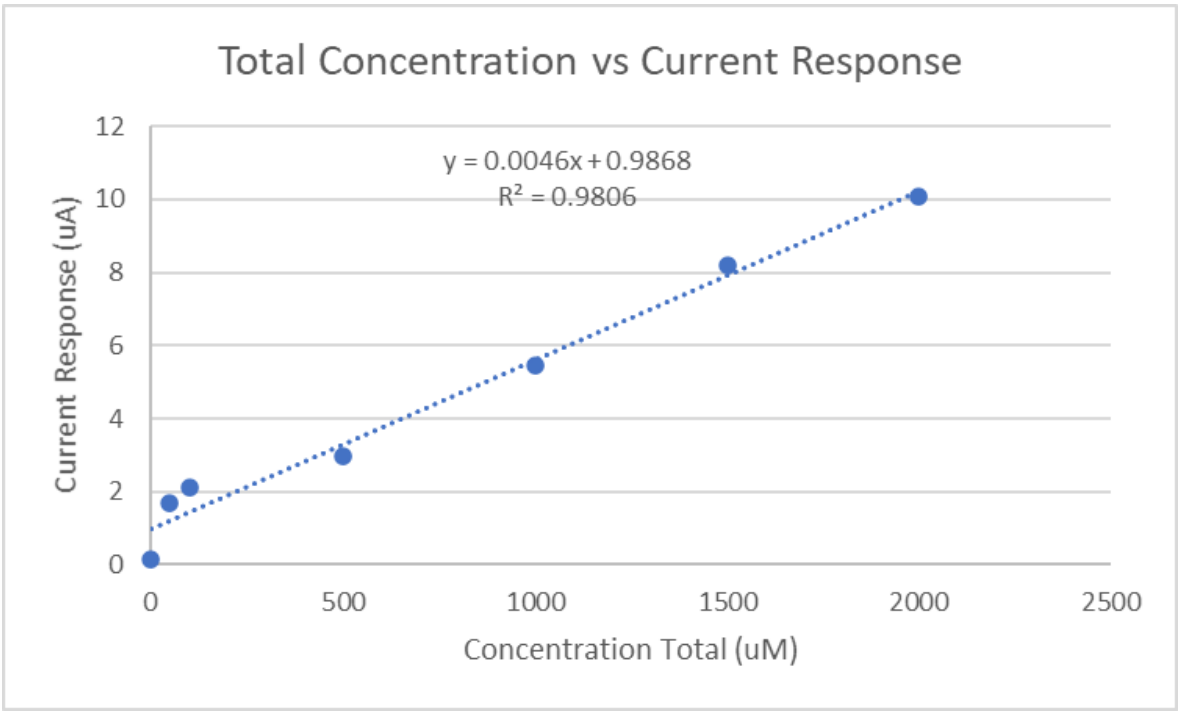


Figure 4: Lineweaver-Burk Plot

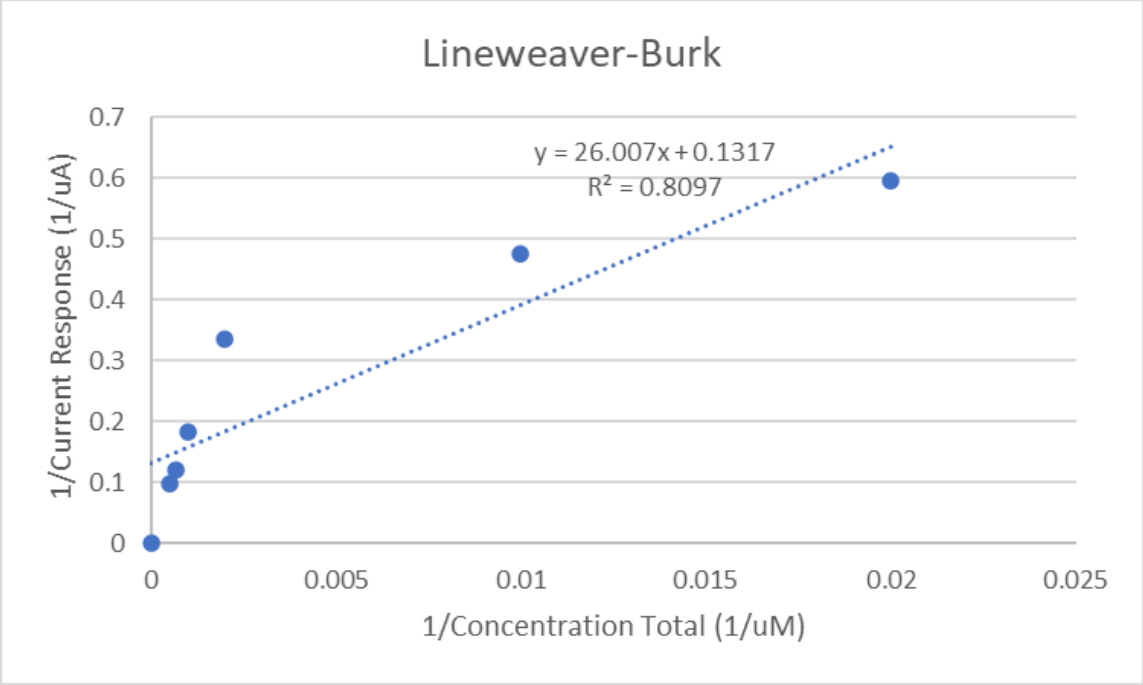
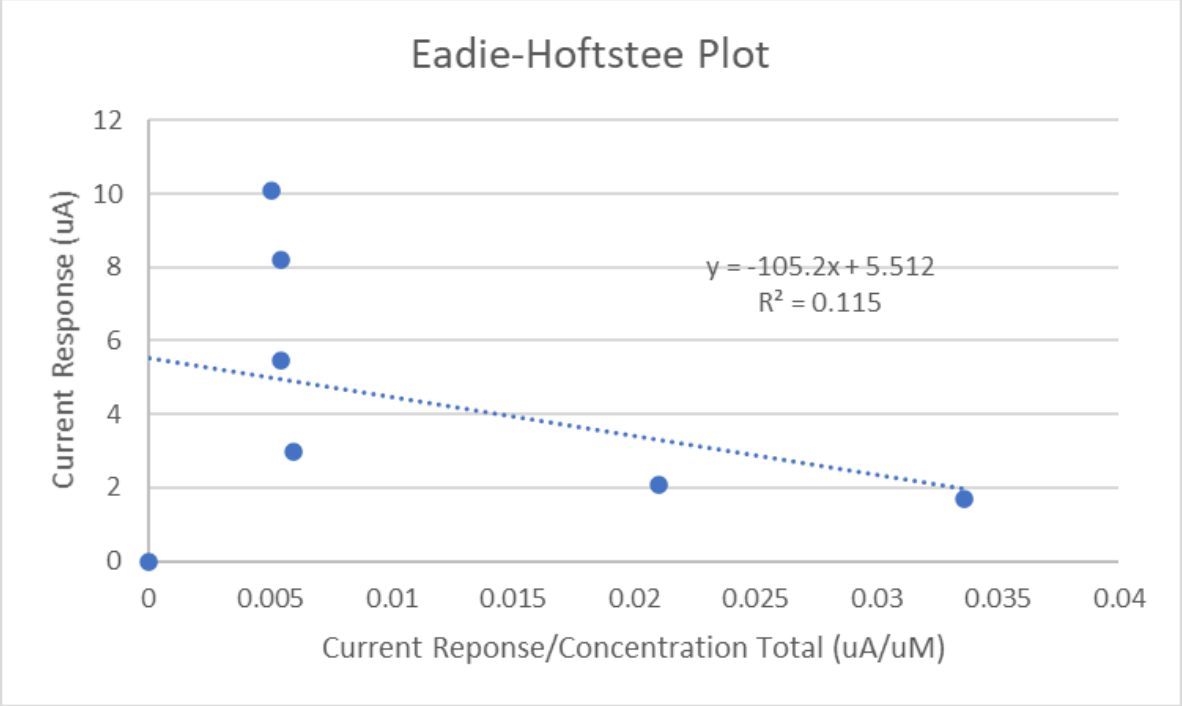


Figure 5: Eadie-Hoftstee Plot



During the initial experiment when the glucose concentration in the solution reached 2000 uM, the response current became steady state. Based on the data collected from Table 1, the Current Response vs. Total Concentration curve was plotted. The slope of the curve is depicted as an orange dotted line shown in Figure 4, which is equivalent to the sensitivity of the sensor. This relationship is represented in equation 1 where *R* is the current response and *a* is the total concentration.

The slope of the plot was found to be 0.0046 uA/uM. The R-square regression value of the plot was 0.9806. Therefore, the sensitivity was 0.0046 uA/uM and the standard deviation of the response current was found through excel to be 3.67 uA. The limit of detection came out to be 2393 uM.

The linear dynamic range of the sensor was from glucose concentrations 50 uM to 2000 uM with current variation from 1.68 uA to 10.1 uA. The response time for the sensor after the addition of the first 50 uM of glucose was 629 sec.

Using the Lineweaver-Burk plot, Eadic-Hofstee plot, and BRENDA, results for K_m, V_{max}, k_{cat}, and $\frac{k_{cat}}{K_m}$ were found. These are shown below in Table 2 and compared to the known value ranges found in the online database "BRENDA".

Table 2: Evaluated Enzyme Kinetics Data Using Different Methods

Different Kinetic Properties of Glucose Oxidase	**Lineweaver-Burk**	**Eadie-Hofstee**	**BRENDA**
K_m	197.47	105.2	0.019-733[mM]
V_{max}	7.593	5.512	0.0098-5.30 [umol/s]
k_{cat}	1.52	1.1024	0.59-318.2[1/s]
$\frac{k_{cat}}{K_m}$	0.0077	0.0104	0.004-323[1/mMs^-1]

Table 3 shows the concentrations of glucose in solution C as detected in the commercially available glucose sensor as well as the sensor developed in the lab. The concentration was determined by the commercial sensor to be 312 mg/dl. This can be converted to 17.540 mM. Using the three electrode biosensor with 5uL of sample C, a current of 17.8 uA was found. This was plugged into the equation found in Figure 3 to get a concentration of 3.655 mM.

Table 3: Mystery Glucose Concentration in Commercial Sensor vs Bio-Sensor

Mystery Solution Label	Concentration as per digital Commercial Sensor (mM)	Concentration as per Developed Bio-sensor (mM)
Solution C	17.540	3.655

5. Discussion

The known solution current response vs. time data was used to evaluate the different performance parameters(FoM) of the lab-developed bio-sensor (Mentioned in Table 4) and the kinetics property of the enzyme(Mentioned in Table 2). The Glucose was detected ampereometrically at a constant potential of +0.6V and pH 7.4 PBS buffer. The experimented data in table 4 is compared with the Table 5 data of different biosensors(based on GOx enzyme and direct oxidation sensing method). The electrochemical sensing method is extensively used for unknown glucose detection. This is a well-established method used across the industry. Glucose sensors based on direct, nonenzymatic, electro-oxidation, and electro-reduction of Glucose are also very popular [24]. The unknown concentration of Glucose was detected by lab developed biosensor from the calibration curve. The same solution is also measured with a commercial digital sensor. Different glucose concentration values are found for the unknown solution C. One possible reason for this difference would be the different calibration scales of both sensors. The commercial sensor may be developed based on blood glucose concentration level, but higher glucose concentration is used in this experiment. Other than this, during the initial setup and preparation of the sensor, air may be entrapped as a bubble in the solution droplet. Hence, a homogeneous membrane can not be formed on the sensor surface. This entrapped air may be one reason for unexpected variation in response to the current data of solution C. If any of the electrodes are moved or touched during the preparation procedure, it may lead to unexpected results. Moreover, some enzymes may have spilled from the sensor surface, which could decrease the signal. As different people work on the sensor at different steps of sensor preparation, some human mistakes may occurred during the experiment. These incidents are probable explanations for the unexpected variation in concentration measured during the data collection from lab-developed biosensors.

The results in Table 4 can be compared to those found in other research articles. While the sensitivity found in this study was 0.0046 uA/uM, the sensitivity using a tapered optical fiber had a sensitivity of 1.06 nm/mM, while a graphene-glucose oxidase biocomposite was found to have a sensitivity of 1.85 μA/mM* cm^2. While this study had a linear dynamic glucose concentration range of 50 uM to 2000 uM, the optical fiber sensor had a linear range of 0 mM - 11 mM, and the graphene-glucose oxidase biocomposite was found to have a very wide linear range of 0.1-27 mM [8][9]. In Table 5, the FoM of different standard electrochemical glucose sensors is listed based on their application[11]. For GOx enzyme-based glucose sensor with Platinum Nanoparticles has a linear dynamic range of 0.1–10 mM.[16]
Where as Au nanoparticle-based glucose sensor with GOx enzyme has a linear dynamic range of 6 μM–5 mM, response time <6 s, and detection limit 3 μM at pH 7 PBS buffer solution[12].

Table 4: Figure of Merits of Glucose Biosensor

Figure of Merit	Corresponding Value
Sensitivity	0.0046 uA/uM
Limit of Detection (LOD)	2393 uM
Standard Deviation	3.67 uA
R-square regression value	0.9806
Linear Dynamic Range	Glucose Concentrations 50 uM to 2000 uM with Current Variation from 1.68 uA to 10.1 uA
Response Time	629 sec

Table 5: Different electrochemical glucose sensor used for various biological samples [11]

Detection	Nano-Component	Response time	Linear Range	Detection Limit	Sample Type	Sample Treatment	Ref
GOx	CNT, Au NP	<6s	6 μM–5 mM	3 μM	Plasma	Diluted (1:4) PBS, pH 7	[12]
GOx	CNT, Ag NP	<10 s	0.5–50 μM	0.1 μM	Serum	Diluted (1:500) 0.1 M BRB, pH 6	[13]
GOx	CNT, AuPt NP	3s	0.01–9.49 mM	0.01 mM	Spiked Serum	Diluted (1:6.25) PBS, pH 7	[14]
GOx	Polyaniline grafted CNTs	~6 s	1–10 mM	0.1 μM	Spiked Serum	Diluted (UNK) PBS, pH 7	[15]
GOx	Pt NP	ND	0.1–10 mM	ND	Cerebrospi-nal Fluid	Untreated	[16]
GOx	Pd NP, PEDOT Nanofibers	ND	0.5–30 mM	75 μM	Serum	Diluted (1:1) PBS, pH 7	[17]
GOx	Fe3O4 NP	ND	6 μM–2.2 mM	6 μM	Serum	Diluted (1:10) PBS, pH 6.8	[18]
GOx	Fe3O4 NP	10 s	0.5–80 μM	0.1 μM	Spiked Blood	Diluted (1:250) PBS, pH 6.5	[19]
Direct Oxidation	CNT (coated with NiO)	ND	0.2–12 mM	0.16 mM	Serum	Diluted (1:200) 0.1 M NaOH	[20]
Direct	CuO/CuOX	<1 s	2 μM–15	0.05 μM	Serum	Diluted (1:50)	[21]

Oxidation	NS		mM			0.1 M, NaOH	
Direct Oxidation	CNT, Pd NP	3 s	0.5–17 mM	0.2 μM	Blood	Diluted (UNK) PBS, pH 7.4	[22]
FET & ConA	CNT	>1 min	1 pM–1 nM	1 pM	Spiked Plasma	Untreated	[23]

In Table 2, different enzyme kinetics data are reported. This data has been found from the Lineweaver-Burk plot, the Eadic-Hofstee plot. Later this data is compared with the 'Brenda' database. The values K_m, k_{cat}, and $\frac{k_{cat}}{K_m}$ fall within the range of the *Brenda* database. But the value of V_{max} is slightly above the range listed on *Brenda*. It may be due to different pH levels of buffer solution between the lab setup(pH 7.4) used and those used to detect the values used on *Brenda*. The enzyme performance is highly dependent on temperature. The enzyme exhibited optimum catalytic activity at pH 5.5 and the temperature optimum for glucose oxidase, catalyzed D-glucose oxidation was 40°C.[25] The temperature at which the lab experiment was performed is not the standard one. Another reason could be that the data on *Brenda* is collected in standard and clean operating conditions. During lab experiments, the solution or biosensor may unintentionally be touched by humans or foreign particles. Another reason could be that air was entrapped in glucose oxidase droplets, which affected the reaction mechanism. These reasons and more could have contributed to the disparity of the value of V_{max}.

The evaluated enzyme kinetics data from the Lineweaver-Burk plot, and the Eadic-Hofstee plot have some different values. This is due to the point distribution of the data on both plots. In the Lineweaver-Burk plot, The data are compressed, and crowding is more at one end, but in the Eadic-Hofstee plot, the data is evenly distributed. From literature 1, It is also found that the accuracy of the Medic-Hofstee plot is higher compared to the Lineweaver-Burk plot. The data found from the Eadic-Hofstee plot are more accurate.In this experiment, due to the unavailability of the actual rate of reaction, the current response is considered proportional to the rate of reaction. The values found in the plot can be considered an apparent value.

The mismatch in the data may be due to developmental errors. To improve the biosensor's functionality, some points need to be considered. During preparation, It should be taken care that bubbles could not form at the sensor surface. Direct human skin touch with biosensors should be avoided. During the experiment, the beaker and the connecting electrodes should be properly cleaned with distilled water. While glucose insertion, Direct touch of the pipette with a working electrode should be avoided.

The typical glucose biosensor used in the health industry is in the range of 65-105 mg/dL from Appendix A. In the lab experiment, a higher glucose concentration is used. This could be one of the reasons for different values in commercial and lab-developed sensors. The sensor used in this experiment is amperometric. Other types of biosensors are also there like potentiometric, thermal, and optical other than Glucose, different biosensors are used in the healthcare industry to detect oxygen, pH, Sodium ion, Urea, Potassium ion, etc. Different types of biosensor have been listed in Appendix A.

6. Conclusion

This lab was important in understanding the process of measuring glucose concentration and current response with a working electrode, platinum counter electrode, and reference electrode connected to a glucose oxidase biosensor. The mechanics and chemical reactions of the biosensor were experienced first-hand for a better understanding of the theory. A comparison between the sensitivity of a commercially available biosensor and the 3 electrode lab biosensor was made. This helped to give a better comprehension of the disparity between lab biosensors and commercially available technology.

During the data collection, there were several unanticipated results in the current response. Unaccounted-for variation in current could be the result of several human error factors in the procedure. It was found in the discussion of the report that by using different methods of data collection and evaluation, the data can be interpreted to be vastly different across the board. When interpreted using the Lineweaver-Burk plot, the R^2value was found to be 0.8097, while it was found to be 0.115 in the Eadie-Hofstee plot. When compared to the known ranges of data in the online database "BRENDA", both methods were within known limits, but still ranged quite a bit. It is understood that data evaluation should be chosen by the plot that best fits the collected data of the experiment. In this lab, the Lineweaver-Burk plot had a better fit, therefore most likely had more accurate results than the Eadie-Hofstee plot.

This laboratory adequately helped to explain the procedure to find the glucose concentration using an electrochemical glucose oxidase biosensor. It also helped to show how the Lineweaver-Burke plot and the Eadie-Hofstee plot can be used to evaluate the data and find the glucose concentration in unknown solutions. By referencing other articles, the class learned other methods and techniques to find the glucose concentration.

Futuristically, it would be interesting to investigate non-electrochemical glucose monitoring systems. Many scientists believe the future of glucose monitoring for diabetes mellitus could be in the form of fluorescent glucose detection. An article from the journal *Analyst* described fiber-optic biosensors that utilize glucose/galactose binding protein labeled with Badan. This was attached via a tag to an acid to the surface of polystyrene beads, and used to read glucose levels via fluorescence. The researchers hope this technology can be used to create a continuous glucose-monitoring system [7]. There is also some study going on smart weable based glucose sensor based on optical sensing. Non-invasive biosensors for indirect evaluation of routinely-measured blood components by sweat analysis have broad potential clinical applications. Research are carried out on the wrist-borne non-invasive glucose monitor (NIGM) device to measure blood glucose (BG) levels using photoplethysmographic (PPG) optical sensors.[26] Continuous glucose monitoring is an important advancement in diabetic treatment research, and could positively influence many lives.

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Appendix A.

1. Table 1-3, Introduction to Bioanalytical Sensor, Alice J. Cunningham, 1998

TABLE 1-3 Typical Biosensors Used in Healthcare

Analyte	Normal Range (in blood)	Chemical Transduction	Examples of Energy Transduction
Sodium ion	136–143 mmol/L	Ionophore complexation	Potentiometric
Potassium ion	3.6–5.0 mmol/L	Ionophore complexation	Potentiometric
Calcium ion	1.15–1.31 mmol/L	Ionophore complexation	Potentiometric
Chloride ion	98–107 mmol/L	Ionophore complexation	Potentiometric
pH	7.31–7.45 blood 5–8 urine	Ionophore pH sensitive chromophore	Potentiometric Optical
Oxygen	80–104 Torr	Gas permeable membrane Perylene or Ru fluorophore	Amperometric Optical(quenching)
Carbon dioxide	33–48 Torr	Gas permeable membrane with bicarbonate solution Membrane with bicarbonate solution and pH chromophore	Potentiometric Optical
Glucose	65–105 mg/dL	Enzymatic catalysis	Amperometric Potentiometric Optical Thermal
L-Lactate	3–7 mg/dL	Enzymatic catalysis	Amperometric Potentiometric Optical
Urea	7–18 mg/dL (BUN)	Enzymatic catalysis (+ phenol)	Potentiometric Thermal Optical