

Chlorophyll and Silk-based Oxygen Producing Biomaterials for Tissue Engineering

Thesis

By Ching-Chieh Russell Wang

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Department of Biomedical Engineering
Tufts University
Medford, Mass.

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SUMMARY

The lack of oxygen is often a limiting factor in various tissue engineering applications. In numerous studies centered on the regeneration of tissues and organs, the lack of oxygen diffusion is often one of the major problems encountered. Oxygen is a vital component in the development and proliferation of cells and vascularization of tissues necessary for tissue regeneration studies. A large body of research has been performed in an effort to address this problem. These studies had varying approaches which include the use of oxygen-containing fluids, angiogenic factors, and cell-matrices that are designed to better facilitate the diffusion of oxygen. More recent studies conducted to address this problem of oxygen diffusion have been based on the implementation of oxygen releasing biomaterials. These studies were centered on implantable biomaterials that release oxygen over a period of time but do not actually produce it. This study, instead of utilizing biomaterials that are rich in oxygen that releases over time, focuses on the production of chlorophyll-based biomaterials that can produce and release oxygen over a prolonged period of time (Harrison 2007).

The main components of the oxygen-releasing biomaterials in this study are plant chlorophyll and silk fibroin. In the search for the ideal oxygen producing system for this study, photosynthesis stood out as the most promising candidate. Photosynthesis is one of the most crucial and common naturally occurring processes in the environment. Photosynthesis is the most significant oxygen producing process in Nature and because of this, has been extensively studied. The fact that photosynthesis has been well studied is one of the reasons why it was chosen for the research presented here. The stability of photosynthesis in Nature makes it a favorable choice for the study as well. This study hopes to maintain the stability of photosynthesis in a synthetic environment.

The choice of silk as the other main component of this oxygen-producing biomaterial is based on research that has shown it to be both flexible in its application space and biologically compatible. The biomaterial required for this study must be compatible enough to implant into living organisms and have a controllable degradation timeline. Silk fibroin has been demonstrated to be a promising biomaterial because of a number of attributes (Perry 2008). Silk is also believed to be less toxic than most synthetic biomaterials. This non-toxicity will be important in maintaining the stability of photosynthesis in the biomaterials as well as play a role in the biocompatibility of the biomaterial when implanted in vivo.

The goal of this study is to produce a novel oxygen-producing biomaterial by incorporating plant chloroplasts into silk fibroin that will release oxygen for a prolonged period of time. Such a biomaterial could be promising in many tissue engineering applications. In addition to tissue engineering applications, the development of an oxygen producing biomaterial would also have a wider range of applications. Such an oxygen-producing biomaterial can be viewed as a type of “artificial leaf” that could find applications in fields from bio-catalysis to bio-sensing.

SIGNIFICANCE AND BACKGROUND

Significance. The main goal of this study is to fabricate an oxygen-producing biomaterial that can successfully produce and release oxygen for prolonged periods of time. Beyond this point, we have two widely different yet equally significant applications in mind. The first application of this oxygen-producing biomaterial involves the utilization of such an oxygen-producing biomaterial in a tissue engineering context. One of the primary problems that tissue engineering studies come across when attempting to successfully regenerate tissues and organs is oxygen diffusion (Harrison 2007). We attempt to address this problem with the fabrication of our oxygen-producing biomaterial. The second application of our oxygen producing biomaterial carries less medical relevance. The seemingly simple goal of creating biomaterials that mimic biological processes is a common goal among countless scientists. By successfully creating a biomaterial that effectively mimics the biological process of photosynthesis, this study could have a major impact on fields from bio-catalysis to bio-sensing (Meunier 2008).

Chloroplast Isolation. A significant portion of the study is dependent on the ability to isolate chloroplasts from plants. The goal is to isolate the photosynthetic complex from leafy plants and utilize this in our biomaterial. Previous studies have also focused on isolating components within the photosynthetic complex for similar applications (Mullet 1980). Studies have focused on the separation of photosystem 1, which is the chlorophyll-containing membrane protein complex, using chemical detergent (Kiley 2005). These studies also attempted to stabilize the photosystem 1 proteins in the solid-state, which is similar to the goal of isolating chloroplasts in silk film in our study. The added complexity of isolating an individual protein complex found within photosynthesis became the primary focus of such studies. Specially designed peptide detergents were used in an attempt to stabilize the photosystem while their

sample dried to its solid state (Kiley 2005).

The desire to isolate individual protein

complexes within

photosynthesis was a result of the direction

of these studies. Previous studies that

focused on isolating the individual protein

complexes rather than the entire

photosynthetic system were focusing on the

generation of photocurrent rather than

oxygen for their final applications (Mullet

1980). More recent studies have focused on the

isolation of the larger component of thylakoids, which are found within chloroplasts (Figure 1).

Chloroplasts are the center for photosynthetic reactions and are known to perform carbon dioxide

conversions very efficiently. Another benefit to isolating entire chloroplasts is the fact that they

can achieve all the primary processes within photosynthesis as well we most of the secondary

processes. These processes include light capture, electron transport, NADPH and ATP synthesis,

and synthesis of 3-carbon phosphorylated compounds. Additionally, they synthesize proteins and

other components, making chloroplasts an arguably self-sufficient organelle (Meunier 2008).

From our assessment of these previous studies, we have chosen to isolate entire chloroplasts for

our study.

Encapsulation of chloroplasts in silk fibroin. Many studies have been performed that

research the encapsulation of bacteria in various synthetic and natural biomaterials. Far fewer

studies have been performed on the encapsulation of chloroplasts. This is because, unlike

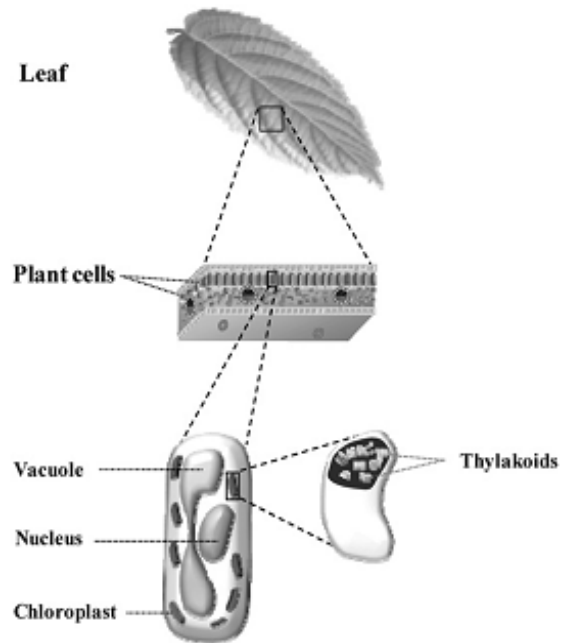


Figure 1. Schematic representation of chloroplast and thylakoid location within a plant (Source: Meunier 2008)

bacteria, photosynthetic organelles like chloroplast have a low resistance to abiotic environments. Of these studies focusing on the encapsulation of chloroplasts, few attempt to encapsulate chloroplasts in organic matrices. Most organic matrices possess poor thermal and mechanical stability, have uncontrolled porosity, and are not always fully biocompatible (Meunier 2008). As a result, most studies centered on the encapsulation of chloroplasts look to inorganic materials to provide a stable environment for the chloroplasts to function (Figure 2). Despite observations from previous studies about the properties of organic matrices, we believe silk fibroin can offer

Encapsulating Matrix Composition	Previously Observed Properties (Meunier 2008)	
Organic	Poor thermal stability (even at low temperature) Poor mechanical stability Uncontrolled porosity Synthesis not always fully compatible	the stability seen commonly only seen in inorganic matrices. Some more recent attempts at
Inorganic	Good thermal stability Good mechanical stability	encapsulating chloroplasts in inorganic

Figure 2. Observed matrix properties from previous chloroplast encapsulation studies

matrices seem to show that despite the matrices may exhibit good thermal and mechanical stability, they still do not create suitable environments for chloroplasts to survive and function for prolonged periods of time. One such study immobilized thylakoid membranes isolated from spinach leaves in polyvinylalcohol- bearing styrylpyridinium (Rouillon 1995). The study aimed to optimize operational conditions like temperature and pH for the chloroplasts. The problem with the polyvinylalcohol used in their study was that components of it eventually leached into the thylakoids that were suspended within it. This turned out to damage the chloroplasts and decrease their lifespan and performance. With silk fibroin, we aim to optimize conditions for the chloroplasts in an environment free of components that may end up being toxic to the chloroplasts.

Oxygen production of the chloroplast incorporated silk fibroin films. Most attempts at regenerating tissues and organs are hampered by the lack of oxygen diffusion at the regeneration site. Attempts at addressing this problem include the use of oxygen-containing fluids, angiogenic factors, and cell-matrices that are designed to better facilitate the diffusion of oxygen. More recent studies conducted to address this problem of oxygen diffusion have been based on the implementation of oxygen releasing biomaterials. Recent research focused on the incorporation of the oxygen rich compound of sodium percarbonate into films of PLGA (Harrison 2007). The study attempted to address the issue oxygen diffusion in the context of tissue regeneration engineering. However, one major flaw to the design of their study was the fact that the biomaterial did not actually produce oxygen. The sodium percarbonate incorporated PLGA was rich in oxygen that would release over a period of time after implantation. Results from the study showed that their biomaterial released oxygen for approximately 24 hours. Once the oxygen contained in the films was completely released, oxygen levels ceased to increase (Harrison 2007). The biomaterial in that study was ineffective in actually producing oxygen over an extended periods of time by merely released all the oxygen it contained over a brief interval. Additionally, the rate of release of the oxygen was not steady. Effective oxygen diffusion in tissue regeneration applications must occur over longer periods of time than 24 hours. It would also be ideal if the rate of oxygen release were more constant and controllable. With the implementation of photosynthetically active chloroplasts as our main oxygen-producing components, we hope to achieve a prolonged period of oxygen release as well as a steady and controllable rate of oxygen release.

Biomaterials	Period of Oxygen Release	Oxygen Release
Sodium percarbonate + PLGA	~ 24 hours	Inconsistent, Not Controlled
Chloroplasts + Silk (proposed)	Over 24 hours	Steady, Controllable

Figure 3: Comparison of oxygen release between recent study and our proposed study

Biocompatibility of the chloroplast incorporated silk fibroin films. The long term goal of this study is to produce a biomaterial that can be used for tissue engineering applications. This means that in addition to being able to produce and release oxygen consistently over a prolonged period of time, the materials used must also be biocompatible. The biomaterial therefore, must have low toxicity levels, must not be rejected by the host organism, and must degrade suitably. A recent study attempted to solve the problem of oxygen diffusion in tissue regeneration for tissue engineering applications with the production of oxygen releasing PLGA film incorporated with sodium percarbonate. The film they produced in their study was successful in releasing oxygen, but its oxygen-releasing components are shown to be slightly toxic and less biocompatible than desired (Harrison 2007). We will attempt to address this issue by using silk fibroin as the material in which to house the oxygen-producing chloroplasts. Studies have shown the silk fibroin is a promising new biomaterial with excellent biocompatibility characteristics. Processed silk fibroin can be non-toxic, easily accepted into biological systems and organisms, and can have a controllable rate of degradation dependent on its processing and post-processing. Our study hopes to not only utilize a more stable and consistent system for producing and releasing oxygen, but also aims to improve upon the biocompatibility and adoptability of the biomaterials used in our silk-based oxygen-producing biomaterial (Figure 4).

Biomaterials	Biocompatibility
Sodium percarbonate + PLGA	Sodium percarbonate can be toxic to surrounding cells and tissues when degraded
Chloroplasts + Silk	Silk and chloroplasts have no known biocompatibility issues

Figure 4: Comparison between biocompatibility of recent study and our proposed study

SPECIFIC AIMS

The long term goal of this study is to produce a novel oxygen-producing biomaterial for tissue engineering applications. *We hypothesize that the fabrication of durable, oxygen-producing biomaterials can be achieved by incorporating plant chloroplasts into silk fibroin films.* To achieve the objective of this study, we will accomplish the following specific aims:

Aim 1: Fabricate oxygen-producing silk film. Our working hypothesis is that silk fibroin films will provide a compatible environment for the plant chloroplasts to photosynthesize. In order to accomplish this goal, we will isolate chloroplasts from baby spinach leaves and then incorporate the isolated chloroplasts into silk fibroin solution. This silk fibroin solution with chloroplasts suspended inside will then be applied to trays in which the solution can be allowed to set into silk fibroin films containing plant chloroplasts. It is expected that silk fibroin films with chloroplasts suspended within them will be created from this procedure. If the procedure does not achieve the expected result, the silk fibroin solution can be modified before the chloroplasts are suspended within the solution. Another possible alternative is to set the silk fibroin and then apply a layer of chloroplast suspension on top of the silk fibroin and let them set this way. The end result, no matter the process, will still be the fabrication of silk fibroin films containing isolated plant chloroplasts.

Aim 2: Assess the release of oxygen of the chloroplast incorporated silk film. Our hypothesis is that a chlorophyll-based oxygen-producing biomaterial will effectively produce and release

oxygen for a prolonged period of time. Once the chloroplast incorporated silk films are produced, their oxygen production and release must be tested. This can be achieved by using the Chlorolab System developed by Hansatech Instruments Ltd. The Chlorolab system allows us to control the intensity of light our photosynthetic samples are exposed to and measures the oxygen concentration of the sample it contains in real-time. Using the Chlorolab system, we will measure the oxygen production of our photosynthetic silk films over a set period of time to determine the amount of oxygen released by the films and also to determine the approximate usage lifetime of the oxygen-producing silk films fabricated in this study. This Chlorolab system can also be used to measure oxygen release from a control sample of chloroplasts suspended in buffer. If possible or necessary the Chlorolab system can also be utilized to measure the oxygen releasing capabilities of other oxygen-releasing biomaterials proposed in previous studies as a basis for comparison. We expect that our oxygen-producing silk films will produce and release oxygen for a longer period of time than oxygen-releasing biomaterials from other studies.

Aim 3 (Future Goal): Test biocompatibility of oxygen-producing silk film. We hypothesized that our chloroplast incorporated silk films will be suitable for tissue engineering applications because of the biocompatible nature of silk fibroin. We will test the biocompatibility of our silk films and silk films incorporated with chloroplasts by subjecting them to in vitro biocompatibility tests as well as in vivo implantation tests. We expect our silk films to be biocompatible and show little toxicity. If our films appear to be less biocompatible than expected, the structure and composition of our initial silk solution will be altered and new films will be tested.

Aim 4 (Future Goal): Test effectiveness of oxygen-producing silk films in vivo. Our hypothesis is that our chloroplast infused silk fibroin films will be able to successfully produce and release oxygen when implanted as a biomaterial. To test this, in vivo implantation tests will be performed to assess the ability of the silk film to produce and release oxygen when implanted under the epidermis of mice. The level of tissue necrosis will be studied for implantations of different films. We will also examine the condition of the tissue over a period of time to determine how long the oxygen-producing silk film can effectively produce and release oxygen. We expect the results from our chloroplast infused silk films to exceed the results seen in other studies using other oxygen releasing biomaterials. If we find that our films are not as effective at producing oxygen in vivo as we expect, we will focus on in vitro tests. The development of an artificial leaf that can produce oxygen in the presence of light over a prolonged period of time would be a significant contribution to efforts currently underway to create biomaterials that mimic biological processes.

RESEARCH DESIGN AND METHODS

The more immediate and specific goal of this study is to fabricate an oxygen-producing biomaterial that will produce and release oxygen over a prolonged period of time. Results from this more immediate goal can potentially have major impacts on the fields of bio-catalysis and bio-sensing. Additional research from results of our more immediate goal lead to the long term goal of this study, which is to fabricate an oxygen-producing biomaterial that can be used for tissue engineering applications, such as tissue and organ regeneration. Our long term goal has a decidedly more medical focus. Our first and second specific aims directly address our more immediate goal for this study, while the third and fourth specific aims are geared more toward our long term goal. This study is flexible and low risk in the sense that while our aims are connected to one another, they are not completely dependent on each other and valuable information can be gained from each individual specific aim of the study (Figure 5).

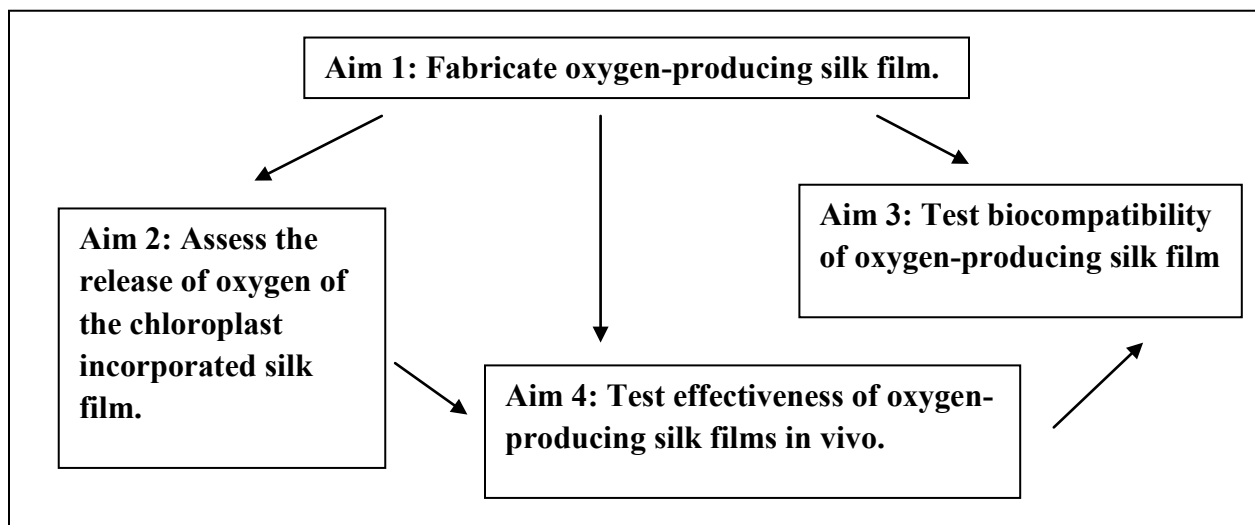


Figure 5: Schematic depicting relationship between aims of the study

Specific Aim 1: Fabricate oxygen-producing silk film.

1.1 Introduction:

There are two main components in the fabrication of our oxygen-producing silk films. The first component is the chloroplasts. We have decided to isolate whole chloroplasts rather than specific systems or proteins within chloroplasts. Our goal is to keep the entire photosynthetic system intact and somewhat self sustainable. The second component of our fabrication is the silk fibroin. Essentially, our first specific aim addressed the combination of these two components.

1.2 Experimental Design:

1.2.1 Chloroplast Isolation

To isolate our chloroplasts we will follow a well documented and proven protocol. To isolate whole chloroplasts we will follow the procedure laid out in the Chloroplast Isolation Kit produced by Sigma-Aldrich® (Sigma). The main components of the Chloroplast Isolation Kit are chloroplast isolation buffer, Percoll, bovine serum albumin, and filter mesh 100. The plant that we will isolate the chloroplasts from will be baby spinach. Baby spinach leaves are rich in chloroplasts and contain fewer veins and components that need to be filtered out along the process. The protocol set out by the isolation kit essentially calls for the homogenization of the spinach leaves in the chloroplast isolation buffer provided by the kit. The homogenized spinach leaves are then passed through a filter and centrifuged to remove unwanted plant components and whole cells. After more centrifuging and passing through a Percoll layer, all the unwanted plant components including broken chloroplasts will have been removed, leaving us with a suspension of spinach chloroplasts (Sigma). This suspension will then be stored at 2°C in the dark to preserve photosynthetic activity until further use (Meunier 2009).

1.2.2 Incorporation of Isolated Chloroplasts into Silk Fibroin Films

Purified silk fibroin will be produced from harvested *Bombyx mori* cocoons.

Sericin, a water-soluble glycoprotein that binds fibroin filaments, will be removed from fibroin strands by boiling the cocoons in a 0.02 M aqueous solution of sodium carbonate. Then, the remaining fibroin bundle will be thoroughly rinsed in Milli-Q water and allowed to dry.

The dry fibroin bundle will then be dissolved in a 9.3 M aqueous solution of lithium bromide at 60°C for 4 hours. The lithium bromide salt will then be extracted from the solution over the following three days through a water-based dialysis process from which the purified silk fibroin solution will then be extracted (Perry 2008). Once the silk fibroin is obtained, the isolated chloroplast suspension will be suspended inside the silk fibroin solution. Once the chloroplasts are evenly suspended within the silk fibroin solution, silk films will be made out of the suspension by spreading set amounts of the suspension over plastic dishes. Initial films used in this study will be smaller in size and be comprised of 1 mL of suspension. The films will be left to set in the dark at 2°C.

1.3 Expected Outcome

The expected outcome from this component of the study is the fabrication of silk fibroin films that encapsulate whole chloroplasts isolated from baby spinach leaves. Depending on what is deemed necessary for subsequent steps in the study, the size and chloroplast concentration of the films can be modified.

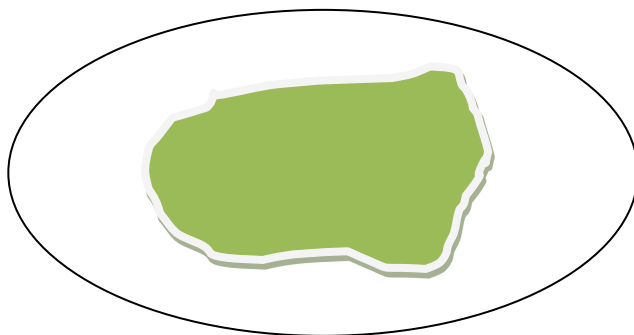


Figure 6. An illustration of a basic chloroplast incorporated silk fibroin film

1.4 Potential Problems and Alternative Strategies

A potential yet unlikely problem that may be faced in this step of the study may be that the silk films do not set correctly when suspended with the isolated chloroplasts. If the procedure does not achieve the expected result, the silk fibroin solution can be modified before the chloroplasts are suspended within the solution. Alternatively, pure silk fibroin can be set as a film first and then a layer of chloroplast suspension can be applied on top of the silk fibroin film and then another layer of pure silk fibroin can be applied on top of the chloroplast layer. This approach would encapsulate the chloroplasts in the silk fibroin in a sandwich fashion. The end result will still be the fabrication of semi-transparent silk fibroin films containing isolated chloroplasts.

Specific Aim 2: Assess the release of oxygen of the chloroplast incorporated silk film.

2.1 Introduction:

We hypothesized that a chlorophyll-based oxygen-producing biomaterial will effectively produce and release oxygen for a prolonged period of time. Once the chloroplast incorporated silk films are produced, their oxygen production and release must be tested.

2.2 Experimental Design:

The assessment of the oxygen release of our chloroplast incorporated silk film will be carried out using the Chlorolab System developed by Hansatech Instruments

Ltd (Figure 7). To measure the oxygen

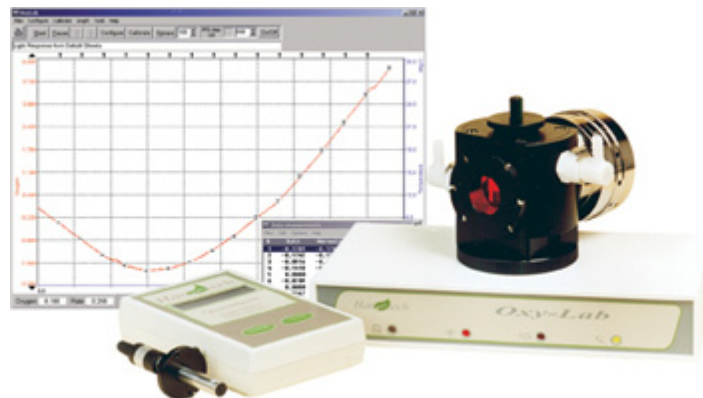


Figure 7: Photograph of Chlorolab System by Hansatech with example of data output interface

evolution of chloroplast incorporated silk films produced in the study, we will follow the procedures outlined in the operations manual of the Chlorolab System. The oxygen production of the films will be tested in an aqueous solution of 10 mM ferrocyanide which will serve as an electron donor (Izawa 1980) and 5 mM ammonium chloride which will serve as the uncoupling agent for photosynthesis (Rouillon 1995). The Chlorolab system allows us to control the intensity



Figure 8: Red LED Light Source



Figure 9: Liquid-Phase Electrode Chamber

of light our films are exposed to under the LED light (Figure 8) and will measure and record the oxygen concentration of the liquid within the electrode chamber (Figure 9) real-time. The resulting data from the Chlorolab system will be data showing the oxygen concentration and temperature of the sample per unit time. From this data, we can calculate the rate of oxygen production beginning from the point at which the light was turned on over a period of time. We will take oxygen production measurements at daily intervals after the fabrication of the chloroplast containing silk films to determine the lifespan of the chloroplasts when set in the silk fibroin. Our data will show us how long the chloroplast can remain functional after being set in the silk film and will also show us the photosynthetic activity of the chloroplasts over a period of time. We will also measure oxygen release from a control sample of chloroplasts suspended in buffer over the same time interval to determine whether the silk fibroin helps to preserve the

chloroplasts function. The Chlorolab system can also be utilized to measure the oxygen releasing capabilities of other oxygen-releasing biomaterials proposed in other studies as a basis for comparison. The data from these readings will be plotted on the computer and normalized for changes in the temperature of the sample. This way, changes in the samples oxygen concentration as a result of increasing or decreasing temperature can be accounted for. It will also be important to note the times at which the LED light will be turned on to determine if changes in oxygen concentration are from photosynthetic activity.

2.3 Expected Outcome:

We expect that our oxygen-producing silk films will produce and release oxygen for a longer period of time than oxygen-releasing biomaterials from other studies. We also expect that our films will produce oxygen at a more consistent rate than the oxygen-releasing biomaterials from other studies.

2.4 Potential Problems and Alternative Strategies:

A potential problem is that the Chlorolab system does not function as expected. In the event of this situation, our first alternative is to contact Hansatech Instruments Ltd. and troubleshoot whatever problem we have. If the problem cannot be resolved this way, another system that measures oxygen concentration in an aqueous environment will be used.

Specific Aim 3 (Future Goal): Test biocompatibility of oxygen-producing silk film.

3.1 Introduction:

We hypothesized that our chloroplast incorporated silk films will be suitable for tissue engineering applications because of the biocompatible nature of the silk fibroin and chloroplasts.

We will test the biocompatibility of our silk films and silk films incorporated with chloroplasts by subjecting them to in vitro biocompatibility tests as well as in vivo implantation tests.

3.2 Experimental Design:

The silk films incorporated with chloroplasts will be subjected to a number of biocompatibility tests. These tests can be run by a number of commercial laboratories and will include tests for, cytotoxicity, sensitization, irritation, and biodegradation (Figure 10).

Biocompatibility Test	Criteria
Cytotoxicity	Determines whether a product or compound will have any toxic effect due to leachables on living cells
Sensitization	Tests for sensitivity to any part of a device during exposure to the body for any length of time
Irritation	Determines how irritable a product or compound is to the body
Biodegradation	Tests how much of the product or compound is absorbed by the body and follows the product or compound through the body after it has been absorbed to determine the effects over time.

Figure 10: Table of biocompatibility tests that will be run on silk films in our study
(Source: <http://www.micromedlabs.com/services/bs.php#biocomp>)

3.3 Expected Outcome:

Because the main components of our chloroplast incorporated silk film have been known to be biocompatible, we expect our silk films to be biocompatible.

Specific Aim 4 (Future Goal): Test effectiveness of oxygen-producing silk films in vivo.

4.1 Introduction:

We hypothesized that our chloroplast infused silk fibroin films will be able to successfully produce and release oxygen when implanted as a biomaterial. To test our hypothesis, in vivo implantation tests will be performed to assess the ability of the silk film to produce and release oxygen when implanted under the epidermis of mice.

4.2 Experimental Design:

Our animal model for this study will be nude mice. We will implant the chloroplast incorporated silk films between the skin and muscle of half our specimens and for the other half, we will implant silk films that do not contain chloroplast as a control. The mice will be allowed to move around and eat normally, and will be subject to typically normal light conditions. Two mice from the non control and control group will be sacrificed daily to assess the condition of the tissue around the implant. The tissue will be examined for necrosis and photographed under standard lighting. Histology of the skin will also be used to assess the level of inflammation, tissue architecture, and necrosis. The results from this study will show the condition of tissue after implantation at day 1, 2, 3, and so on. Inflammation of tissue around the implantation site will be rated on a predetermined scale and percentage of tissue around the implantation site undergoing necrosis will be determined and recorded for each day. Once all data is gathered, the results will be plotted to see if there is a trend in the regeneration or degradation of the tissue. From the condition of the tissue, we should be able to determine the level and effectiveness of oxygen diffusion from the implanted silk films.

4.3 Expected Outcome:

We expect the results from our chloroplast infused silk films to exceed the results seen in other studies using other oxygen releasing biomaterials. Under normal lighting conditions, the films should be able to photosynthesize and steadily produce and release oxygen each day for a prolonged period of time.

4.4 Potential Problems and Alternative Strategies:

A potential problem is that our films are not as effective at producing oxygen in vivo as we expect. In this case, we can attempt to alter the chemical composition of the base silk fibroin solution that we use to produce our films to see if we get improved results. We can also try altering the physical structure of our films like making them porous so that the mice might accept the film in their bodies with more ease. If none of these approaches help to improve the effectiveness of the silk films in vivo, we will focus on in vitro tests. The development of an artificial leaf that can produce oxygen in the presence of light over a prolonged period of time would still be a significant contribution to efforts currently underway to create biomaterials that mimic biological processes and would also have a major impact on fields from bio-catalysis to bio-sensing.

RESULTS

We were successful in the isolation of intact chloroplasts from baby spinach leaves using the procedure outline in the Chloroplast Isolation Kit produced by Sigma-Aldrich®. The baby spinach leaves used in this study were prewashed and prepackaged versions found in local supermarkets. To ensure that the chloroplasts obtained for our study were still functioning and active, chloroplast isolation was only performed on spinach leaves purchased on the day of isolation. Isolated chloroplasts were stored in suspension and successfully incorporated into silk fibroin solution. Chloroplast-containing silk fibroin solution was successfully set onto plastic dishes and films were made contained 1 mL of chloroplast suspension each (Figure 11). The silk fibroin solution containing the isolated chloroplasts was successfully set into films that could be peeled from the plastic plates on which they set and tested for photosynthetic activity.

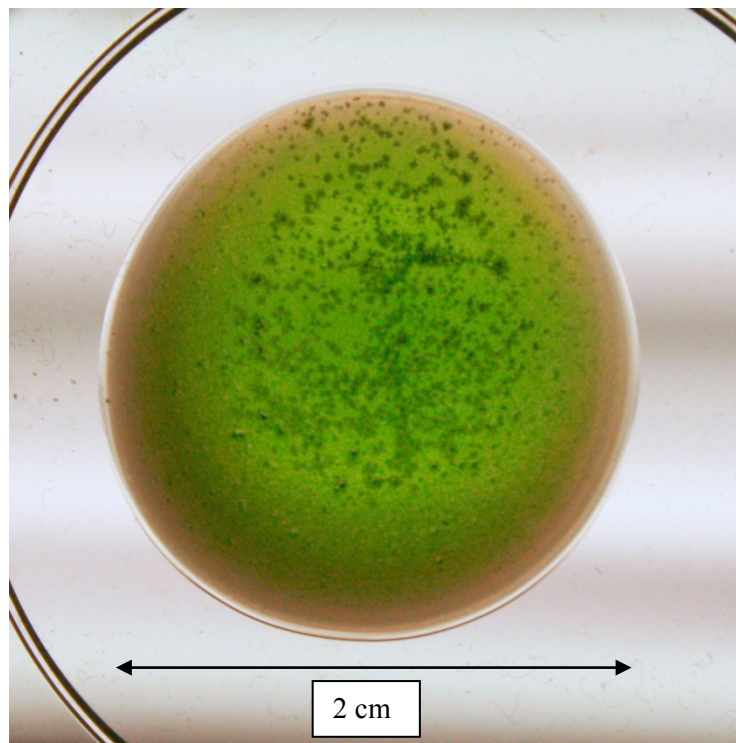


Figure 11. Photograph of chloroplast incorporated silk fibroin film set on plastic plate

The photosynthetic activity of the chloroplast incorporated silk film was assessed using the previously described Chlorolab system developed by Hansatech Instruments Ltd. Multiple batches of chloroplast incorporated silk fibroin films were produced and oxygen production was measured at different time points after the production of the films. Oxygen production readings were taken for the first batch of chloroplast incorporated silk fibroin films as well as for isolated chloroplasts that were suspended in buffer. The test was performed two days after the chloroplasts were isolated from baby spinach leaves. This test was to determine the ability of silk fibroin film to preserve the activity of the isolated chloroplasts. Results from these first tests showed that the oxygen production of the chloroplast incorporated silk fibroin films significantly exceeded the oxygen produced by chloroplasts suspended in buffer solution (Figure 12).

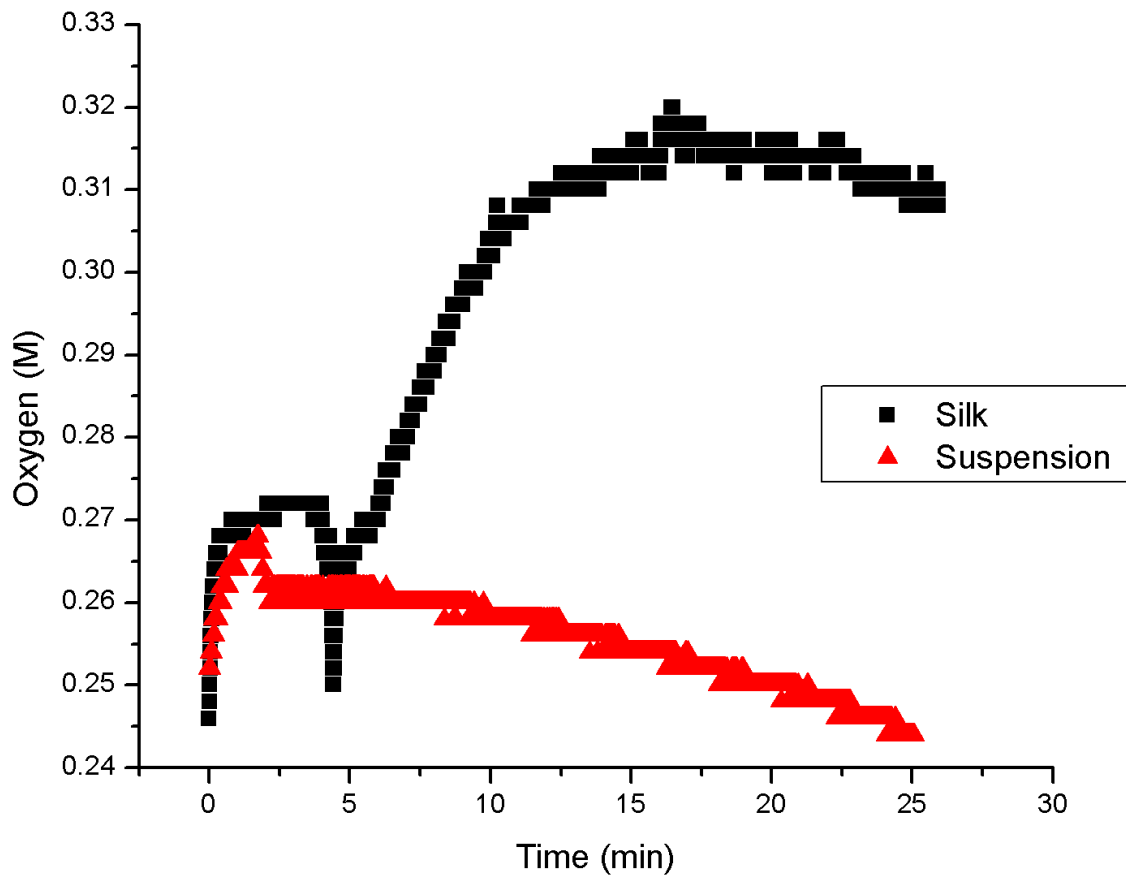


Figure 12. Oxygen production of chloroplast incorporated silk fibroin film versus oxygen production of chloroplast suspension

The oxygen production readings were taken two days after isolation and incorporation of the chloroplasts into the silk fibroin solution and buffer. The results from the first tests show that the silk fibroin solution incorporated with chloroplasts was able to produce oxygen even two days after the chloroplasts were removed from the baby spinach leaves. The isolated chloroplasts that were just suspended in buffer did not exhibit any oxygen producing capabilities just two days after they were isolated from the baby spinach leaves. The results from the first batch of films were taken using a liquid-phase electrode chamber containing water. The water gradually dissolved the silk fibroin film, releasing the incorporated chloroplasts into the electrode chamber.

After the first batch of films demonstrated the preservation of photosynthetic activity of the chloroplasts incorporated into silk fibroin, additional sets of films were made using the same procedures. Testing of the films produced after the first batch of films was conducted slightly differently. When testing for photosynthetic activity, the electrode chambers were filled with buffer instead of water. This kept the films intact rather than break them down as water did. By taking readings from intact chloroplast incorporated films, we could examine the ability of the films themselves to produce oxygen. The isolated chloroplasts varied slightly from batch to batch as a result of the different sources of baby spinach leaves. Variations in the chloroplast incorporated films also arose from the preparation process. The second chloroplast isolation yielded chloroplasts that exhibited significantly less activity than the first batch. One day after the chloroplast isolation, oxygen production readings were taken on a sample of chloroplasts suspended in buffer (Figure 13). Despite a small initial rise in oxygen concentration, photosynthetic activity of the chloroplast suspension diminished rapidly. Seven days after the isolation of chloroplasts and the incorporation of the isolated chloroplasts into silk fibroin films, the chloroplast incorporated films were tested (Figure 14). The goal of this test was to examine

the long-term ability for the silk film to preserve the activity of the isolated chloroplasts. Further tests were performed on the chloroplast containing silk films 14 days after fabrication which showed that the chloroplasts no longer demonstrated photosynthetic activity after this period of time (Figure 15).

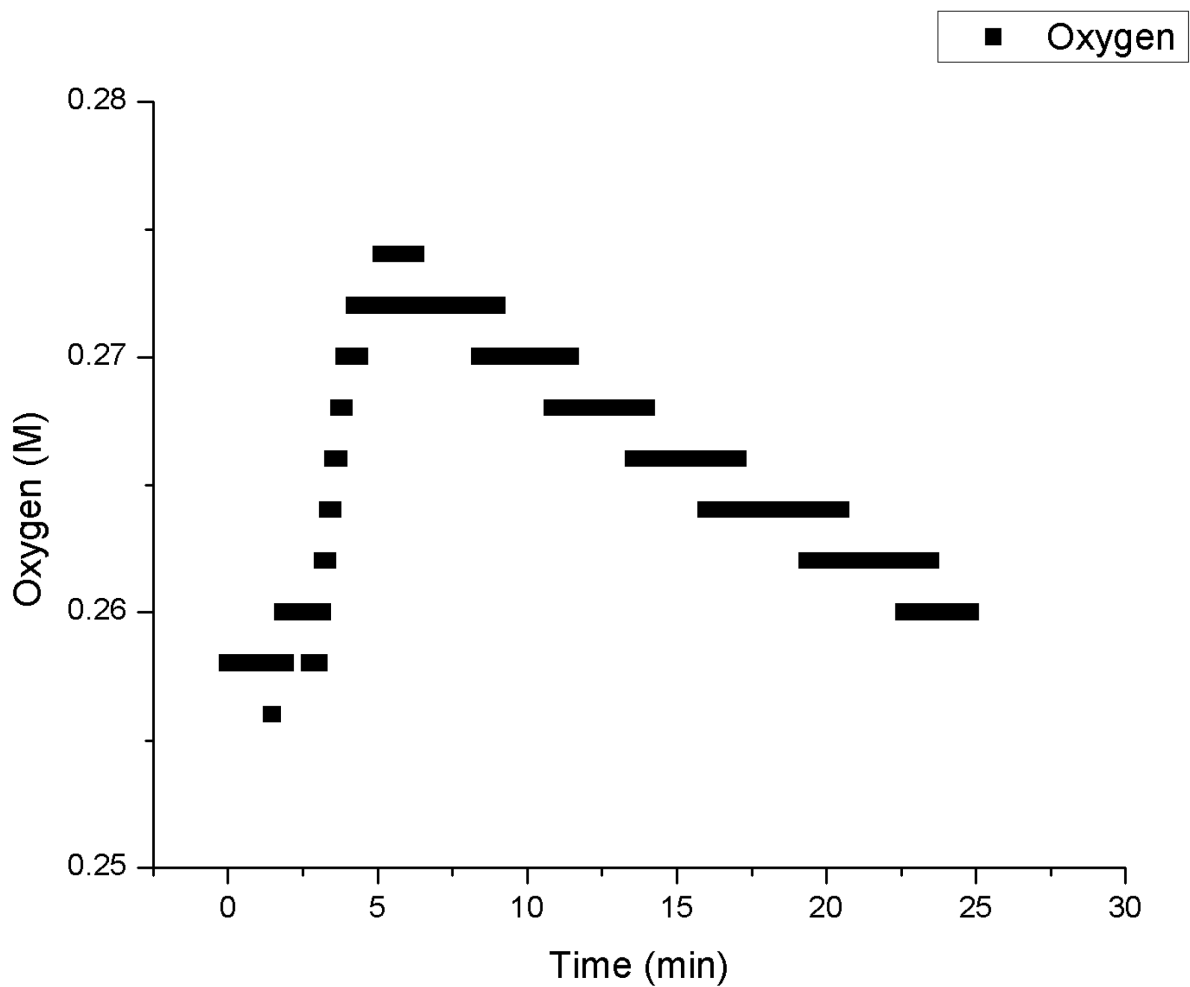


Figure 13. Oxygen production of chloroplast suspension (Batch 2 – Day 1)

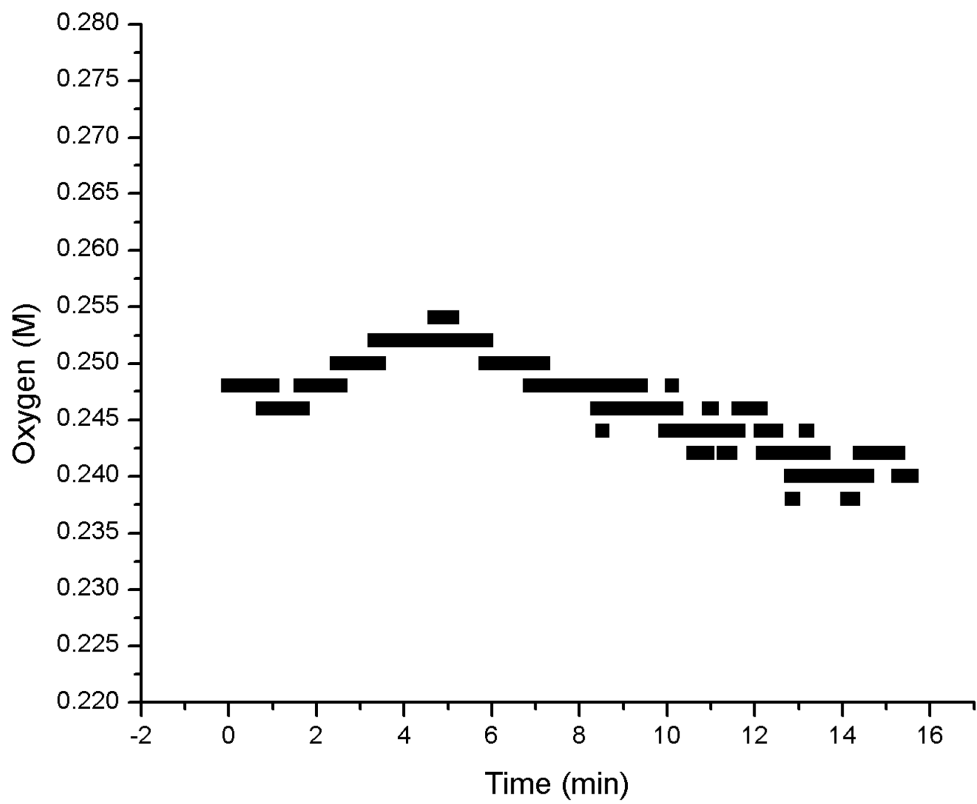
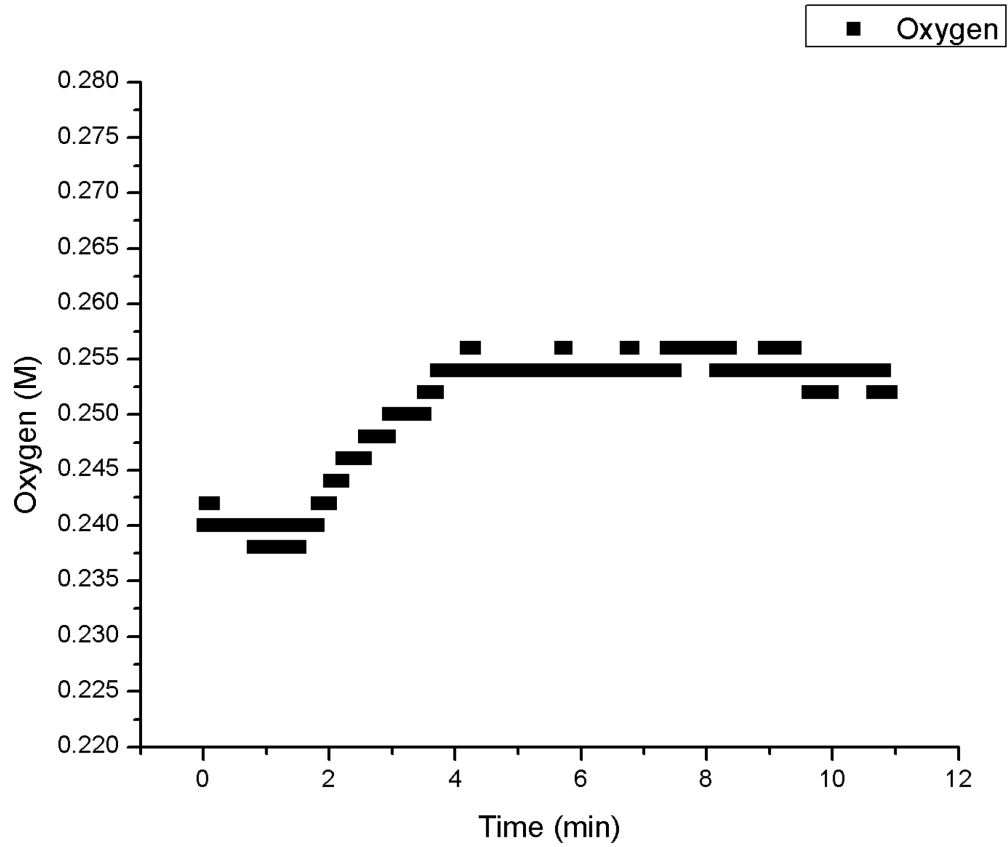


Figure 14. Oxygen production of chloroplast incorporated silk fibroin film (Batch 2 – Day 7)

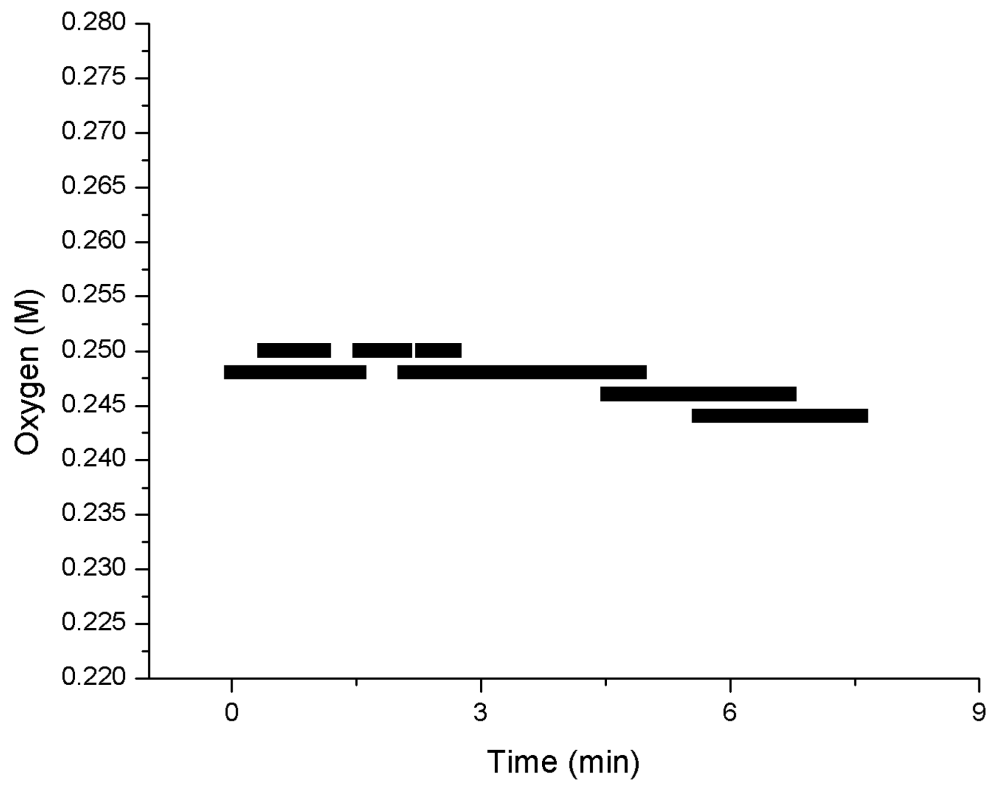
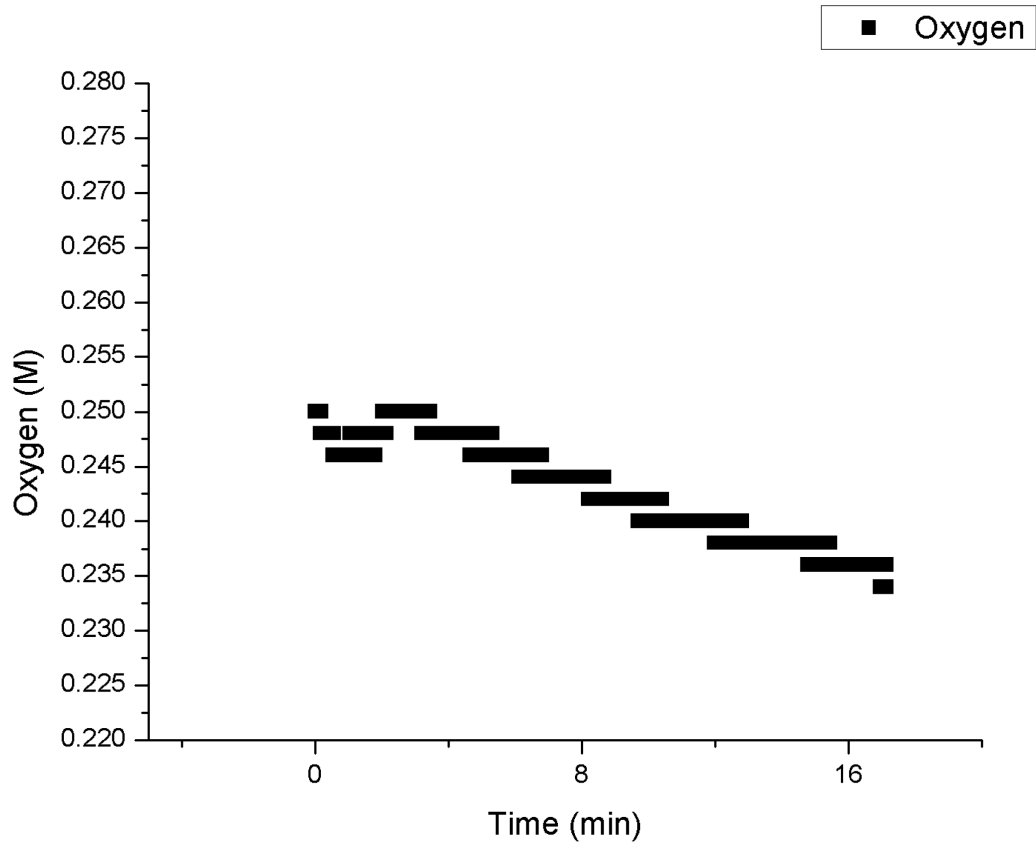


Figure 15. Oxygen production of chloroplast incorporated silk fibroin film (Batch 2 – Day 14)

Upon completion of analyzing the second batch of films, time was spent practicing and revising the chloroplast isolation process. It was noticed that variations in the chloroplasts isolated from the baby spinach leaves resulted from inconsistencies in the blending and filtering of the spinach leaves. Numerous batches of chloroplasts were isolated with the purpose of producing a consistent chloroplast suspension. Inconsistencies in the blending of the leaves, filtering of plant solution, and centrifuging often resulted in poor or unstable readings. Some patches of chloroplasts produced films that would not set into films correctly.

After we were able to produce consistent batches of isolated chloroplasts and effectively set them into silk fibroin films, more tests were run to determine the effectiveness of the chloroplast incorporated films in producing oxygen. The films were tested on a daily basis after they were produced to determine the effect of time on the ability of the films to produce oxygen. One such batch of chloroplast incorporated silk fibroin films was tested daily after chloroplast isolation up until five days after isolation when the films no longer demonstrated any oxygen-producing capabilities. The film tested after one day after chloroplast isolation demonstrated the most impressive oxygen production (Figure 16). Readings taken from the second day showed significant oxygen production at levels less than those of day one (Figure 17). Readings taken from day three still showed oxygen production but at inferior values than those measured on day two (Figure 18). Readings taken on day four exhibited even less oxygen production (Figure 19). On the fifth day of testing, no oxygen production was detected (Figure 20).

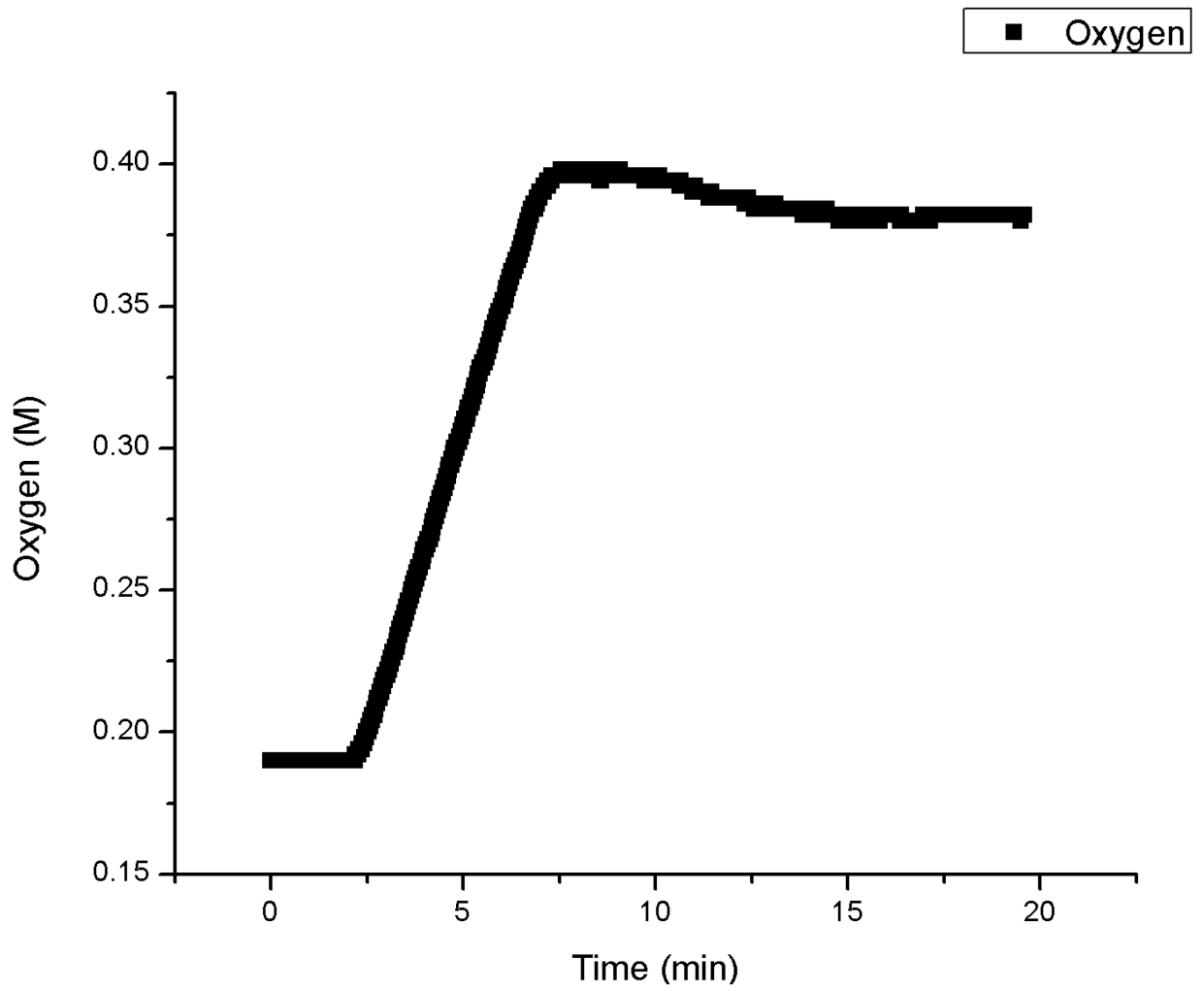


Figure 16. Oxygen production of chloroplast incorporated silk fibroin film (Batch 3 – Day 1)

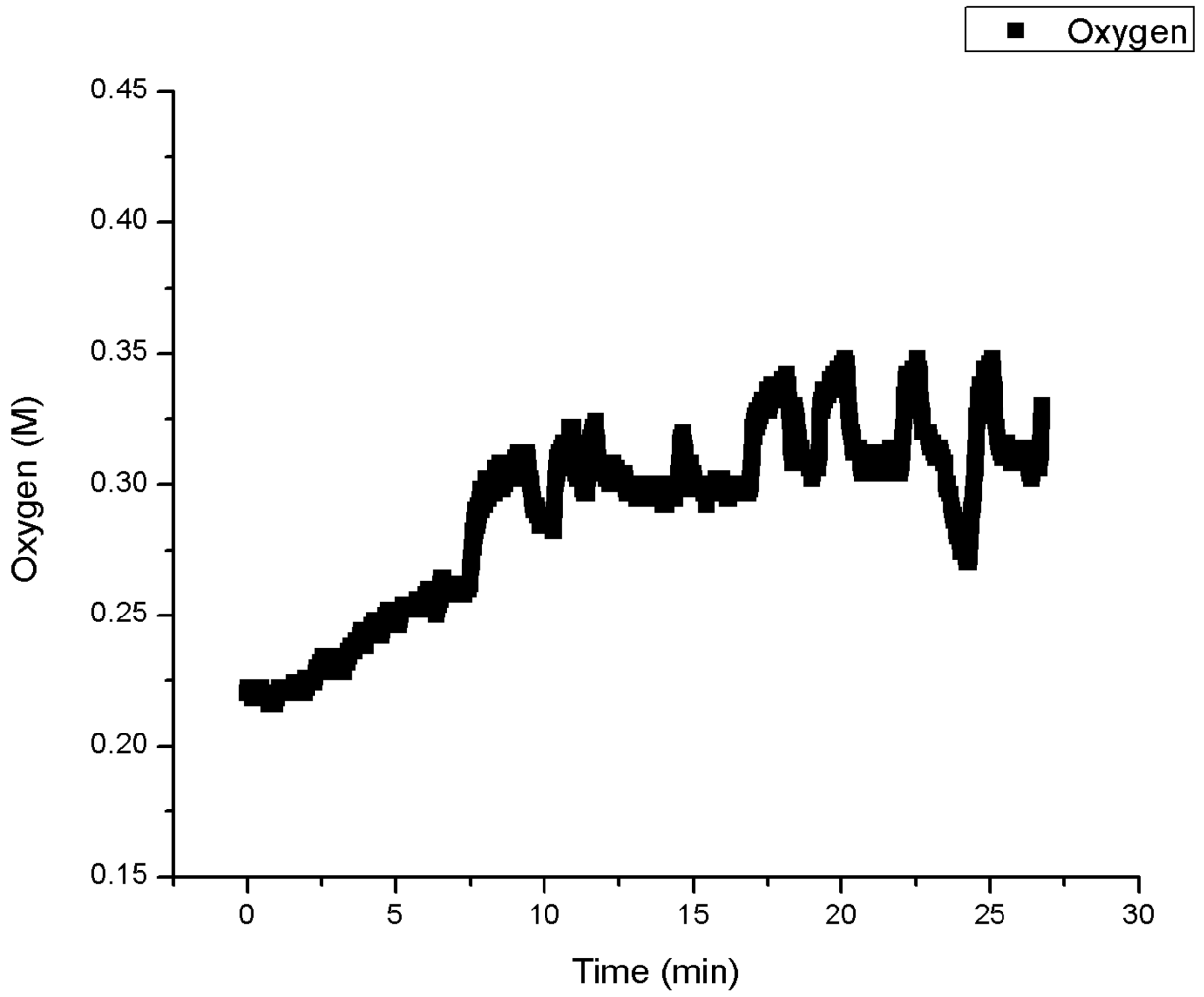


Figure 17. Oxygen production of chloroplast incorporated silk fibroin film (Batch 3 – Day 2)

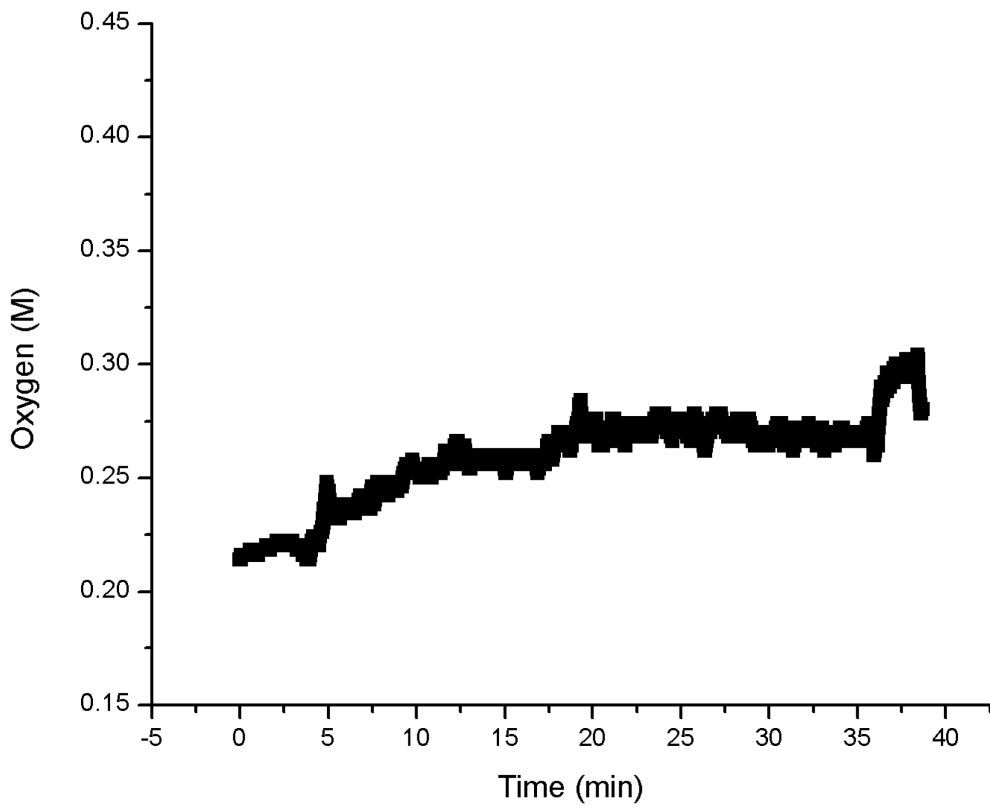
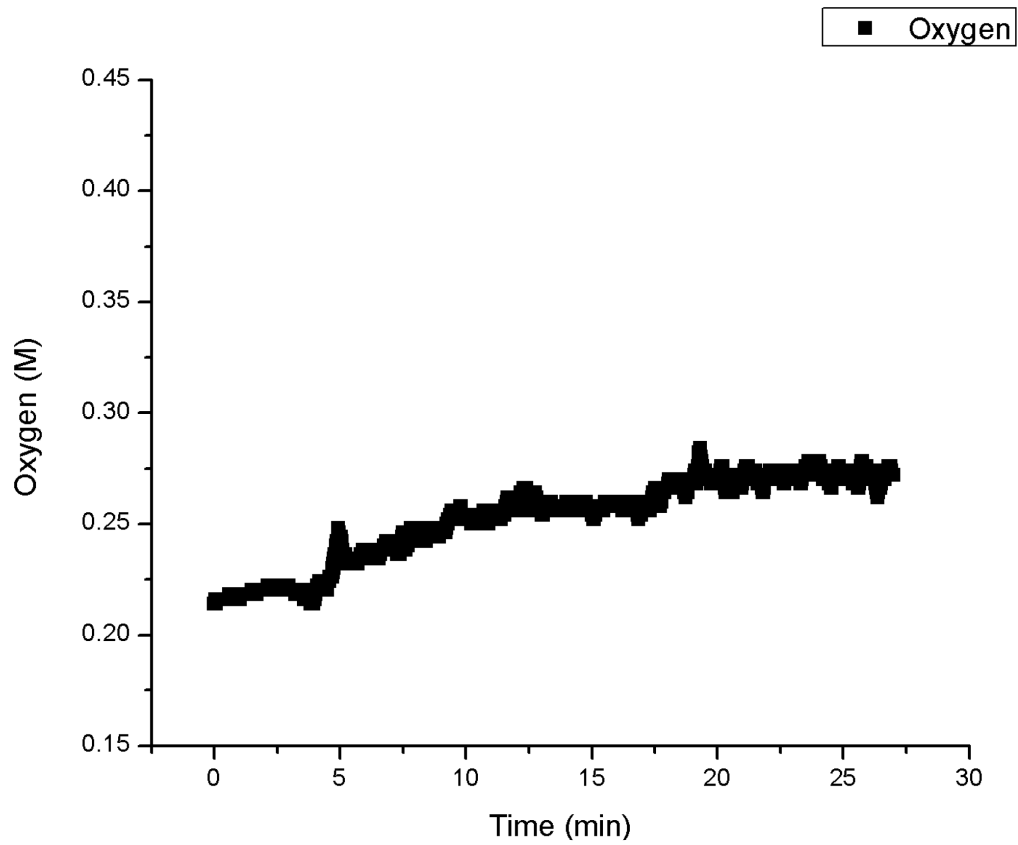


Figure 18. Oxygen production of chloroplast incorporated silk fibroin film (Batch 3 – Day 3)

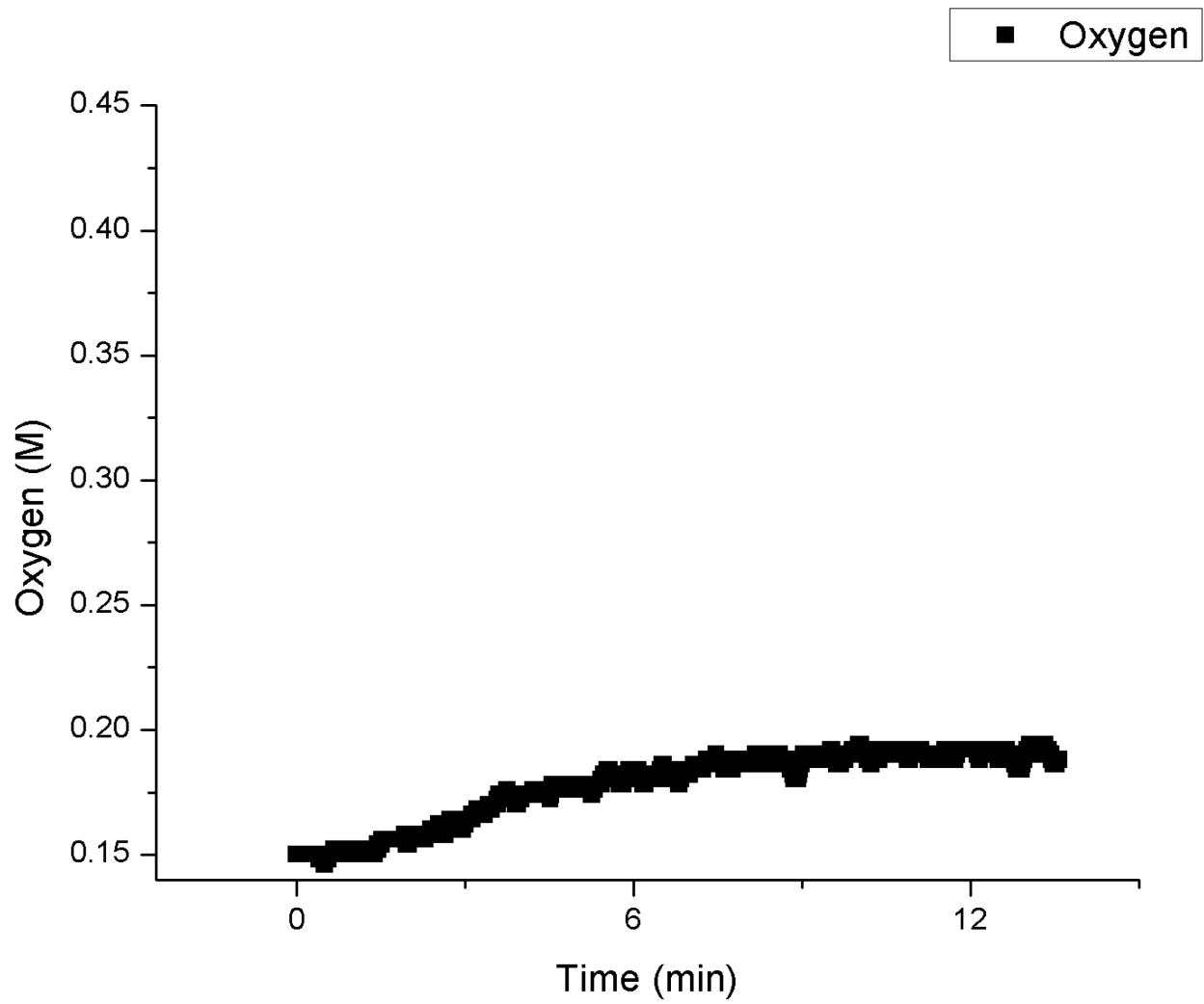


Figure 19. Oxygen production of chloroplast incorporated silk fibroin film (Batch 3 – Day 4)

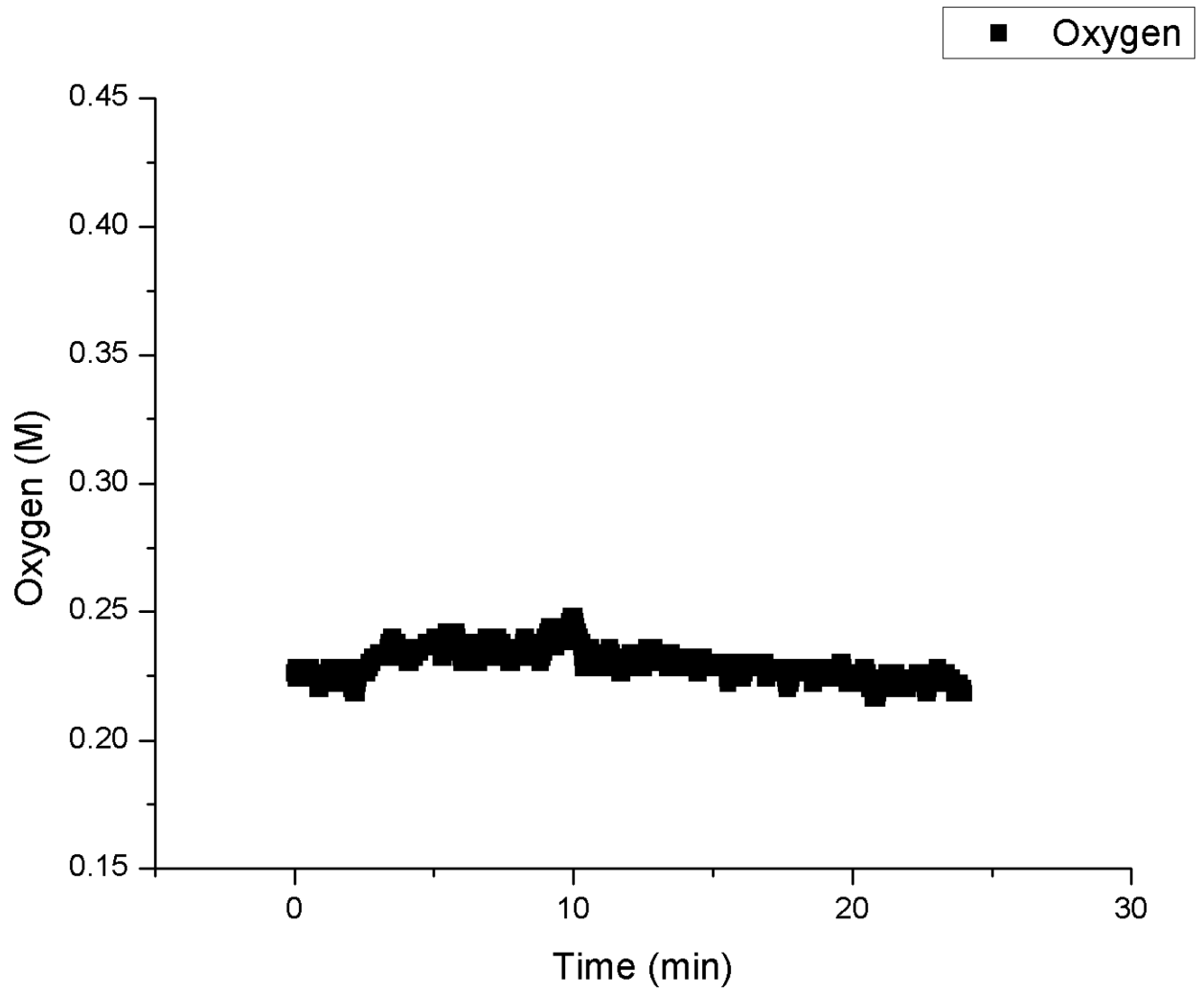


Figure 20. Oxygen production of chloroplast incorporated silk fibroin film (Batch 3 – Day 5)

DISCUSSION

We were successful in completing the first and second specific aims, which were the immediate goals of this study. We were able to successfully fabricate chloroplast incorporated silk fibroin films for the purpose of oxygen production. The two part method essentially consisted of the isolation of the chloroplasts from baby spinach leaves and the suspension of the chloroplasts in silk fibroin solution that would later be plated and set into a film. The initial results of the chloroplast isolation and film setting were extremely varied and inconsistent. The quality of the chloroplasts varied based on the types of spinach leaves available at the time of purchase. Because we did not plant the spinach ourselves, the freshness of the spinach leaves seemed to fluctuate based on when and where the leaves were purchased. Also, the guidelines we followed for our isolation process seemed to leave room for interpretation at certain points. For example, a process which requires the blending of the spinach leaves in a blender called for the blender to be used for a few seconds. No blade speed was specified and also it was difficult to determine how fine the eventual spinach puree should be. Following the guidelines too rigidly sometimes resulted in large chunks of leaf that chloroplasts could not be isolated from. Also, blending the spinach leaves for too long seemed to result in too many broken chloroplasts and not enough intact chloroplasts. One batch of films we fabricated used chloroplasts that were obtained in an isolation process that did not filter well and the films ended up containing extra plant components that kept many of the films from setting. After enough batches of films were made, our familiarity with the isolation and film setting process increased and the data collected from the films became more consistent.

We were also able to successfully assess the oxygen production of all the fabricated films as well as chloroplast suspension. The Chlorolab System we used was relatively easy to use and

straight forward for our purposes. The Chlorolab System monitored the oxygen concentration in an aqueous environment and this suitable enough to effectively monitor the oxygen producing activity of the films we tested.

The results we obtained from our tests demonstrate that the isolated chloroplasts incorporated into the silk fibroin maintained their photosynthetic activity for a prolonged period of time. The isolated chloroplasts that were suspended in buffer lost their photosynthetic ability relatively quickly. While our tests do show that silk fibroin solution can be used to encapsulate chloroplasts and preserve their photosynthetic activity they also showed that even chloroplasts incorporated into silk fibroin lose their oxygen producing capabilities over time. Additionally, the results obtained from our chloroplast incorporated films varied greatly based on the batch of isolated chloroplasts they were obtained from. When the isolation process and the spinach leaves used were ideal, the silk films would be very effectively produce oxygen. However, there were numerous batches of films that were produced that yielded very little photosynthetic activity. These inconsistencies in photosynthetic performance can likely be attributed to the chloroplasts used or the Chlorolab System itself. We noticed that when we encountered inconsistencies along the isolation process, the isolated chloroplasts were often less active than expected. Also, the amount of oxygen produced always seemed to vary with each batch of spinach leaves used to isolate chloroplasts from. Ideally, a more specific isolation process and more consistently fresh source of spinach would be used to ensure consistently fresh and active isolated chloroplasts.

The Chlorolab System we used to measure oxygen production was sensitive enough to show that our films were producing oxygen and to measure the evolution of oxygen levels in the chamber. However, the nature in which the readings were taken seems to invite inconsistencies in the data received from the tests. The oxygen sensing electrode is located at the bottom of the

chamber and a small stirrer is located directly on top of the electrode to ensure that contents in the chamber circulate continuously. During our tests, we noticed that the stirrer would sometimes become dislodged from its spinning position or get obstructed by silk fibroin film circulating around the chamber. These events often resulted in major drops in the oxygen level being sensed by the electrode. In addition, the electrode at the bottom of the chamber needed to be prepared and calibrated prior to each test session. We observed that after some preparations and calibrations, the device did not always function exactly as it had other times. It seemed that the accuracy of the device sometimes varied from time to time. Ideally, the oxygen monitoring system would perform consistently each and every time it was used. Also, it would have been appropriate to measure oxygen production in environments that are not aqueous. However, this would require purchasing an entirely different oxygen level detecting system.

This study was successful in achieving the first two specific aims and in accomplishing these specific aims, we were able to show that it is possible to isolate chloroplasts from higher plants and incorporate them into silk fibroin solution to produce an oxygen producing biomaterial. While we were able to produce effective films more consistently over time, this study has shown that more optimization must be done to improve the quality of the chloroplasts isolated from plants, to improve the quality of the silk fibroin film incorporated with these chloroplasts, as well as to improve the system to test the effectiveness of the films in producing oxygen.

CONCLUSION

Our preliminary results show that an oxygen producing biomaterial can be fabricated by silk fibroin films incorporated with chloroplasts isolated from higher plants. While the initial results were not completely consistent in terms of level of oxygen production, we have shown that photosynthetic activity can be preserved and activated in chloroplasts that have been incorporated into silk fibroin. It has been shown that more time is necessary to optimize the chloroplast isolation process and the accuracy of the data gathering methods can be improved.

We were unable to address any of the long term specific aims of the study. However, the successful achievement of the first two specific aims which are the immediate goals of the study set a foundation for continuation of the project and realization of the long term goals of this study.

FUTURE WORK

Future work on the project would require the optimization and establishment of a rigid protocol for the isolation of chloroplasts and the fabrication of the silk fibroin films incorporating the isolated chloroplasts. The ability to achieve little variation in the quality of the films produced would result in much more consistent test results. The method in which the oxygen production of the films is measured can also be optimized and improved. There should be little variation in the way the device performs so ensure accuracy and consistency of the data.

Once the films and oxygen production monitoring are optimized, future work can focus on the third and fourth specific aims outlined by this project. Testing of the biocompatibility and eventual in vivo testing of the films would be major advancements toward the production of an oxygen producing biomaterial for tissue engineering purposes.

Additional future directions with this study may not even have to be completely medically focused. The successful production of a biomaterial that possesses no biocompatible properties, yet effectively produces oxygen through photosynthesis over prolonged periods of time, would equate to the creation of an artificial leaf. Such a creation would have a huge impact on the fields of bio-catalysis and bio-sensing and possibly even contribute to the effort to reduce greenhouse gases in the atmosphere and the overall effort to curb global warming.

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