Spring 10-2003

Increasing the Stability of Pancreatic Islets During Transportation

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SENIOR PROJECT - APPROVAL

Name: Joshua Brock Thomas

College: Engineering
Department: Chemical

Faculty Mentor: Dr. Paul Frymier

PROJECT TITLE: Increasing the Stability of Pancreatic Islets During Transportation

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: ___________________________, Faculty Mentor

Date: 10/30/03

Comments (Optional):

SEE ATTACHED LETTER
April 30, 2003

To Whom It May Concern:

It has been an absolute pleasure to work with Mr. Thomas. If I had a lab full of equally qualified and motivated students, life would be much easier. He has been a credit to the program as well as to the University at large. I think the opportunity to work on an independent research project was a great learning experience for Mr. Thomas. I wish him every opportunity for success in his future research career.

Sincerely,

[Signature]

Paul Frymier
Associate Professor of Chemical Engineering
Increasing the Stability of Pancreatic Islets During Transportation

Senior Honors Project

University of Tennessee
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J. Brock Thomas

B.S. Chemical Engineering, Honors

May 2003
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Summary

Pancreatic islets, also called islets of Langerhans, are clusters of β-cells that produce glucose-level-controlling insulin. If the body has an autoimmune response to these β-cells and begins destroying them, Type 1 diabetes (insulin-dependent) results.
Transplantation of healthy islets into the body has been a measure that has proven to be successful at treating diabetes. Transplantation of the islets has occurred using naked islets and encapsulated islets. Naked islets require immunosuppression, whereas encapsulated islets are protected from the immune system.

Islets are encapsulated using a wide variety of biocompatible polymer systems. Calcium alginate is one of many natural polysaccharides that are used in encapsulating islets. The commercially available form of alginate is sodium alginate which, when added to water, forms a viscous liquid mixture. When the islet cells are added to this viscous liquid and the mixture is dropped into a solution containing calcium chloride, gelled beads are formed. Cells are trapped in the beads formed, providing a protective barrier for the cells.

This project's goal is to investigate the use of this protective coating as a way of sustaining the activity of the islets during transportation from digestion location to transplantation location. A genetically engineered bioluminescent bacterium designated PM6 was chosen as an initial model system in order to determine if the removal of the cells from the encapsulation material would be likely to cause damage to the cells.

PM6 produces light during its normal growth phase with the ability to act as an instantaneous indication of cellular activity. PM6 was encapsulated in gel beads using calcium alginate. Later, the cells were removed from encapsulation by placing the beads in a solution of sodium chloride. The cell density and light emitted by previously encapsulated PM6 was compared to that of PM6 grown under liquid culture conditions. The lag phase is slightly longer for the encapsulated cells, but the time dependent cell
density and light emission by the previously encapsulated bacteria are essentially the same as that of unencapsulated PM6.

The next step of this project is to look at the stabilizing effects of removing immortalized endothelial kidney cells from the calcium alginate. These cells are considered more sensitive than the bacteria used in this initial study but are easier to maintain in culture than islets. If positive results are obtained for the kidney cells, the study will proceed to the pancreatic islet cell line.
Background

Pancreatic Islets

Pancreatic islets are often referred to as the islets of Langerhans. These islets are clusters of insulin producing and secreting $\beta$-cells found in the pancreas. Insulin is the hormone necessary to regulate the glucose level within the body. When these $\beta$-cells are destroyed by an autoimmune response, the body becomes insulin deficient. This disease is known as Type I diabetes and accounts for five to ten percent of the diagnosed cases of diabetes in the United States.$^1$ The number of people that suffer from Type I diabetes is approximately 0.85 – 1.7 million.$^1$ The most prevalent treatment for Type I diabetes is through some form of insulin dosage. Insulin must be administered daily, either through numerous injections or by an insulin pump, in order to control the blood-glucose level. Secondary complications, such as nephropathy, neuropathy, and cardiovascular problems,$^2$ can be prevented by intense insulin therapy; however, patients taking insulin have an increased risk in experiencing hypoglycemic episodes.

Isolation of Pancreatic Islets

Warnock and Rajotte first developed the method of isolating pancreatic islets, and Lanza et al. later modified the method.$^{3,4}$ The pancreas is injected with cold University of Wisconsin organ preservation solution$^5$ and removed from the body. The organ is then injected with a crude solution of collagenase via the pancreatic duct system. The enzyme digests the pancreas, and the dissociated tissue is washed with cold medium. Utilizing the density difference between the islets and the exocrine tissue, the islets are separated,
collected, and washed several times. The separated islets are placed in either an α-MEM or Ham's F12 based medium. The compact appearance of the islets is regained after approximately 24-48 hours in culture.

**Encapsulation and Transplantation of Pancreatic Islets**

Whole organ transplantation of the pancreas requires an extensive surgical procedure. Therefore, it is desirable to only transplant the insulin-producing islets. Encapsulation of the islets into a biocompatible polymer that continue to allow the secretion of insulin has been successful in sustaining normoglycemia. Due to the large volume of the encapsulated islet grafts, the peritoneal cavity was designated as the ideal transplantation site. A small incision is made in the abdomen, and the encapsulated islets are transplanted to the cavity. Encapsulation eliminates the need for immunosuppression, and transplantation in the peritoneal cavity reduces the risks associated with a full organ transplant. However, most of the islet transplants are still performed using naked islets because of the lack of government approval for encapsulated islets.

During transportation from the digestion location to the transplantation site, the activity of some of the islets is lost due to unknown causes. These causes could be extreme temperature variation or even shock and stress experienced while in transit. The objective of this project is to determine if encapsulating the islets in a biocompatible material will retain the activity of the islets during transportation. The basis of this biocompatible material will be alginate.
**Alginate**

Alginate is an anionic polysaccharide derivative of brown seaweed. Alginites have hydro-gel forming properties with many di-, tri-, or multivalent cations. A semi permeable gel is formed when the alginate gel is cross-linked with one of these cations. Because of its mild gelling and good biocompatibility, alginate gel has long been used in the food and pharmaceutical industries. Alginates are linear copolymers of 1,4-linked \( \alpha \)-L-guluronic acid (G) and \( \beta \)-D-mannuronic acid (M) arranged as chains in alternating block wise patterns. The relative amounts of guluronic and mannuronic acid in the alginate has a significant impact on porosity, swelling behavior, stability, biodegradation ability, gel strength, immunological characteristics, biocompatibility, and diffusion.

**Bioluminescent Bacterium Designated PM6**

Due to the lack of availability of pancreatic islet cells, the preliminary studies to determine if the encapsulation and separation processes were harmful were performed on a surrogate cell line. This cell line is a bioluminescent bacterium designated PM6 that was created by researchers at Syracuse University. The bacterium is a genetically modified bacterium that is derived from a *Pseudomonad* strain isolated in activated sludge. The activated sludge microorganisms were mated with *E. coli* carrying the pUTK2 plasmid. The pUTK2 plasmid contains a *lux* transposon and a tetracycline resistant gene. The *lux* transposon contains the five genes necessary to catalyze light and a constitutive promoter, which allows the bacterium to produce light under normal conditions.

The reason this bacterium was chosen is because it is widely available in the
laboratory, and other researchers working in the laboratory are familiar with growth and assay procedures for PM6. Since the bacterium emits light under normal growing conditions, this characteristic could be utilized in an assay that determines if the bacteria survived the encapsulation and separation procedure.

**Work Performed**

*Equipment and Materials Used*

The cell density of the PM6 bacteria was determined using a Beckman spectrophotometer DU520 at a wavelength of 600 nm. The light emission was measured using a Sirius Luminometer. An Innova 4000 (New Brunswick Scientific) incubator/shaker was used to grow the PM6 in culture. A 1.8% w/v solution of alginic acid, sodium salt (Sigma—from macrocystis pyrifera (Kelp) low viscosity [9005-38-3]) in distilled water was the source of the alginate, and a 0.05 M solution calcium chloride (Fischer Scientific) in distilled water served as the divalent cation used in the hydro-gel bead formation. A 2.0 M solution of sodium chloride (Fischer Scientific) distilled water was the source of the sodium ions to break the cross-linking between calcium and alginate. The sodium chloride and calcium chloride solutions were pH adjusted using hydrochloric acid and sodium hydroxide. Difco nutrient broth (Becton Dickinson) prepared according to the instructions of the manufacturer was the growth medium used in growing the PM6. A 2.5 g/200 mL solution of tetracycline was also used in the growth of the bacteria. The bacterium has a tetracycline resistance gene, and the presence of tetracycline allows the growth of the bacteria in pure culture.
**Production of a Growth and Light Emission Curve for Unencapsulated PM6**

Unencapsulated PM6 was first grown to determine a growth and light emission curve in normal liquid culture. 100 μL of frozen PM6 was transferred to a 1 L flask containing 100 mL of an 8 g/L solution of nutrient broth. 80 μL of a 2.5 mg/200 mL solution of tetracycline was added to the flask. The flask was placed in the incubator/shaker at 30°C and 200 rpm. Cell density and light emission readings were obtained every 15 minutes starting at approximately 4.5 hours from inoculation. Readings were taken until the optical density of the solution reached 2.6-2.8.

![Cell density and light emission curves for unencapsulated PM6 at 30°C and 200 rpm.](image)

**Figure 1:** Cell density and light emission curves for unencapsulated PM6 at 30°C and 200 rpm.
**Encapsulation of PM6 Using Calcium Alginate**

PM6 was grown in liquid culture following the previously mentioned procedure. When the cells had reached an \( \text{OD}_{600\text{nm}} \) of approximately 0.9-1.0, the cells were cooled to 4°C. 2 mL of the refrigerated cells were spun down and activated using 1 mL of fresh 8 g/L nutrient broth for 20 minutes. After the activation time had expired, the cells were spun down and 500 \( \mu \text{L} \) of alginate was added to the micro centrifuge tube. The mixture was agitated to ensure the cell pellet had dissolved in the alginate.

The source of alginate used in this project was alginic acid. The divalent cation used as the cross linking agent was \( \text{Ca}^{2+} \), obtained from the compound calcium chloride. Gel beads containing encapsulated PM6 were made by dropping this 500 \( \mu \text{L} \) of 1.8% w/v solution of sodium alginate containing spun down PM6 (2 mL of \( \text{OD}_{600\text{nm}} \) refrigerated cells) from a 5 cc syringe and 23 G 1½” needle into a 150 mL solution of 0.05 M calcium chloride. The divalent \( \text{Ca}^{2+} \) cross links the alginate immediately to form a gel bead containing encapsulated PM6. The beads and calcium chloride are stirred at 300 rpm for approximately 5 minutes to allow complete gelation.

\[
2 \text{Na(Alginate)} + \text{Ca}^{2+} \rightarrow \text{Ca(Alginate)}_2 + 2 \text{Na}^+
\]

The gel beads were then removed from the calcium chloride solution by decantation. The gel beads were placed in 200 mL of a 2.0 M solution of sodium chloride. The sodium chloride and gel beads were stirred at 600 rpm until the PM6 separate from the gel beads. The first data set below represents a 4.5-hour interaction time between the encapsulated PM6 and the 2.0 M sodium chloride solution. The second set of data below represents a 2-hour interaction time between the encapsulated PM6 and the sodium chloride. Once the cells have separated from the gel beads, the solution is
centrifuged for 25 minutes at 12000 rpm. The sodium chloride is decanted from the sodium alginate and PM6 remaining at the bottom of the centrifuge bottle. 100 mL of an autoclaved 8 g/L solution of nutrient broth containing 80 μL of 2.5 g/200 mL tetracycline was transferred to the centrifuge bottle. The solution was agitated to ensure the PM6 and gel were returned to a homogeneous mixture with the nutrient broth.

**Production of a Growth and Light Emission Curve for Encapsulated PM6**

The nutrient broth, sodium alginate, and PM6 solution were transferred to a 1 L flask and placed in the incubator/shaker at 30°C and 200 rpm. Light emission and optical density readings were taken for the solution.

![Graph of Cell Density and Light Emission Curve for Encapsulated PM6](image)

**Figure 2:** Cell density and light emission curve for previously encapsulated PM6 at 30°C and 200 rpm, 4.5 hour sodium chloride interaction.
Figure 2: Cell density and light emission curve for previously encapsulated PM6 at 30°C and 200 rpm, 2 hour sodium chloride interaction.

Discussion of Results

The encapsulation and separation procedure used with bioluminescent bacterium PM6 did not inhibit light production or growth. The previously encapsulated bacteria exhibited cell density and light emission values similar to those of unencapsulated bacteria, which can be noted from a comparison of Figure 1 and Figure 2. The previously encapsulated bacteria were carefully examined under a microscope to determine if the bacteria were present in the same form as that of unencapsulated bacteria. The cells appeared to be the same as that of unencapsulated bacteria. If the cells were damaged in the process, one would have expected the cells to neither produce light nor grow.
The interaction time is a significant factor in the lag time of the bacteria. A 4.5-hour interaction produced an approximately 18 hour lag phase, whereas a 2-hour interaction time resulted in approximately a 7.5 hour lag phase. This is most likely due to an extreme osmotic pressure gradient caused by the high salt concentration.

**Recommendation for Future Work**

The next cell line that needs to be studied is immortalized endothelial kidney cells. These cells are readily available within the University of Tennessee Center for Biotechnology. The kidney cells would be more sensitive than the bacteria used in this study, and would provide one additional stepping-stone before encapsulation and separation of primary pancreatic islets, which are more difficult to obtain and culture, are examined. The form of alginic acid used in future experiments should be one that has been purified and is preferably of a higher guluronic acid content because the guluronic and mannuronic acid ratio directly affects the diffusion properties of the bead. The composition of the alginate affects diffusion, which will become a significant factor in retaining the cell’s activity while encapsulated. Information regarding key factors that degrade the activity of the islets during transportation should be obtained. One may find that encapsulation will not prevent the determined factors from negatively affecting the activity. A source of primary islets must also be determined in order for the study to proceed past the endothelial kidney cell line.
References

Increasing the Stability of Pancreatic Islets

J. Brock Thomas
B.S. Chemical Engineering May 2003
Mentor: Dr. Paul Frymier
Pancreatic Islet Cells

- Referred to as Islets of Langerhans
- Beta cells that secrete insulin when the body experiences an increase in the glucose level
- The destruction of these cells by an autoimmune response results in Type 1 Diabetes (Insulin-Dependent)
Isolation of Pancreatic Islets

- The pancreas is infused with University of Wisconsin organ preservation solution.
- The organ is removed from the body and infused with a crude solution of collagenase.
- The islets are collected using density based purification and placed in tissue culture.
Islet Transplantation

- The isolated islets are transplanted into the peritoneal membrane
- The immune system is suppressed to prevent rejection of the cells

Bioartifical Pancreas

- Entrapment of the isolated cells in a biocompatible polymer system
- Transplantation of the entrapped islets does not require immunosuppression
Objective of Project

- Increase the survival rate of the isolated islets during transportation from point of digestion to point of transplantation
- Utilize the method used to encapsulate islets for bioartificial transplantation to maintain the functionality during transportation
- Exhibit that the cells retain their activity when removed from their protective encapsulation material
Alginate

- Serves as the biocompatible encapsulation material
- Gels under mild physiological conditions
- Naturally occurring polysaccharide found in brown algae
- Available commercially as alginic acid or sodium alginate
Gelling Procedure

- 2% w/v sodium alginate is dropped approximately 10 cm from a syringe and needle into a 150 mL 0.05 M solution of CaCl$_2$.
- The polymer is cross-linked to form a gel bead.
- $2 \text{Na(Alginate)} + \text{Ca}^{++} \rightarrow \text{Ca(Alginate)₂} + 2 \text{Na}^+$

Cell Separation

- The beads are removed from the CaCl$_2$ after 5 minutes and placed in a 200 mL 2.0 M solution of NaCl.
- The reverse of the above reaction occurs, and the encapsulated cells fall out of the gel solution after approximately one hour.
- The mixture is centrifuged to separate the NaCl from the remaining gel and cells.
Cell Lines

◆ PM6 Bioluminescent Bacteria
  ■ Determine if cell separation method destroys cells
  ■ Positive results will indicate the ability of the cells to grow and produce light after gelling, separation, and culture

◆ Endothelial Kidney Cells
  ■ Continue to determine if cell separation method destroys cells
  ■ Positive results will indicate the ability of the cells to reattach after gelling, separation, and culture
Cell Lines (continued)

◆ Pancreatic Islet Cells

- Continue to determine if cell separation method destroys the activity of the cells
- Positive results will indicate the ability of the cells to produce insulin after gelling, separation, and culture
- Determine if encapsulation provides stability against the elements encountered during transportation
PM6 Bioluminescent Bacteria

- Created through directed evolution
- Contains the \textit{lux} transposon which catalyzes light-producing reactions
- Emits bioluminescence under normal growing conditions
Cell Inoculation and Growth

- 100 µL of frozen PM6 cells are inoculated into 100 mL of 8 mg/mL nutrient broth
- The inoculated cells are then incubated and shaken at 30°C
- The growth of the bacteria and light emittance is exhibited in Figure 1
Figure 1: Growth Curve and Light Emitted for PM6

RLUs and Abs as a Function of Time for PM6-03/06/2003
Immobilized Cells Results

- 2 mL of refrigerated PM6 with an OD of 0.9 was spun down and mixed with 500 μL of sodium alginate
- The PM6 cells and sodium alginate were gelled
- The cells were removed out of encapsulation using sodium chloride and placed in growth medium
- Figure 2 represents the growth curve and light emitted for PM6 after having been encapsulated and released from the calcium alginate
- Lag phase is increased, but growth and light emitted follows the same pattern as cultured cells
Figure 2: Growth Curve and Light Emitted for Encapsulated PM6

RLUs and Abs as a Function of Time for Previously Encapsulated PM6-04/02/2003

Time (hours since start)
Conclusions

- Gelling and separation of cells does not destroy the activity and the viability of the PM6 bioluminescent bacteria.
- Encapsulated cells exhibit similar growth and light emitted patterns as those that are cultured.
- Endothelial kidney cells will now be encapsulated to determine if a more sensitive cell line can withstand the cell separation step.
Acknowledgements

◆ Dr. Paul Frymier, professor of chemical engineering
◆ Center for Environmental Biotechnology, lab space, equipment, and supplies
◆ Dr. Thomas Broadhead, director of honors program
Figure 1: Cell density and light emission curves for unencapsulated PM6 at 30°C and 200 rpm.

**Encapsulation of PM6 Using Calcium Alginate**

PM6 was grown in liquid culture following the previously mentioned procedure. When the cells had reached an OD\textsubscript{600 nm} of approximately 0.9-1.0, the cells were cooled to 4°C. 2 mL of the refrigerated cells were spun down and activated using 1 mL of fresh 8 g/L nutrient broth for 20 minutes. After the activation time had expired, the cells were spun down and 500 \(\mu\)L of alginate was added to the micro centrifuge tube. The mixture was agitated to ensure the cell pellet had dissolved in the alginate.

The source of alginate used in this project was alginic acid. The divalent cation used as the cross linking agent was Ca\textsuperscript{2+}, obtained from the compound calcium chloride.

Gel beads containing encapsulated PM6 were made by dropping this 500 \(\mu\)L of 1.8% w/v solution of sodium alginate containing spun down PM6 (2 mL of OD\textsubscript{600 nm}}