Instituto Tecnológico y de Estudios Superiores de Monterrey

Monterrey Campus

School of Engineering and Sciences

Master of Science in Nanotechnology

Thesis proposal

Development of a Microfluidic Platform for the Detection of Glucose

by

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Campus Monterrey

The committee members, hereby, recommend that the proposal presented by Cinthia Janet Ramírez Murillo to be accepted to develop the thesis project as a partial requirement for the degree of **Master of Science in Nanotechnology**.

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Abstract

We will develop an electrochemical carbon-based biosensor to monitor the viability of cells in culture in real-time by measuring glucose concentration in the media. Most of the cell viability assays that have been used up to this point are destructive, thus the assay is performed at the end of the experiment/treatment of the cells. Attempts to monitor cultures in real-time have been approached with optical sensors, which are more expensive than electrochemical sensors and less viable for miniature applications such as lab-on-a-chip. With an electrochemical sensor, we will be able to measure glucose in real-time, without damaging the cells. In this project, a carbon-based electrode will be designed and fabricated via photolithography and pyrolysis while exploring the performance improvements that can be achieved by modifying the electrode with addition of carbon structures to improve sensitivity. We believe electrochemical sensing methods are a more viable, cost-effective option to monitor cultures in real-time.

1. Introduction

Electrochemistry is the study of chemical processes driven by electrical forces and electrical energy generated by chemical reactions. Electrochemical methods have been widely used for a number of applications such as measuring pH, food monitoring, water quality assessments, manufacturing, automotive, environmental monitoring and medical diagnostics. One of the growing applications of these sensors is in point-of-care diagnostics, where "[e]lectrochemical sensing strategies have the potential to achieve rapid, sensitive, selective, and low-cost detection of biomolecular analytes relevant to clinical diagnosis and monitoring treatment of disease." (Labib et al., 2016) There are several variations in electrochemical sensors depending on the analyte they are designed for. If the analyte is electroactive, an electrical signal is generated without modification of the electrode surface. If the analyte is not electroactive, electrochemical sensors work by having a recognition element that interacts with the analyte, producing an electroactive substance. In some cases, this recognition element also amplifies the signal. Recognition elements can be enzymes, DNA strands, antibodies or cells. There are two main types of interaction between the recognition elements and the analyte: catalytic interactions and binding interactions. In the case where the recognition element is an enzyme, this is a catalytic interaction in which the enzyme speeds up a reduction-oxidation reaction and the signal generated by the electroactive products (or the electron transfer) is measured. In the case of binding interactions, the electrical signal is generated by the presence of the analyte at the electrode surface (Cui, 2017) .

Currently, the more common methods to perform cell viability assays are optical, which depend on addition of a dye to the cells and the fluorescence or absorbance of the sample is analyzed depending on the dye used. Some examples of these are Tetrazolium reduction assays, Resazurin reduction assays, Protease viability marker assay, the ATP assay and the Clonogenic or Colony Formation assay (Riss et al., 2016) . Mainly due to the cytotoxicity of the dye, these protocols have to be carried out at the end of the experiment, after the cells have been cultured and treated for a number of days in the incubator. Furthermore, optical sensors have not been limited to only provide end-point cell viability assays, but there are also optical sensors that have been developed to monitor cell-cultures in real-time, since they are non-destructive and non-invasive optical methods are very promising for biological applications where a sterile environment is necessary. An optical sensor for monitoring glucose concentration in real-

time was developed based on an oxygen sensor (Tric et al., 2017). However, these sensors are expensive, the solution where the analyte is needs to be clear, and they are less viable for miniaturization or lab-on-a-chip applications.

In the cases where the analyte is dynamic and provides information about cell metabolism, having to wait until the end of a culture period to evaluate the effects of a treatment on cell viability drastically limits the information that a researcher can have to analyze and determine whether a certain drug is effective at stopping cancer cell proliferation, for example. Another disadvantage of the endpoint methods is that they require manual processing once the incubation time is over in order to obtain any valuable results. Cell media containing cell debris must be carefully dispensed from the culture dish, the cells remaining on the plate have to be incubated with a dye in order to be observed and quantified. Colonies have to be counted or an image processing protocol needs to be developed in order to obtain quantitative results. The sensor proposed would allow the scientist to monitor his/her culture in real-time and be able to not only know the final state of the culture but the entire process without having to manipulate or dye the cells at the end of each experiment. The following proposal presents the problem definition and motivation, hypothesis and research questions, objectives, related work, methodology and work plan that will be followed in order to create a platform for the detection of glucose in cell culture.

2. Problem Definition and Motivation

Through this project, we are planning to develop a platform for the detection of glucose in a bioreactor. Cell-culture is one of the main procedures that biologists, physicians, bioengineers and other scientists use for biocompatibility assessments. When testing a new drug, or a new material used in the manufacture of a medical device, new biomaterials to seed the cells or when performing a biopsy for a patient, cell-culture is carried out. Although widely used, the process of culturing cells is far from trivial. Cells need to be seeded very carefully to ensure not only their survival but assure they are not disturbed or damaged in any way to avoid phenotype changes. Another key component of cell-culture is that it needs to be carried out in an aseptic environment at all times to ensure they are not contaminated with virus or bacteria. In this last aspect, the biggest threat besides a contaminated lab or incubator is the human who is culturing them. Although protective equipment is used for cell-culture, the process of manipulating cells is so delicate that any small movement that was not premeditated can introduce undesired agents from the human manipulating them to the culture. Since cells are then incubated at a highly humid (~95% relative humidity), warm (37 °C), close to pH neutral (~7.4) environment, it is optimal not only for animal cell growth but also for the propagation of bacteria and fungi. Without mentioning the work load to the scientist performing the time-consuming cell-culture activities, the aspects of human manipulation of the cells involve a series of risks for the cultures that sometimes create loss of time and money to the laboratory.

Since their rise in the 1940's the use of bioreactors has increased in laboratories, starting with the fed-batch bioreactors which still involved the intervention of a human in order to change the media periodically and the more advanced perfusion bioreactors. Perfusion bioreactors minimize the human intervention necessary for the cells to survive throughout the culture. Media flows continuously from the media reservoirs to bioreactor chambers where cells are seeded onto a pre-fabricated biomaterial. The media flow is driven by peristaltic pumps at flow rates below 10 mL/min. This system reduces the risks

involved in the traditional cell-culture methods by minimizing the manipulation of cells. Since the main purpose of cell-culture is to test cell behavior or response to different stimuli, cell-survival assays are used to evaluate what percentage of cells, relative to the number of cells seeded, have survived under the presence of a certain drug or chemical. At this point, most of the assays used for this purpose are destructive, meaning that the cells have to be seeded, cultured for a few days to ensure no contamination is present, incubated while exposed to the chemical being studied for a period of time (typically 7-14 days), the dead cells removed from the culture flask, live cells are dyed and the results can be analyzed at the end of the experiment. During the time of the experiment, in most cases no quantitative assessment can be performed to ensure that cells are showing any specific behavior when exposed to the chemical of interest or that the treated cells are behaving differently when compared to the control.

Through the development of a sensor for the detection of glucose, we attempt to provide a solution to this problem by producing a sensor that can be integrated into a bioreactor and provide the user with real-time information about the glucose levels in the media flowing to the cells. This information can be traced to cell metabolism information that can aid scientists to know time-dependent effects of their treated cells during the experiment. The first phase of the project will be to synthesize a carbon—based electrode that can be functionalized by immobilizing the enzyme glucose oxidase onto the electrode surface. Then we will test whether we can successfully detect an electrical signal in a liquid containing glucose (i.e. cell culture media). The key motivations for using an electrochemical sensor for this application are that it allows for real-time measurements since the assessment of cell viability will now be non-destructive and it does not require the addition of a dye or reactant that damages the cells, it is not as expensive to produce as an optical sensor, and it is viable for miniaturization.

3. Hypothesis and Research Questions

The hypothesis of this work is that using nanotechnology to fabricate a sensing platform, we can improve the limit of detection of glucose to monitor cell metabolism in a miniature perfusion bioreactor. Some of the research questions that arise from this hypothesis are: what type of design should be followed to optimize the cell-culture monitoring? Although the scope of this project does not include the integration of the sensing platform with the bioreactor, there are aspects of the initial design that will need to be considered in light of the final application for this sensor. Some of these questions are: should this be an in—line platform where media flows from the bioreactor chamber to the sensor or should the sensing platform be directly integrated in the chamber? In specific, how will the glucose measurements be traced to a cell viability reported in percentage? What model should be followed to represent the electrochemical interaction between glucose and the electrode?

4. Objectives

The general objective of this work is to develop a microfluidic platform for the detection of glucose that can be integrated into a perfusion bioreactor to achieve real-time measurements of glucose levels, thus monitoring the cell metabolism during experiments while they are treated to different concentrations of a drug of interest.

The particular goals to achieve as this research work is conducted are:

- Designing an electrode for the measurement of glucose in cell-culture media.
- Modeling the electrochemical interaction of the analyte at the surface of the electrode.
- Fabrication, testing and optimization of the electrode developed for the measurement of glucose in cell-culture media.
- Comparing experimental results with modeling.

5. Related Work

Among the most widely studied electrochemical sensors are glucose sensors. They are commercially available and can be optimized for different applications by the electrode material, the agent immobilized on the electrode surface, selective coatings or membranes placed on the surface, etc. The most common approach to measure glucose is by immobilizing glucose oxidase on the surface of the sensing platform. The interaction between the analyte and the enzyme glucose oxidase on the electrode surface follows this equation. Biosensors for continuous glucose monitoring are based mainly on the following equations:

$$\beta\text{−D−Glucose} + \text{GODox} \rightarrow \beta\text{−D−glucono−}\delta\text{−lactone} + \text{GODred} \quad (1)$$

$$\text{GODred} + O_2 \rightarrow \text{GODox} + H_2O_2 \quad (2)$$

$$\beta\text{−D−Glucono−}\delta\text{−lactone} + H_2O \rightarrow \text{gluconic acid} \quad (3)$$

where GODox and GODred refer to the oxidized and reduced glucose oxidase, respectively (Tric et al., 2017). Figure 1 shows a schematic representation of the reactions above.

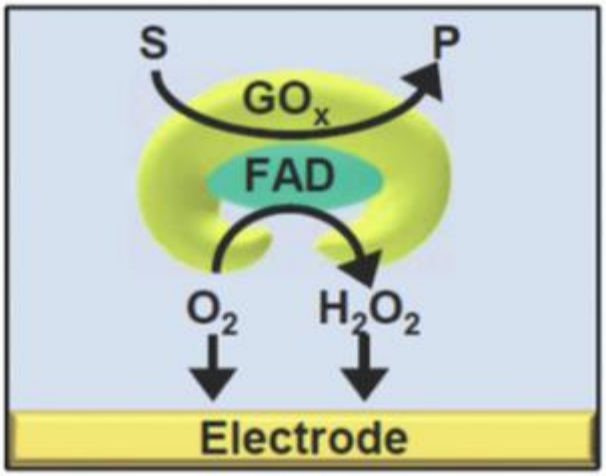


Figure 1. Schematic representation of electrochemical interactions at the electrode surface of an enzymatic glucose sensor. Used without permission (Lee et al., 2018).

The schematic and reactions above are representative of the first-generation electrochemical glucose biosensors. Given that this type of sensors is cheaper than optical sensors, they have been widely exploited and developed. More recent studies of electrochemical sensors deal with modifications on the

electrode surface to improve its properties rather than synthesis of the electrode itself. One of the remaining areas of improvement of electrochemical sensors is the effective electron transfer from the redox enzyme to the electrode surface. The modifications include addition of metal nanoparticles such as gold or platinum or the use of carbon nanomaterial-based coatings.

Some studies demonstrate that use of carbon-based nanostructures for the design of electrodes improves the electron transfer from the active center of the redox enzyme to the electrode surface. One example of this is the work by Lina Mikoliunaite *et al.* This study compares the amperometric response of three modified electrodes and a regular uncoated graphite rod electrode. The coating consisted of a polycarbonate membrane modified with Single-walled carbon nanotubes with reduced graphene oxide (SWCNT-rGO). One side of the membrane was called the conductive side because it was completely covered with SWCNTs. On the other side (nonconductive), only a few SWCNTs were deposited. The first electrode tested was covered with the membrane and the conductive side was on top, nonconductive side was in contact with the electrode. On the second electrode, the membrane was turned halfway, leaving the nonconductive part inside and then placed on the electrode. On the third experimental electrode, the membrane was again turned halfway but this time the conductive part was left inside before placing it on the electrode. Lastly, the control electrode consisted of only the graphite rod electrode with glucose oxidase, without any membrane coating. There was almost no current when the electrode was covered with the membrane folded with the conductive part inside. The results showed similar amperometric responses for the other three electrodes as shown in Figure 2. This means that the SWCNT on the polycarbonate membrane effectively allowed for electron transfer from the active center of glucose oxidase to the electrode surface since the presence of the membrane coating with the conductive side in contact with either the redox enzyme or both the redox enzyme and the electrode surface did not reduce the amperometric response of the graphite rod electrode. The outcome of this hypothesis testing was “that the polycarbonate membrane with single-walled carbon nanotubes and reduced graphene oxide layer do not reduce the effectiveness of electron transfer from the active center towards the electrode”. The authors

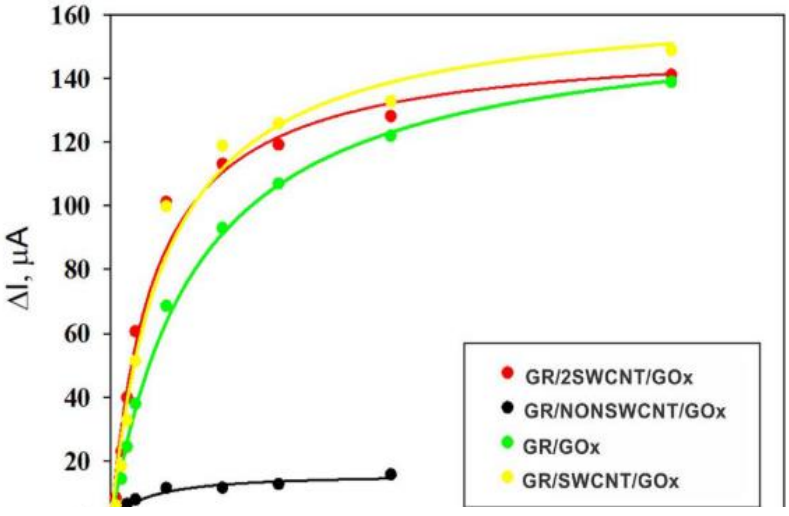


Figure 2. The amperometric response of the electrodes differently modified with polycarbonate membrane having SWCNT-rGO coating. Used without permission (Mikoliunaite et al., 2017).

conclude that it is expected that SWCNT-rGO coatings modified with enzymes without carbon black electrode could be used as flexible glucose biosensors in the future. (Mikoliunaite et a., 2017)

Another work that presents the use of carbon nanomaterials for the improvement of glucose electrodes is the development of a highly sensitive electrode for glucose and lactate monitoring in cell-culture using carbon nanotubes. The work presented compares non-nanostructured and nanostructured electrodes and it shows that "direct electron-transfer between the protein and the electrode is not possible without nanostructuration." The experiments involved the use of a bare graphite electrode functionalized with glucose oxidase and a graphite electrode where multi-walled carbon nanotubes deposited between the electrode surface and the enzyme glucose oxidase. Figures 3 and 4 show images of the bare electrode and the nanostructured electrode, respectively. While the image of the bare electrode shows the small aggregates which characterize graphite, Figure 4 shows the wrapped strands formed by carbon nanotubes on the electrode surface. It is explained by the authors that "the ends of carbon nanotubes, which terminate with a carboxylic group, are quite hydrophilic, but the walls, which comprise the majority of the tube, are highly hydrophobic." The electron field emission properties of the carbon nanotubes were also studied based on carbon nanotube alignment on the electrode surface. The simulations developed in the study allowed to conclude that the majority of the total current was contributed from the nanotube sidewall rather than the tip of the nanotube. They also showed that the electrical current from the sidewall was produced at lower angles with respect to the substrate.

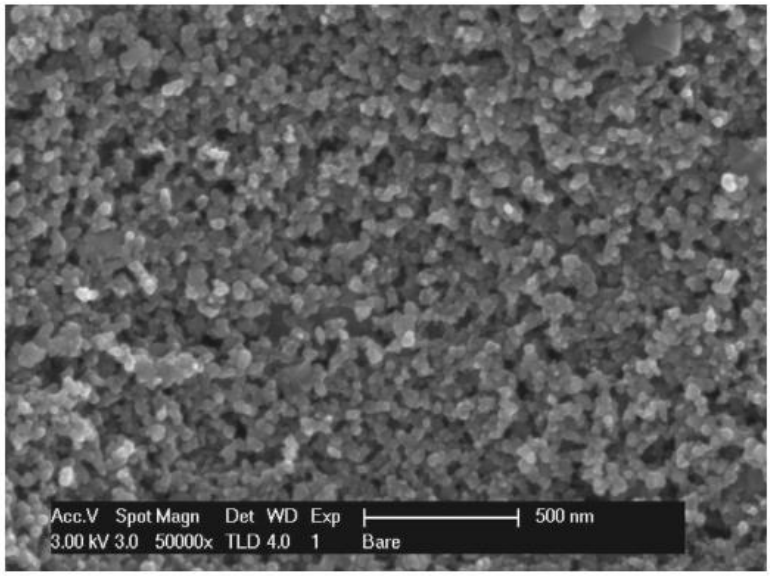


Figure 3. SEM image of the surface of a bare electrode. Used without permission (Boero et al., 2011).

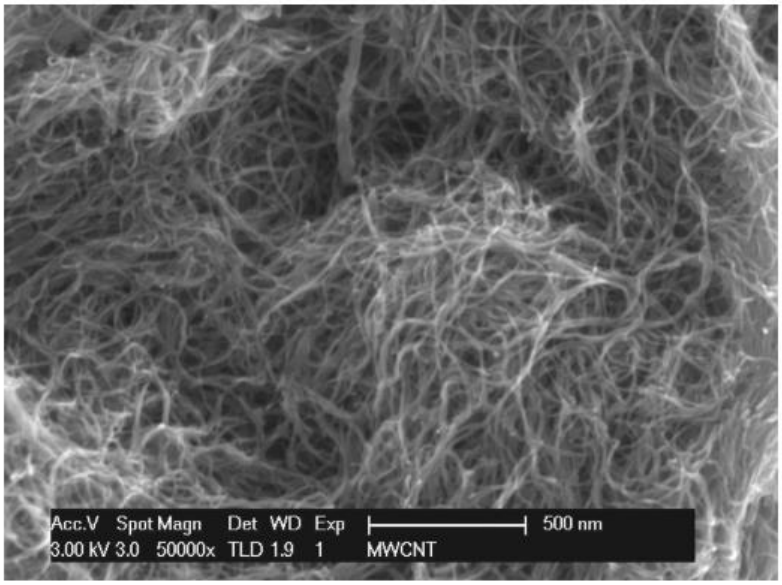


Figure 4. SEM image of the surface of a nanostructured electrode. Used without permission (Boero et al., 2011).

As observed in figure 5, when using a bare electrode (graphite, screen-printed electrode without multi-walled carbon nanotubes) no current was detected from the different glucose concentrations. According to the authors, "[t]he detected current is in the order of nA for bare electrode, and signal to noise ratio is too high to sufficiently distinguish different concentrations of the substrate" (Boero et al., 2011). On the other hand, the nanostructured electrodes on which multi-walled carbon nanotubes had been deposited detected the different concentrations of glucose with an appropriate sensitivity. The reported sensitivity of the nanostructured sensor was 27.7 $\mu A\ mM^{-1}\ cm^{-2}$ and the linear range of the electrode was 0.073-4 mM. Although the sensitivity and the limit of detection reported are within the order of magnitude of previous studies, this work further confirms that the use of carbon nanostructures is effective in electron transfer from the redox enzyme to the electrode surface of a biosensor.

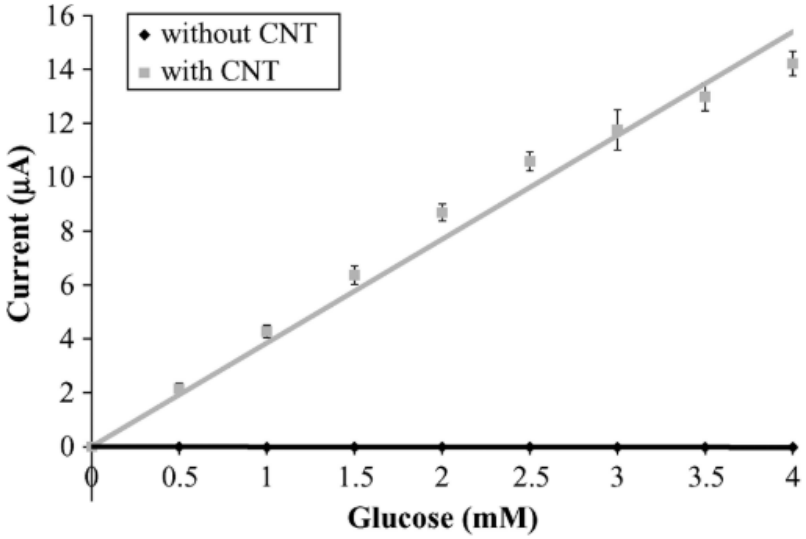


Figure 5. Glucose detection for non-nanostructured and nanostructured electrodes. Used without permission (Boero et al., 2011).

6. Methodology

The methodology that will be followed for this project is to complete and submit a fully detailed project proposal, there will be a design phase of the project in order to propose different electrode geometries guided by the characteristics that will be of selectivity and specificity required for the application of this electrode in a cell-culture environment. Once a set of designs is proposed, we will model the physical phenomena in each of the designs using COMSOL software to determine which design meets our requirements more closely. Based on the outcomes of the modeling phase, we will select the geometries that are most suitable for the application and fabricate them. The process to fabricate these electrodes will involve identification of adequate precursors for carbon electrodes, photolithography, and pyrolysis in order to vaporize the non-carbon components of the precursor.

To initiate the fabrication process, we will create designs of electrode patterns using AutoCAD software and print them on to a glass surface. As shown on figure 6, the photolithography process begins with deposition of a photoresist onto a silicon wafer, this wafer is then placed into a spin coater which holds the wafer in place through a vacuum while a shaft spins to spread an even layer of photoresist over the wafer. After baking, the wafer with the UV mask prepared from the printing patterns is exposed to UV-light in order to transfer the pattern to the wafer. Once the wafer is baked a second time, it is soaked in the adequate photoresist developer. During exposure, if a negative photoresist such as SU-8 was used, the pattern exposed to light has been cross-linked and it will not be washed away during development. At the end of this process, the wafer with SU-8 will be baked for a longer time than the previous baking for the heteroatoms to evaporate, leaving behind a structure composed of carbon. Figure 7 shows a view of the specific patterns that are formed on the silicon wafer throughout the photolithography process. As far as the particle deposition on steps G and H in this figure, this project will explore the use of pyrolyzed organic material rather than gold to enhance electrode performance.

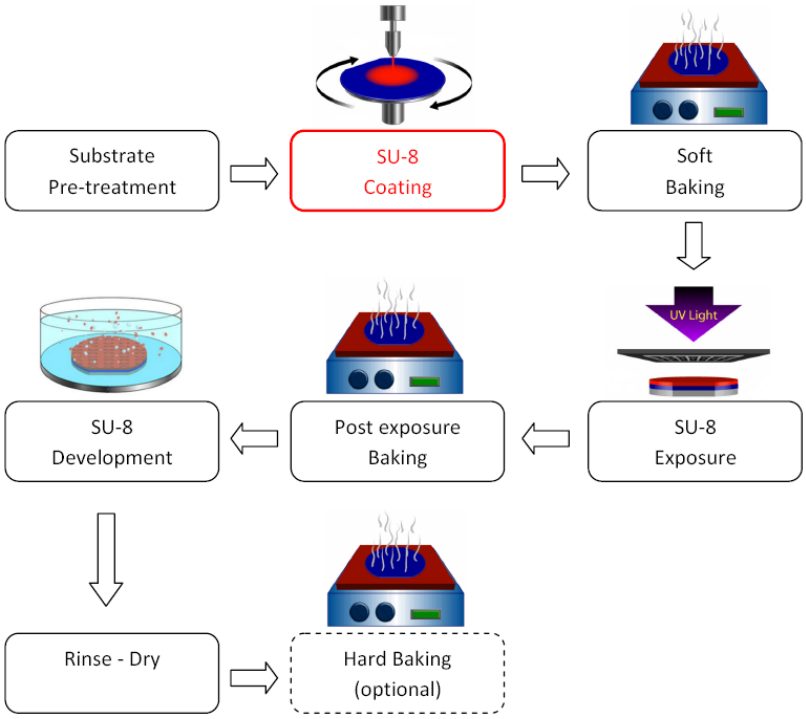


Figure 6. Schematic representation of photolithography process. Used without permission (ELVEFLOW, n.d.).

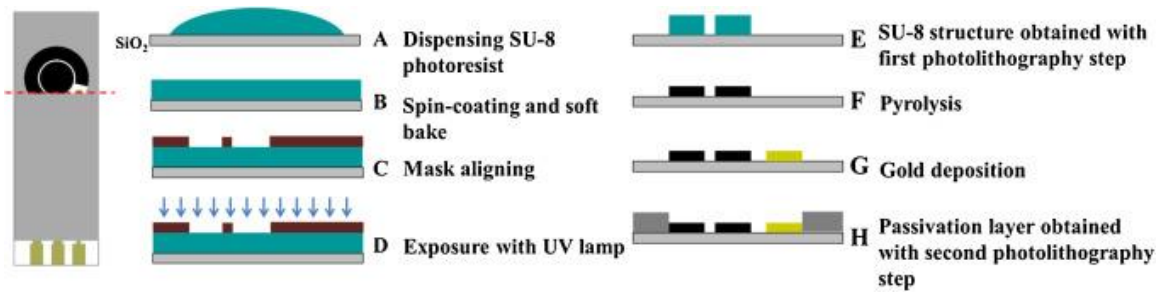
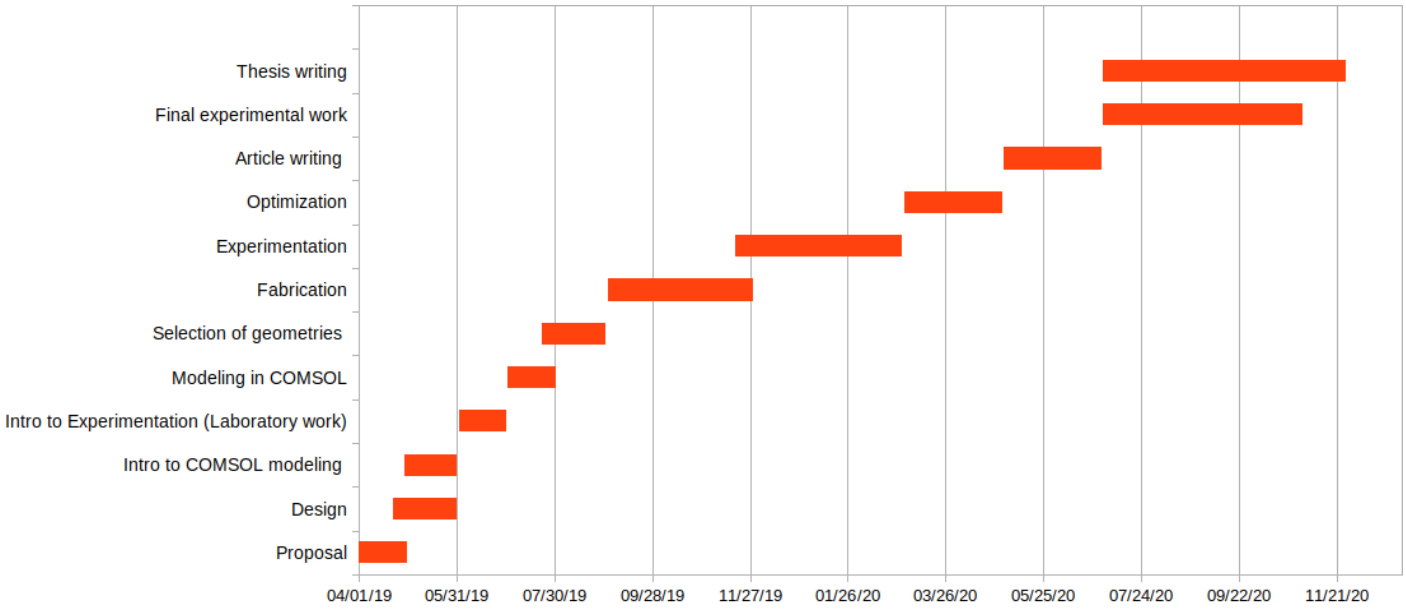


Figure 7. Side-view of the photolithography process wafer transformation. Used without permission (Hassan et al., 2017).

Following the fabrication process, we will also begin testing the electrodes produced. Initially, the electrodes will be tested through cyclic voltammetry using the redox couple ferri-ferrocyanide in order to obtain qualitative information about the electrode and whether it effectively allows for electron transfer. Once this is established, enzyme glucose oxidase will be immobilized onto the electrode surface and tests will now be conducted with glucose as our analyte. It is important to mention that although the final goal of this sensor is to utilize it in a microfluidic platform, the experimental designs will begin at the macro scale (i.e. tests in a beaker) with cell-culture media (or a solution containing glucose). After the tests are completed in macro scale, we will begin with the miniaturization phase of the project. This will involve creating electrodes with dimensions that can be integrated into a microfluidic platform. After testing is completed at the miniaturization phase, we will compare our results to the predictions obtained from the modeling phase. This information will allow us to optimize the electrodes for the next iterations.

7. Work Plan

The following Gantt chart shows the schedule of activities to develop a microfluidic platform for the detection of glucose as detailed in the methodology section.



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