GRENOBLE INP UGA Phelma

Biosensors and High-Throughput Analysis

ELECTROCHEMICAL GLUCOSE ASSAY

Report

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1 Introduction

Glucose biosensor is one of the most studied and developed biosensor. It is called *enzyme electrodes* and they were first described by Clark and Lyons in 1962.

Glucose is of special importance because of its involvement in human metabolic processes. In particular, people suffering from diabetes mellitus do not produce sufficient insulin in their pancreas to control adequately the level of glucose in their blood. Doses of insulin have to be administered, and it is vital that the diabetic regularly monitors the level of glucose in the blood.

Glucose biosensors are based on the fact that the enzyme glucose oxidase catalyses the oxidation of glucose to glucono-lactone. This chemical reaction is shown in Fig 1. There are different forms of glucose that exist because of mutarotation, but it will not be developed in this report.

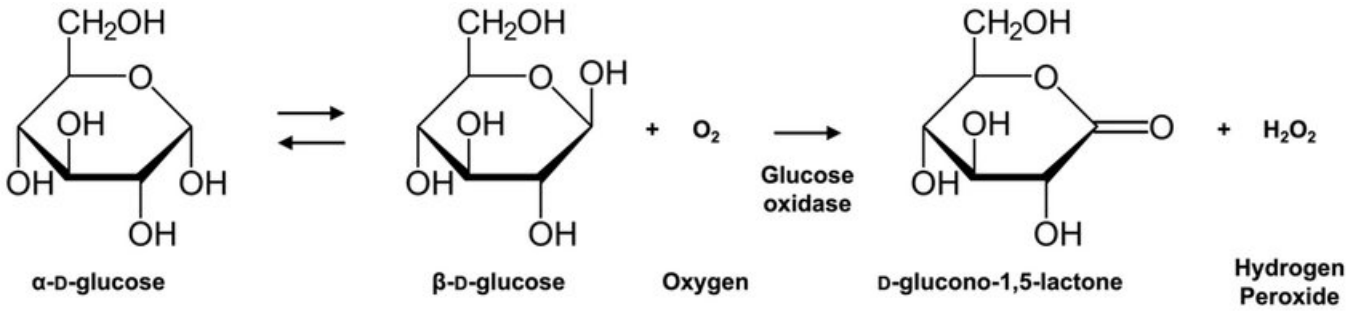

Figure 1: Reaction scheme of glucose oxidation catalyzed by glucose oxidase. Adapted from [1].

The consumption of oxygen is followed by electrochemical reduction at a platinum electrode. The oxygen is reduced into hydrogen peroxide. A voltage is applied between the platinum cathode and the silver anode, sufficient to reduce the oxygen. The cell current is proportional to the oxygen concentration. The concentration of glucose is proportional to the decrease in current. This is summarized on this scheme in Fig 2.

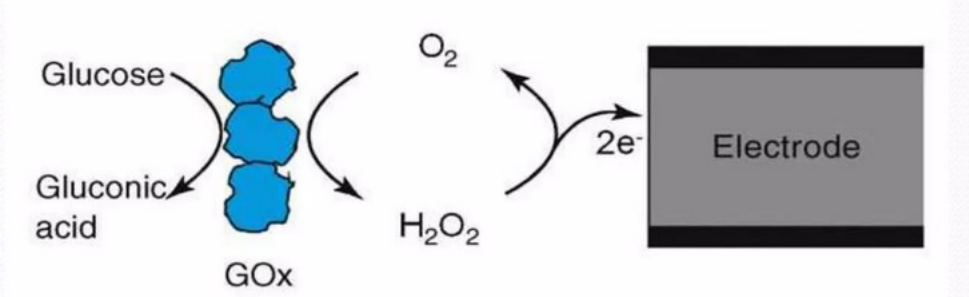

Figure 2: Reaction scheme of glucose oxidation catalyzed by glucose oxidase with the electrodes.

Now that the measurement principles were explained let's dive into how we organized ourselves for the experimental set-up.

2 Materials & Methods

The electrochemical assay done to monitor glucose levels at different situations needed the utilization of several materials and devices. These structures created to assess the different situations will be also explained in detail.

2.1 Materials

Pontentiostat used in a 3-way electrode configuration: working, counter and reference electrode. These electrodes are enclosed in a screen printed configuration which are plugged in the potentiostat. A rubber stopper fitted with two palladium wires and a silver wire covered with silver chloride. Buffer solution with 50 mM of sodium acetate which has been adjusted to pH 5.1 with HCl. Glucose solution to perform all the experiments (Concentration: 100 g/L). Glucose oxidase solution of 100 U/mL. This U nomenclature means that 1 Unit contains enough enzymatic activity to oxidase 1 μmole of the analyte (glucose) in 1 mn at pH 5.1 at 35 ºC. All solutions done are formed in a 50 mL Eppendorf tube which is also used to assess the electrochemical parameters, as the screen printed electrode stick will be submerged in the solutions. The different solutions created are prepared with micropipettes of adequate volume and with dosing gun with different volume pipettes.

2.2 Experimental methods

The volumes used are not the ones suggested by the guide, as they were not big enough for the system of electrodes to work with. 18 mL of buffer were added to the Eppendorf tube and then 360 μL of glucose. The system of electrodes-potentiostat, using the rubber stopper, is introduced into the tube to have the three electrodes submerged. This all configuration is visible in Figure 3.

Figure 3: Eppendorf tube with solution and the electrodes-potentiostat submerged in it.

First of all a initial calibration is done by applying a voltage of 0.7 V to the working electrode a duration of 50 seconds and register the current intensity. This initial calibration is done to ensure that the later experiments done with the actual chemical reaction happening will produce valuable data. Once this is accomplished, 30 μL of enzyme solution of 3U. To achieve this enzyme activity in the amount of buffer solution created before, 21.6 μL of the base enzyme solution is

added into the Eppendorf tube. Immediately after the enzyme addition a stopwatch is launched to perform the first part of the experiment.

2.2.1 First experiment: Successive chrono-amperometries

For the successive recordings a voltage of 0.7 V is applied to the working electrode at 1 minute, 3 minutes and 5 minutes during 50 seconds each, after the injection of the enzyme. We put 18 mL of buffer solution and 360 µL of glucose solution. We added 1080 µL of enzyme solution. All these data is then transferred into a spreed sheet to plot the intensity recordings as a function of $t^{0.5}$. This time axis modification is done to compute the required time to achieve the current limited by diffusion, the time at which the current will no longer increase with function as a saturation is achieved.

2.2.2 Second experiment: Standarization curve

To perform the second experiment a new Eppendorf tube of 50 mL is needed. 18 mL of the buffer solution is introduced into the tube, as well as 1.08 mL of enzyme solution. The difference between this experiment and the first one performed is that in this one the injections are of glucose. This glucose is what will initiate the chemical reaction. The experimental setup is the same as the one depicted previously, visible in Figure 3.

Once the setup has been fixed, a voltage of 0.7 V is applied to the working electrode during the time previously computed as the time needed to achieve current limited by diffusion, and the current is measured then. Now a series of glucose solution injections will be added. Each injection is composed with a volume of 180 μL of the given solution. Current will be measured by applying the same 0.7 V to the working electrode once the time to current limited by diffusion is achieved. This process is repeated ten times until a total 1.8 mL of glucose solution is injected into the buffer-enzyme solution.

2.2.3 Third experiment: Glucose level in a commercial juice

A final assessment is done with the same setup explained before, where the potentiostat-electrodes system is put inside the Eppendorf tube, but in this case the glucose-based solution used with the buffer-enzyme solution is a commercial juice. The same voltage of 0.7 V at the working electrode is applied an then the current is measured at different stages.

3 Results

3.1 First experiment: Successive chrono-amperometries to determine measurement time and incubation time.

3.1.1 Chrono-amperometries at 1mn/3mn/5mn

As explained in the Materials and Methods part, the first experiment is to register the current intensity during 50 s at 1 mn, 3 mn and 5 mn after having added enzyme solution (we start the stopwatch as soon as the enzyme is added). These three curves are shown on Figure 4, Figure 5, Figure 6.

By analyzing the plots, no notable difference can be mentioned between the different assays done at the different times. This can be explained due to the fact that in this first experiment the injection is of enzyme solution. This enzyme induces the oxidation of the glucose solution,

producing the explained H_2O_2, but is not until the voltage is applied that the oxidation of this hydrogen peroxide can occur, which is the redox reaction that generates the electrons that cause this current intensity. So, at either times of voltage injection (1mn, 3mn and 5mn), the level of current will be very similar.

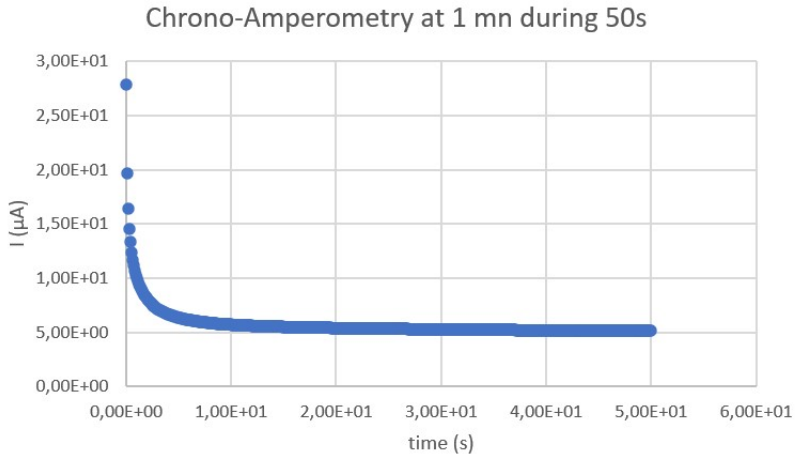

Figure 4: Chrono-Amperometry of I vs t during 50s at 1 mn.

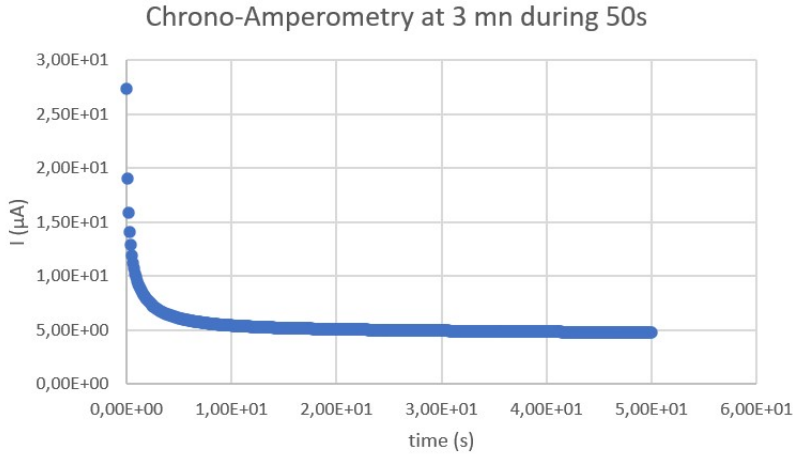

Figure 5: Chrono-Amperometry of I vs t during 50s at 3 mn.

This similarity of currents at different times can be more clearly seen in Figure 7, where all are plotted at the same time. The overlapping of current levels shows this similarity. The only small and barely notable difference is at initial time, in which the first current level decreases with the time of injection.

3.1.2 Determination of the required time to obtain current limited by diffusion

Now that these chrono-amperometries are plotted, we will plot I vs $t^{\frac{-1}{2}}$ to determine the required time to obtain current limited by diffusion. These plots are in Fig 8.

The required time to obtain current limited by diffusion is when the behaviour becomes linear. This happens when 1/sqrt(t) = 0.5 $s^{-1/2}$, so t = 4 s. Therefore, for the second part, we will have to wait approximately 5 seconds before doing the measurement to ensure that the measurements are valuable.

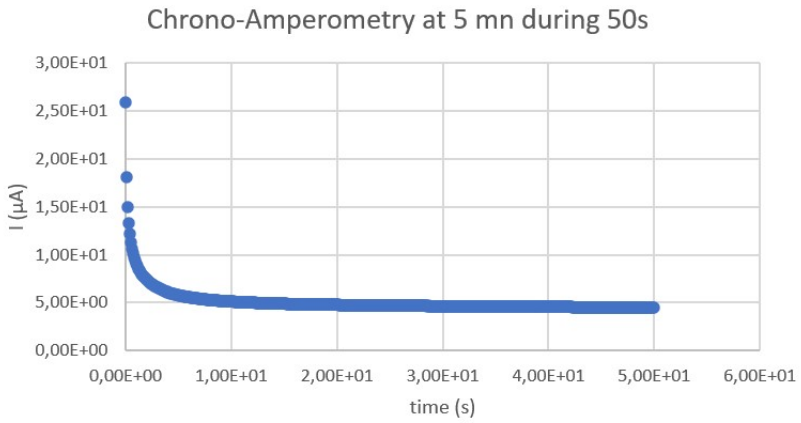

Figure 6: Chrono-Amperometry of I vs t during 50s at 5 mn.

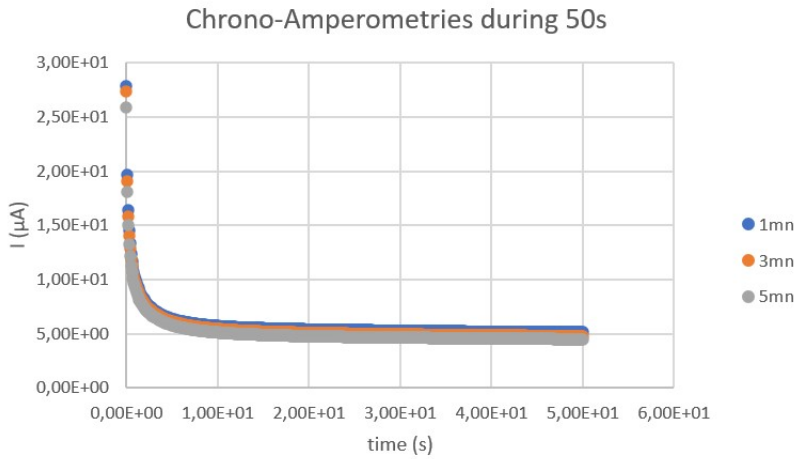

Figure 7: Chrono-Amperometry of I vs t during 50s at 1 mn / 3 mn / 5 mn.

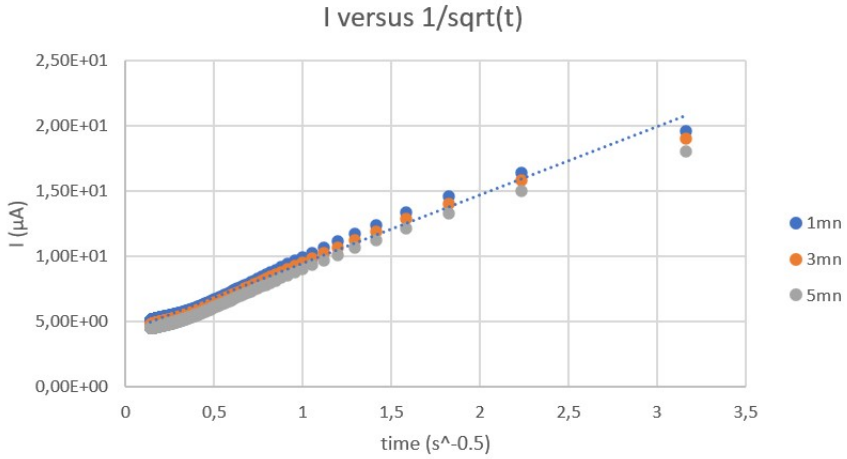

Figure 8: I versus $t^{\frac{-1}{2}}$ for 1 mn / 3 mn / 5 mn.

3.1.3 Amount of hydrogen peroxide having reacted during one chrono-amperometry

Given the 1:1 stoichiometry between glucose and hydrogen peroxide, the amount of hydrogen peroxide which reacted in one amperometry can be determined from the enzyme's catalitic activity as follow : $[H_2O_2] = \frac{108\mu mol/mn}{60s/mn} * 50s = 90\mu$mol. Moreover, the time for complete consumption of glucose was found to be 111 seconds since the amount of available glucose was 200 μmol and the

enzyme activity, 108 U.

3.2 Second experiment: Standardization curve

This experiment, as explained in the Materials and Methods part, was based on assessing how the glucose added to a buffer-enzyme solution changed the amperometric behaviour. As the goal of any standardization procedure, the concentration of our analyte (glucose) was compared with the output of the electrochemical assay, the current intensity. This current level for each injection, or at increasing glucose concentration, was taken was the current value at a time higher than the required time to obtain current limited by diffusion, computed in the previous experiment (more than 4 seconds). This curve can be seen in Figure 9.

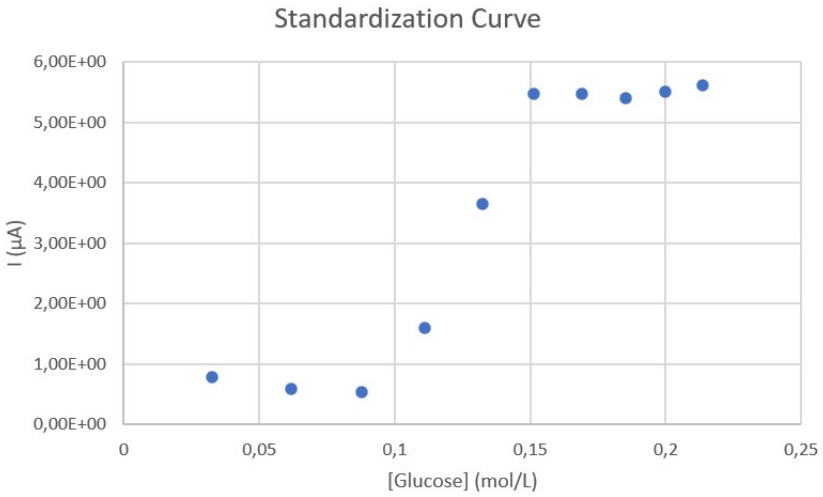

Figure 9: Standardization Curve

It can be seen how the level of current increases with higher amount of glucose. This was expected, as more concentration of glucose in a glucose oxidase enzymatic environment produces more amount of H_2O_2. This increase in hydrogen peroxide induces the freeing of more electrons, that increase consequently the current recorded. The plateau that is visible when surpassing the 0,15 M of glucose added can be related to the arrival to the point in which all enzymatic molecules are already occupied, meaning that even when adding more analyte, the output current stays constant as no more H_2O_2 is being produced. This plateau could be increased if the enzyme concentration in the enzyme solution is also increased, as the number of enzymatic sites would be higher, allowing for more glucose binding and hydrogen peroxide production.

3.3 Third experiment: Glucose level in a commercial juice

The goal of this final experiment was to assess a more real situation, by using a juice as the glucose solution. The chronoamperometry result of this experiment can be seen in Figure 10. As the buffer-enzyme solution was the same as the one used in the previous experiment the current vs time behaviour is the same with the initial injection. We obtain a current of I = 5 μA. Therefore it would mean by regard of the standardization curve that [gluc] = 0,14 mol/L, or Cm = 25.2 g/L.

An interesting approach was to assess how the injection of more juice solution during the recording time affected the current values (Fig 11). The different peaks after the initial stabilization happen due to this successive juice injection. It can be seen how the current level at which they

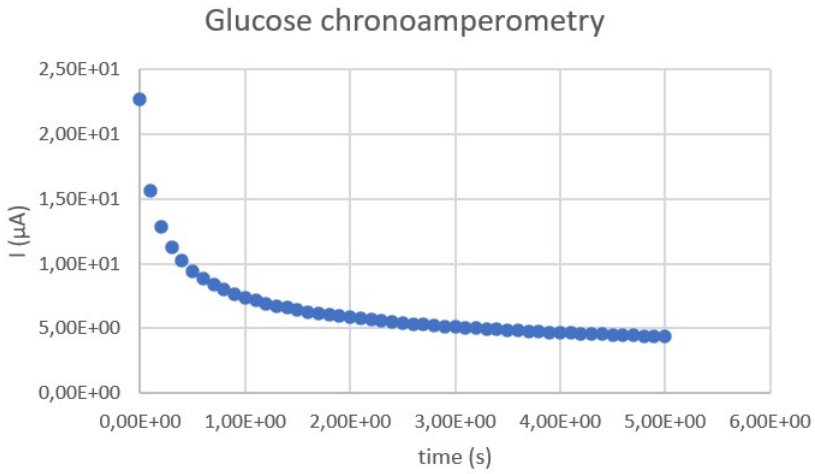

Figure 10: Glucose Chrono-Amperometry

arrive as a peak value is practically the same for all three injections, although the small difference could be related to the fact that the injection were done not using the exact same volume all the time.

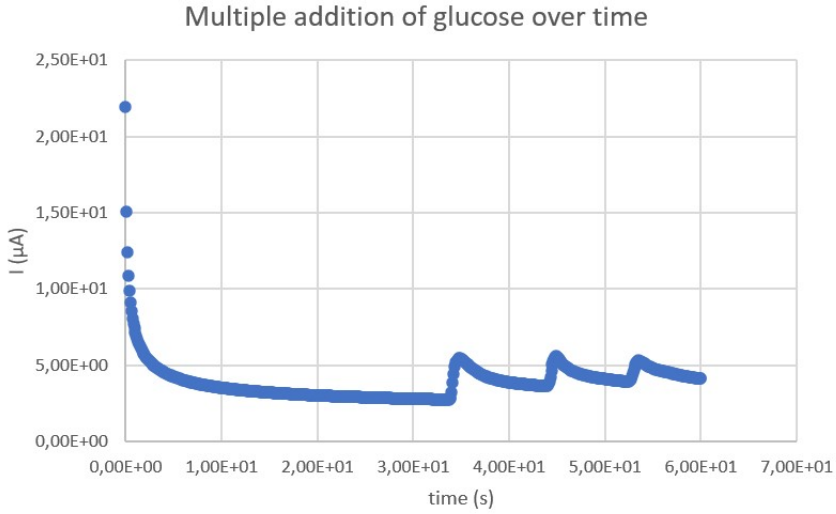

Figure 11: Multiple addition of glucose

4 Discussion

4.1 Comparison with the other group

In Figure 12 the results of the chrono-amperometries at different times since the enzyme injection can be seen. It is possible that the other groups prepared the this assay more perfectly, as their results are better than ours. The current level is higher during all 50 seconds the later the recording took place. This was the expected and theoretical result, as more time after the enzyme injection leads to more hydrogen peroxide formation, leading to higher current.

Regarding the computation of the time to obtain current limited by diffusion, which the results can be seen in Figure 13, the outcome is practically identical to ours, as the linearity of the plot

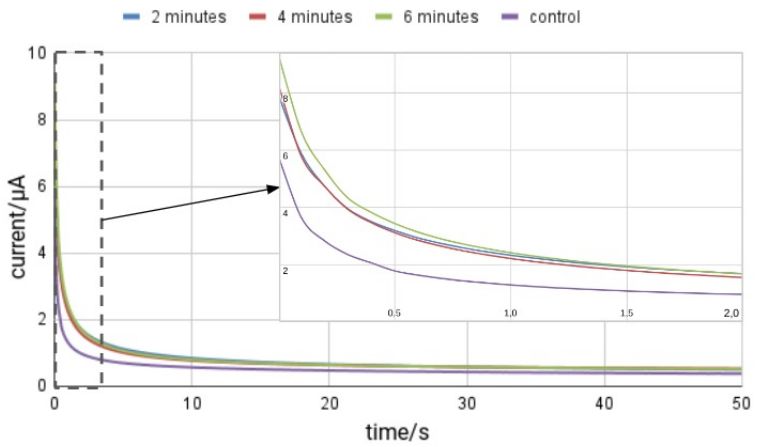

Figure 12: Chrono-Amperometry of other group

starts at approximately the same time as in our assay, 4 seconds. This leads to the same required time to obtain current limited by diffusion.

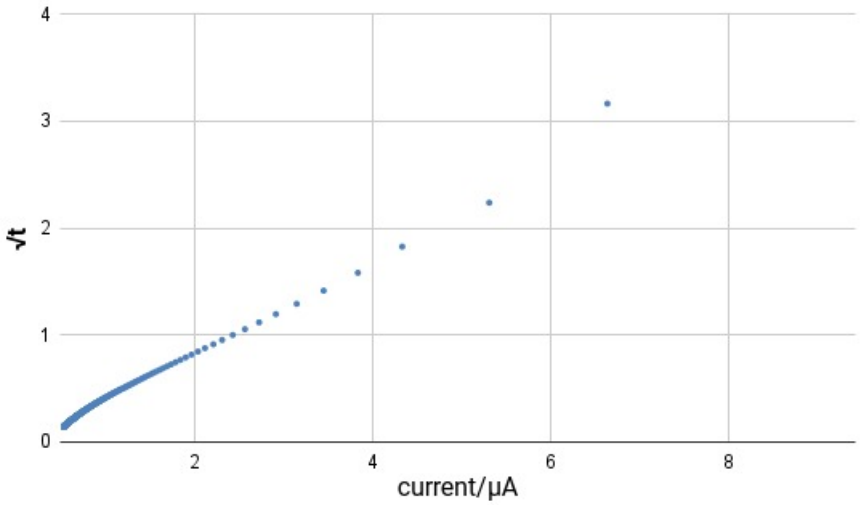

Figure 13: Current vs $t^{\frac{-1}{2}}$ of the other group

Comparing the results obtained in the second experiment, the standardization curve assay, the results can be seen in Figure 14. The showcase is different to our interpretation, as we showed the value of current, at time higher than the required one to obtain it limited by diffusion, as a function of the glucose concentration. This concentration increased with the succesive injections. The other group showed this assays result with the different chrono-amperometries of the different glucose volumes in the buffer-enzyme solution, but the results are the same. Initially the current increases greatly as the analyte concentration, but after the 6th injection approximately this current stays almost the same, arriving at the same enzymatic saturation that we explained in the corresponding results section.

Finally, the juice assay was more of a relaxed guided experiment, so the comparison of results are not really valuable to be compared. Although the results the other group got, visible in Figure 15, are the ones expected, as the current when assessing with juice solution is much bigger than

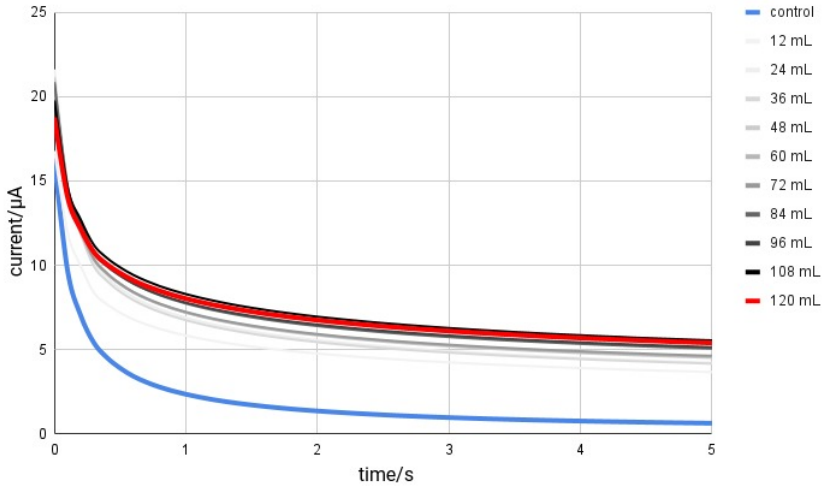

Figure 14: Chrono-Amperometry of glucose solution injections of the other group

the control one, as control does not have analyte (which explains the approximation to 0 A that occurs) and juice is high-glucose content.

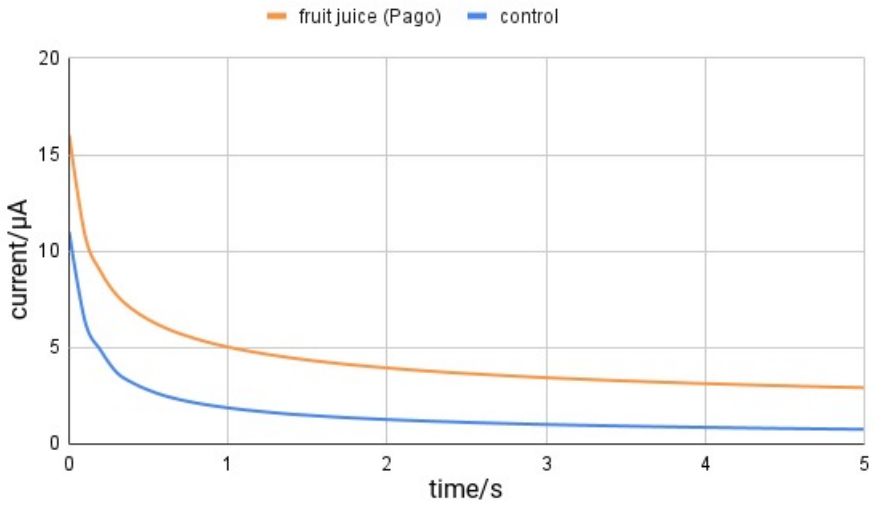

Figure 15: Chrono-Amperometry

4.2 The velocity of the enzymatic reaction

Enzymes are catalysts, not consumed by the reaction and the precise amount is not crucial for the operation of the biosensor. However, there are limiting factors. If we consider the Michaelis-Menten equation, the rate of reaction is directly proportional to the enzyme concentration (Fig 16).

We could have analyzed more deeply the velocity of the enzymatic reaction by varying the enzyme concentration.

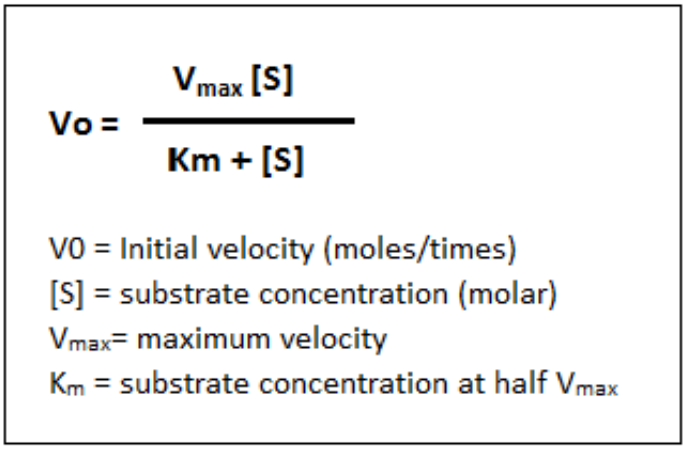

Figure 16: Michaelis-Menten equation

4.3 The possible effect of the mutarotation on the measurement

Glucose is a reducing sugar that undergoes mutarotation. If the assay that is used only measures one form of glucose, it may not accurately reflect the total concentration of glucose in the sample. Additionally, if the glucose concentration in the sample is changing rapidly due to mutarotation, the measurement may not be accurate or precise. We observed a precise measurement with steady values. Therefore, it might be concluded that mutarotation does not have a big impact on the measurement.

5 Conclusion

This practical has allowed us to understand how the glucose biosensors are working. It is really interesting as it completes our lectures on biosensors. Furthermore, we saw the chrono-amperometry technique that is often used in electrochemistry. It allows us to determine the required time to obtain current limited by diffusion. This enables us to make the standardization curve and then to determine the glucose concentration of a juice.

To go further, improvements are being made on the glucose biosensors as it is so important in the health of diabetes patients. We studied first generation glucose biosensors, but newer biosensors were made after as explained in [2]. Second generation biosensors were made by replacing oxygen with non-physiological electron acceptors, redox mediators. Third generation biosensors were the one based on direct transfer between the enzyme and the electrode. These improvements are still thriving and very actual in the growing world of biosensors.

References

[1] Suzuki, Lee, Loew, Takahashi-Inose, Okuda-Shimazaki, Kojima, Mori, Wakako Tsugawa, and Koji Sode. Engineered glucose oxidase capable of quasi-direct electron transfer after a quick-and-easy modification with a mediator. *International Journal of Molecular Sciences*, 21:1137, 02 2020.

[2] Lee SY Yoo EH. Glucose biosensors: an overview of use in clinical practice. 2010.