Antioxidant Properties of Polymeric Coatings on Cell Encapsulation Platforms

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Abstract

Type 1 diabetes (TID) is an autoimmune disease characterized by β-cell death. Current treatment for TID is exogenous insulin therapy. Clinical islet transplantation has shown promising results in treating this disease, but there are still challenges to overcome. Reactive oxygen species (ROS) is a major player in β-cell death. To modulate ROS, antioxidant biomaterials in microencapsulation platforms were investigated. Alginate encapsulated cells were coated with antioxidant materials and exposed to hydrogen peroxide to test ROS protection. For the polymer coatings, alternating layers of PAMAM and alginate were used. It was found that these coatings were able to reduce the amount of ROS present. Further, alginate beads containing MIN6 cells were coated and showed that these coatings didn't affect viability of encapsulated cells and insulin release isn't blocked by the coatings. When exposed to hydrogen peroxide, coated beads showed more viable cells compared to non-coated beads. To further investigate the antioxidant capabilities of these coatings, dexamethasone was used as a model drug and loaded into PAMAM. Dexamethasone was loaded and released from PAMAM when tested in solution. This work will be translated to bead coatings and drug release kinetics will be tested.

Background

Type 1 diabetes (T1D) affects over 1 million Americans with more than 30,000 new cases diagnosed every year.[1] T1D is an autoimmune disease characterized by the attack to insulin producing β-cells of the pancreas. β-cells, in the islets of Langerhans, are destroyed by the immune system, which leads to decreased insulin production and hyperglycemia. Hyperglycemia, increased blood sugar levels, and hypoglycemia, decreased blood sugar levels, are both common in people with T1D. Irregular blood glucose levels lead to complications such as cardiovascular diseases, retinopathy, nephropathy, and neuropathy.[1,2] Currently, there is no cure for this disease and the only way to treat it is via exogenous insulin (either by injection or by a pump). This treatment helps to delay and prevent the onset of complications caused by T1D, but is not a perfect solution. Some of the disadvantages of insulin treatment include patient compliance, insulin resistance, and risk of hypoglycemia.

The transplantation of new β-cells, either by whole pancreata or isolated islets is currently being research and has been showing promising results.[3,4] Clinical islet transplantation (CIT), the introduction of allogeneic islets to the body is a potential cure for TID patients. CIT has progressed over the last 12 years and now shows that multiple donors can provide insulin independence for select patients.[5] The current procedure for CIT includes the transplantation of islets through infusion into the intraportal vein, followed by a rigorous immunosuppressive regimen.[5] The problem with CIT is that patients have to be on immunosuppressant drugs for their entire life and that 60% of transplanted islets are lost at an early stage.[5] The rejection of islets and the loss of β-cell mass is due to four main mechanisms: non-specific inflammatory host reactions, allo-rejection given to self/non-self-discrimination, TID associated auto-reactivity, and poor engraftment of the islets within the liver site.[6]

In order to improve islet viability and protect the islets from potential dangers, researchers are looking into polymeric encapsulation of islets. According to one article, an ideal encapsulation system would be a gel like material that is noncytotoxic, provides a barrier to the immune system, allows proper diffusion of nutrients and waste through the barrier, is biocompatible, and is chemically and mechanically stable.[5] A popular polymer to encapsulate islets is alginate. Alginate is a naturally occurring anionic polymer derived from brown algae.[5] It has many desirable properties including biocompatibility, low toxicity, and low cost.[5] One problem faced

when encapsulating cells in a permeable matrix is the intrusion of low molecular weight toxins and reactive oxygen species (ROS). ROS is a term that includes oxygen radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), and hydroperoxyl ($HO_2^{\cdot}$) radicals, and certain non-radical oxidizing agents, such as hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) that can be converted easily to into radicals.[6] ROS are found in inflammatory environments and lead to direct β-cell death and immune cell recruitment and activation. ROS trigger the innate immune system by promoting cells to attack and destroy the β-cells by producing cytokines and toxic soluble agents including more ROS.[7–9] The adaptive immune system is also triggered by the ROS produced by the innate immune cells and promotes the differentiation of adaptive immune cells into effector cells.[10] There are two main types of ROS that play a role in β-cell death: intracellular ROS and extracellular ROS. In the extracellular environment ROS damage β-cells via oxidation of lipoproteins and overproduction of Matrix Metalloproteases (MMPs). Intracellular ROS are produced by immune cells as well as β-cells when the cells are under stress. Intracellular ROS plays a role in β-cell death by activating intracellular pathways leading to necrosis and apoptosis.

To effectively protect the encapsulated β-cells, a redesign to the polymeric encapsulation method is needed. A new method is the introduction of ultrathin, nanoscale coatings on the polymeric encapsulated biomaterial. This would be done by adding the polymer layers on the outside on the biomaterial. A polymer that can be used to serve this purpose in poly (amidoamine) (PAMAM). PAMAM is a dendrimer that is synthesized by the divergent method. As dendrimers, they are composed of three main components: a central atom, branches extending from the atom, and functional groups on the end of the branches.[10] By adding these coatings to the biomaterial, the β-cells are better protected from extracellular ROS. However, intracellular ROS created by the islets inside the polymeric encapsulating biomaterial still pose a threat to the survival of the β-cells. To help combat this, antioxidant loaded polymer coatings will be added to the outside of the polymeric encapsulating biomaterial. This can be done with PAMAM because it is considered a unimolecular micelle, with a hollow internal lipophilic structure, which entraps low-water soluble drugs, and an outer hydrophilic surface for functionalization.[10] This allows PAMAM to encapsulate various agents by hydrogen bonding drugs to the internal tertiary amines. Therefore, encapsulation of antioxidants, such as vitamins, coenzyme Q10 and lipoic acid in the PAMAM branches and subsequently the polymeric biomaterial should be feasible.

Focus

In this paper, I will explore the antioxidant properties of PAMAM and test its properties when applied as coatings on alginate and glass beads. Also, I will look at the formation of polymeric encapsulation of islets with drug loaded PAMAM coatings. Specifically, the fabrication of this biomaterial and the release of antioxidants from the PAMAM coatings. Dexamethasone (DEX) will be used as a model for drug (antioxidant) release.

Methods

Fabrication of PAMAM Coated Glass Beads

Acid washed-glass beads were purchased from Sigma Aldrich (75μm). Glass beads were quantified via weighing and 300 mg of glass beads were aliquoted per group into a 2 mL microcentrifuge tube. The coatings were based on electrostatic interactions, hydrogen bonding, and covalent crosslinking between silica groups because silica beads are presumed to be negatively charged.

To increase the number of hydroxyl (OH) groups on the surface of the beads and electrostatic bonding, the beads were plasma etched with 0.1 M NaOH at 80°C for 1 min and then washed 3 times with MOPS buffer. Coatings were then added to the glass beads.

To coat the beads alternating layers of PAMAM (1^{st} and 3^{rd} layers) and alginate (2^{nd} and 4^{th} layers) were incubated with the beads. Incubation was done for 10 minutes at 37°C and washed 3 times in between layers with MOPS buffer.

Hydrogen Peroxide Assay on PAMAM Coated Glass Beads

After coating, beads were left incubating for a few hours before testing antioxidant activity. Beads were washed with MOPS and then exposed to a 25μM solution of hydrogen peroxide for 2 hours at 37°C with shaking every 10-15 minutes. After 2 hours of incubation, 50 microliters of solution (n=3) was collected to quantify the amount of hydrogen peroxide left in solution using a Hydrogen Peroxide Colorimetric Dye (Enzo Life Sciences). The hydrogen peroxide sample (50 microliters) was mixed with 100 microliters of hydrogen peroxide colorimetric dye in a 96 well plate and incubated for 30 min for color to fully develop. Absorbance was read at 585 nm.

Encapsulation of MIN6 Cells in Alginate Beads

A day before bead fabrication, prepare the alginate needed at 1.6% in MOPS buffer. Next, sterilize the alginate using a 0.2μm syringe filter. Then add 6 million MIN6 cells per mL of alginate. Set up the encapsulation stand and needle in the biosafety cabinet. The encapsulation stand includes the bead generator. The bead generator is a device that has three openings, one for the alginate to come in, one for it to come out and one on the side where air is pushed through. Place a petri dish with 50mM $BaCl_2$ MOPS below the needle where the beads will come out and connect the syringe filled with alginate to the encapsulation stand. Attach the syringe to the top of the bead generator and steadily press down on the plunger of the syringe until all the alginate is expelled. After 5 minutes in the $BaCl_2$ MOPS solution, begin transferring the beads to a 50 mL conical vial. At 10 minutes, remove the $BaCl_2$ MOPS solution and wash beads with MOPS.

Hydrogen Peroxide Assay of MIN6 Encapsulated Coated Beads

After the beads had 4 alternating layers of PAMAM and alginate, they were washed with MOPS and then exposed to a 25µM solution of hydrogen peroxide for 2 hours at 37°C with shaking every 10-15 minutes. After 2 hours of incubation, the beads were washed three times with MOPS and then 180µL of DMEM media and 20µL of Alamar Blue was added. This was incubated at 37°C for 4 hours. Then, 150µL from each well was taken and read fluorescently by the spectrophotometer with an excitation on 560nm and emission of 590nm.

GSIR Assay of PAMAM Coated Alginate Beads

A Glucose Stimulated Insulin Response (GSIR) Assay was performed on coated alginate PAMAM beads (4 layers and 0 layers) with encapsulated MIN 6 cells. First, the beads were counted (15 beads per well) and placed in a 48 well plate (n=3). Next, the beads were exposed to the hydrogen peroxide assay as explained above. Although after the 2-hour incubation, the beads were washed and then treated with 300µL of 1.67 mM of low glucose Krebs buffer for 30 minutes at 37°C. Following that the liquid was discarded and 300µL of low glucose Krebs buffer was added and incubated at 37°C for 2 hours. The liquid was collected and stored for later analysis. Next, 300µL of 16.7mM of high glucose Krebs buffer was added and incubated at 37°C for 2 hours. The liquid was then collected for later analysis. Then, 300µL of low glucose Krebs buffer was added and incubated at 37°C for 2 hours. Again, the liquid was collected and stored for later analysis.

ELISA Assay of PAMAM Coated Alginate Beads

For this assay, the samples collected from the GSIR are used and analyzed to determine viability and functionality of the cells. First, the samples are added to the ELISA plate according to the glucose it was stimulated with (dilutions of 5x for low glucose and dilutions of 10x for high glucose). Next, 100µL of the enzyme conjugate buffer is added to each well. The plate is then incubated on a shaker at 800 rpm for two hours. After incubation, the plates are washed 6 times with an ELISA plate washer. Then, 200µL of TMB is added as well as 50µL of stop solution to each well. Then the plate is read with the spectrophotometer at 450 nm.

Fabrication of PAMAM Loaded with DEX

Dissolve excess amounts of DEX in methanol. Then, add PAMAM solutions in methanol to this solution and leave on a magnetic stirrer for 24 hours at room temperature. Next, evaporate the solvent with Argon and then reconstitute it with water. Then, stir the solution for 6 hours to remove excess DEX. Lastly, The centrifuged the samples and freeze dry them for 24 hours.[11]

Fabrication of PAMAM-DEX Coated Alginate Microbeads

Once the alginate beads and PAMAM-DEX polymer has been fabricated, we can translate the layer by layer technologies previously developed in the lab to coat alginate beads with PAMAM-DEX coatings. The layer by layer technique is done by coating a negative layer (alginate or CONP-PAA) and then a positive layer (PAMAM-DEX). In order to do this, the beads need to be incubated with the respective solution, then washed three times between layers. The feasibility of layer deposition on beads is shown using a fluorescently labeled PAMAM and imaged via confocal microscopy.

Drug Release in Solution

PAMAM and DEX will be dissolved in methanol and mixed thoroughly. DEX will be dissolved evenly throughout this solution and within the cavities of PAMAM. Then the solution will be dried and resuspended in a buffer of either water or 3-(N-morpholino) propane sulfonic acid (MOPS). In this buffer, PAMAM will dissolve, but DEX will not because it has low solubility in water.[12] This will cause water-insoluble DEX to be entrapped within PAMAM.[11] Next, the solution will be filtered to remove the non-encapsulated DEX.

Encapsulation of DEX within PAMAM will be characterized through Fourier Transform Infrared Spectroscopy (FTIR) to evaluate the changes in chemistry as compared to PAMAM and DEX alone.

The PAMAM-DEX complex will be evaluated regarding encapsulation efficiency and drug release profile. PAMAM-DEX will be evaluated in solution using an <28,000 kDa Amicon filter. PAMAM-DEX will be incubated in various buffers including MOPS buffer and 1% BKC solution (used to mimic carrier proteins in the body).[13] Then, DEX concentrations will be quantified via absorbance readings.

Drug Release in Beads

PAMAM-DEX coated alginate microbeads will be used to determine the drug release kinetics of the PAMAM-DEX coated beads in MOPS buffer and 1% BKC solution. The beads will be incubated in their respective solutions for 10 minute periods. After 10 minutes, the remaining liquid will be kept and plated for absorbance readings. Between periods the beads will be washed 3 times with MOPS.

Results and Discussion

Hydrogen Peroxide Assay of Coated Glass Beads

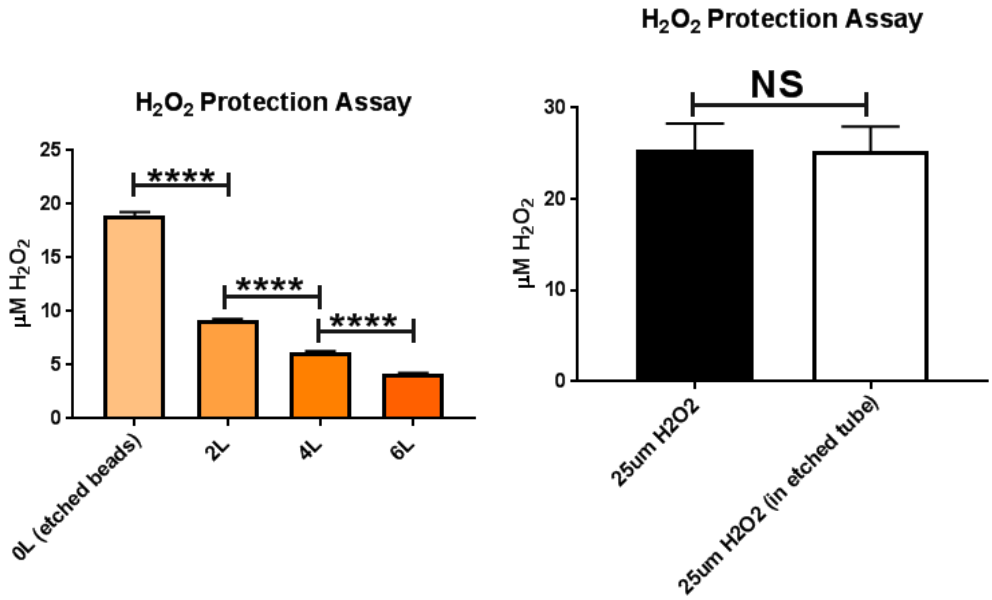

Figure 1 and 2: Hydrogen Peroxide Assay of Coated Glass Beads

In Figure 1, the hydrogen peroxide assay on coated glass beads demonstrated that the PAMAM alginate coatings were able to protect the beads and reduce the amount of hydrogen peroxide in the solution. There is a significant difference of hydrogen peroxide concentration seen in the solution after being exposed to the different number of PAMAM alginate coatings. With more coatings the amount of hydrogen peroxide present after incubation significantly decreased possibly meaning that the PAMAM is acting as an antioxidant. In Figure 2, the antioxidant capacity of the etching process was used as a control. It was found that there was no significant difference between the 25µM of hydrogen peroxide and 25µM of hydrogen peroxide in an etched tube. This is important because it means that the etching process was not a factor in the decrease in micromolar of hydrogen peroxide from the samples.

Fluorescently Tagged PAMAM and Alginate Coatings on Beads

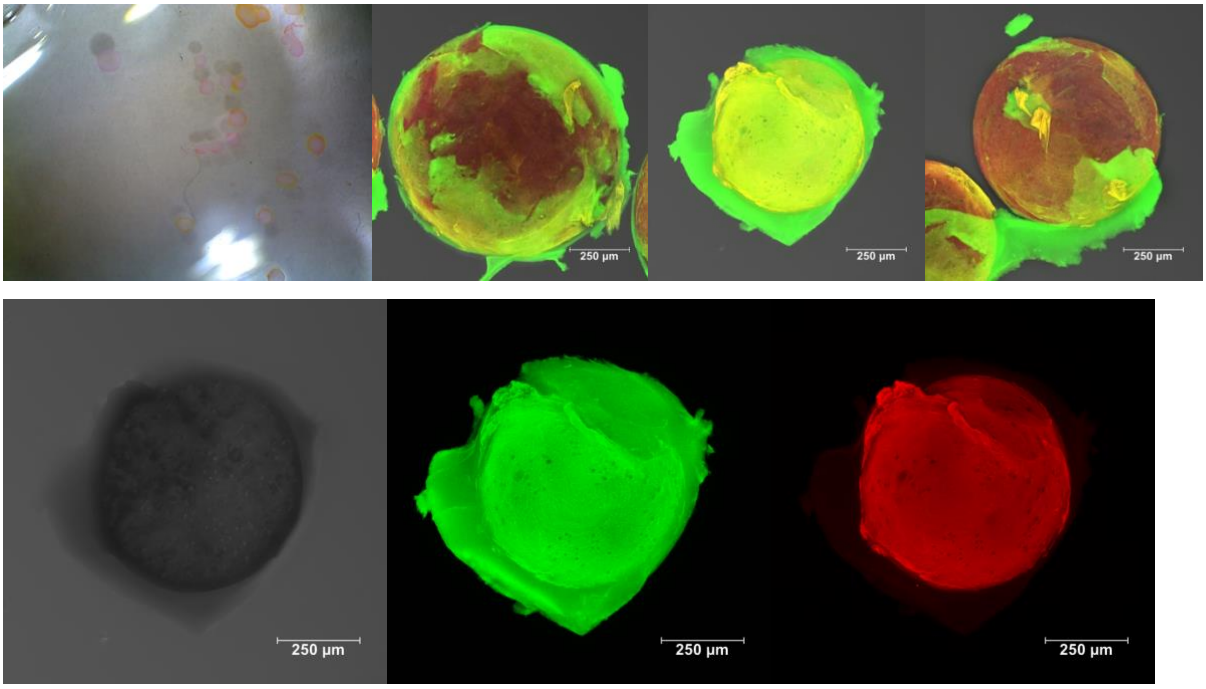

Figure 3: Fluorescently tagged PAMAM(red) and Alginate (green) beads

Figure 3 shows the fluorescently tagged PAMAM and alginate coated beads with MIN6 cells. The PAMAM was tagged with ROX and is seen as red and the alginate was tagged with carboxyfluorescein and is green. This image shows that the bead was indeed coated with alternating layers of PAMAM and alginate. During the coatings the beads tended to clump

together causing imperfect coatings and layers around the beads. In future experiments, the coatings on these beads will be improved by better mixing methods.

Hydrogen Peroxide Assay of MIN6 Encapsulated Coated Beads

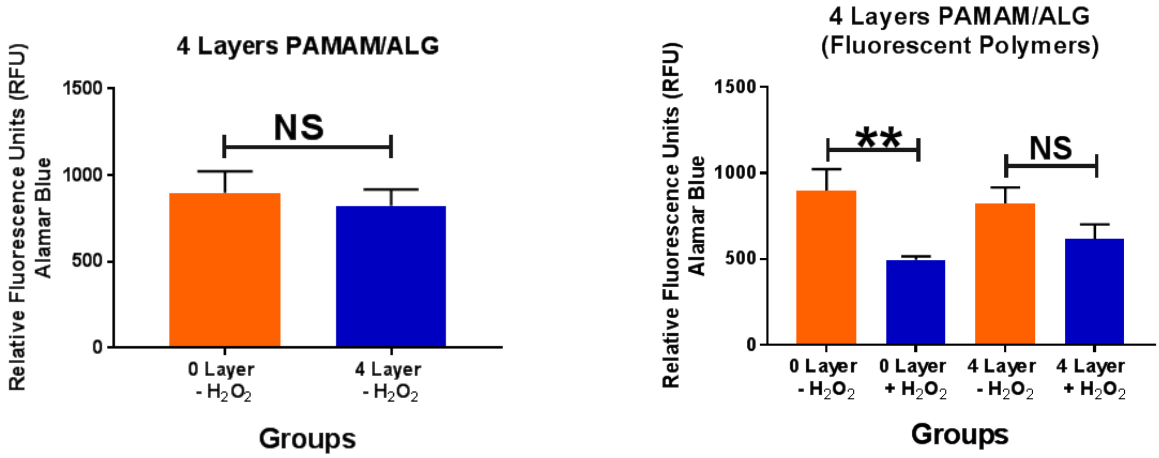

Figure 4 and 5: Relative Fluorescent Units from Alamar Blue Assay on Hydrogen Peroxide Treated and Nontreated MIN6 Encapsulated 0 layer and 4 layer Coated Beads

The beads as seen in Figure 3, were treated with hydrogen peroxide and the results were compared to the 0 Layer beads. Figure 4 shows that there is no significant difference in the fluorescent reading of the alamar blue assay between the 0 layer beads and the 4 layer beads that were not treated with hydrogen peroxide. Alamar blue is used to measure metabolic activity, so the fact that the 0 layer beads and the 4 layer beads had no difference in fluorescent means that the metabolic activity was not altered in a significant way. This means that the PAMAM alginate coatings did not affect the MIN6 cells and their viability. In Figure 5, the addition of hydrogen peroxide on the coatings was assessed through alamar blue fluorescent readings. There was a significant difference of the relative fluorescent between the hydrogen peroxide 0 layer beads and the non-hydrogen peroxide treated 0 layer beads. This is expected because hydrogen peroxide is a ROS and is known to kill cells and therefore decrease cell metabolic activity. There was no significant difference between the hydrogen peroxide treated 4 layer beads and the nontreated 4 layer beads. This means that the metabolic activity of the MIN6 cells was not affected by the coatings. This result suggests that the coatings may be able to protect the cells from the hydrogen peroxide.

ELISA Assay of MIN6 Encapsulated Coated Beads

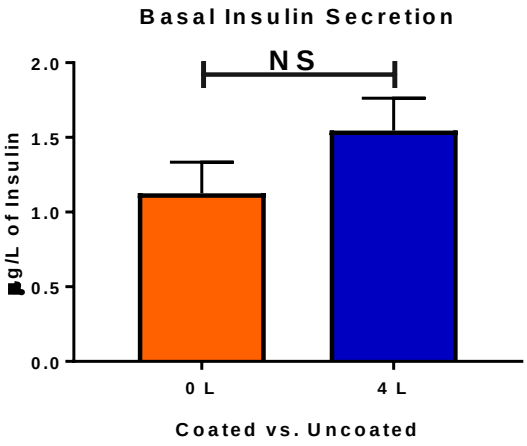

Figure 6: Basal Insulin Secretion of Coated and Uncoated Beads

To determine the functionality of these coatings on cells a GSIR was completed on MIN6 encapsulated coated beads. An ELISA was completed to analyze the GSIR to quantify the insulin release by MIN6 cells when exposed to high and low glucose. From Figure 6, It can be seen that the 0 layer beads and the 4 layer beads both had similar basal levels of insulin released. This means that the coatings were not blocking the secretion of insulin and that the MIN6 cells still had functionality.

Since the PAMAM and alginate coatings showed promising results, the antioxidant loading capacity of PAMAM was looked at using DEX as a model drug.

FTIR of PAMAM-DEX

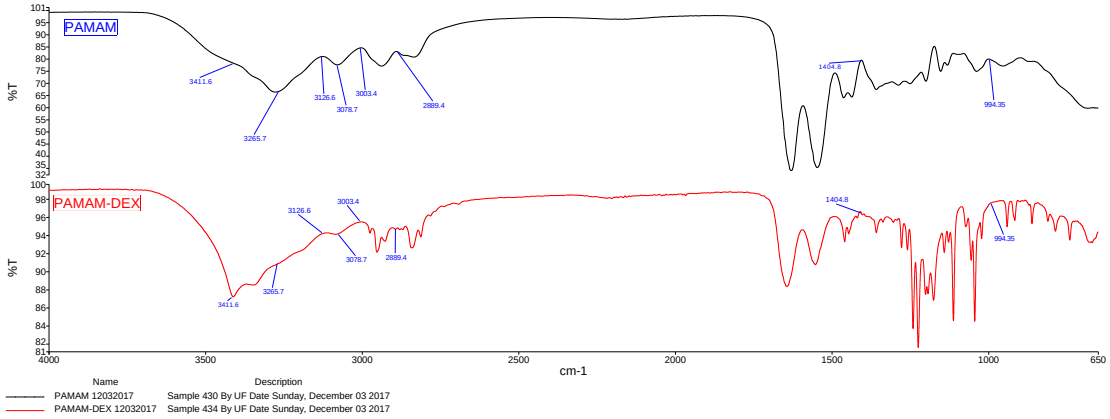

Figure 7: FTIR of PAMAM (blue) and PAMAM-DEX (red)

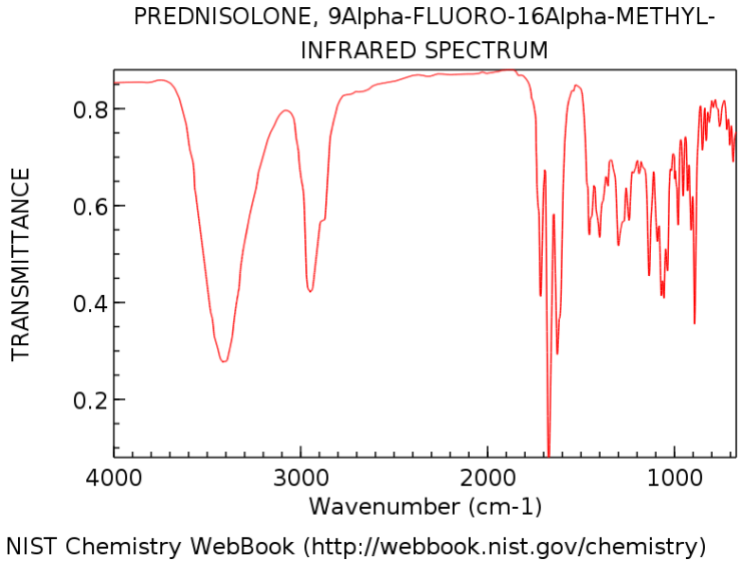

Figure 8: FT-IR of Dexamethasone[12]

The FTIR of PAMAM compared to the FTIR of PAMAM-DEX (Figure 7) shows major differences. These differences can be seen at the blue points which leads us to the conclusion that PAMAM and DEX are both present in the PAMAM-DEX complex. Figure 8, the FTIR of DEX, also shows some similarities to the PAMAM-DEX FTIR.

<u>PAMAM-DEX Drug Release in Solution</u>

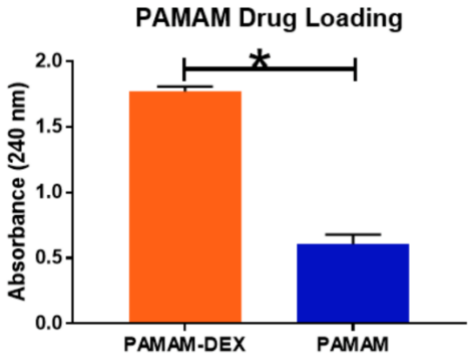

Figure 9: DEX/PAMAM Absorbance Read on Spectrophotometer at 240 nm

The PAMAM-DEX drug release in solution (Figure 9) showed a significant difference in absorbance values. DEX encapsulation was assessed via absorbance readings. PAMAM-DEX (absorbance at 240 nm) was detected in the PAMAM-DEX formulation and to a

lesser extent in the PAMAM alone. There was a signal detected from PAMAM alone due to wavelength overlap. However, DEX has a distinct signal at 240 nm which is not absorbed by the PAMAM alone and this can be seen by the increased signal absorbance. It can then be concluded that DEX is actually released from the PAMAM in solution. This result lead to the investigation of the release of DEX from PAMAM-DEX coated beads.

PAMAM-DEX Drug Release in Beads

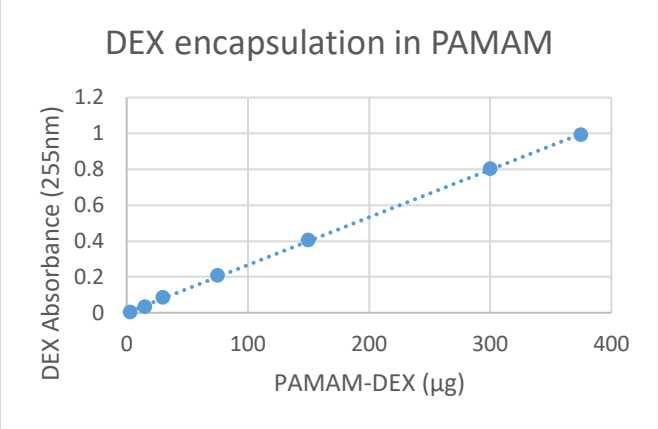

Figure 10: DEX Absorbance at Different Concentrations of PAMAM-DEX

The PAMAM-DEX drug release in beads lead to a couple of complications. There was no detectable absorbance of DEX released from the PAMAM-DEX complex in beads. For that reason, we assessed the concentration of PAMAM-DEX complex needed to obtain a detectable amount of DEX via absorbance readings. We performed an experiment measuring DEX at different concentrations of the PAMAM-DEX complex (data normalized to PAMAM alone), data shown in Figure 10. This data indicates that DEX is detectable at a range of concentrations from 3µg to 375µg. It is also possible that the beads were not loaded with enough PAMAM, and therefore, the low concentrations of DEX released from the beads could not be read with the spectrophotometer. To address this problem future experiments will use High-Performance Liquid Chromatography (HPLC) to measure the DEX concentrations released from the beads. HPLC is able to read smaller concentrations and therefore will determine if the DEX is actually being released from the PAMAM. Another possibility for this is that the DEX may have been released from the PAMAM prior to this study. The beads that were used were not made in the same day, so it is possible that some or all the DEX was released from PAMAM. So, in future studies, the beads will be made and tested on the same day. It is known that the PAMAM contained DEX because the FTIR showed the specific differences.

PAMAM was used as a polymer coating because of its architecture and its ability to be easily functionalized. The DEX is able to be trapped in the branches of the PAMAM and released to the

environment. DEX is a model drug, and in future experiments will be replaced with antioxidants such as, Vitamins A, E, C, and D, as well as, coenzyme Q10 and lipoic acid.

The overall goal of this study and future studies is to transplant coated beads or islets into the body. The body has a pH of ~7.4 at normal conditions, but during inflammation the pH is lower. At a low pH the branches of the PAMAM polymer expand increasing the size of the cavities allowing more of the drug to be released. Since the release drugs from PAMAM increases as the pH decreases, the initial difference in pH allows for more release of the drug. When the inflammation goes down, the release of the drug will occur at a more consistent rate.

Conclusion

Overall, this study showed that PAMAM and alginate can be coated on alginate and glass beads. The experiments done on the coated glass beads lead to the result that PAMAM was able to decrease the amount of hydrogen peroxide in the solution suggesting that the PAMAM coatings can act as an antioxidant. It was also found that the coatings did not affect the metabolic activity of the encapsulated MIN6 cells. The GSIR/ELISA also showed that there was still functionality of the cells with the layers present. More experiments will be done to further determine the antioxidant capacity of the PAMAM coatings and their effects on cells. In the future, these beads will be transplanted into B6 mice and coated/non-coated beads will be compared.

The PAMAM-DEX experiments showed that dexamethasone can be encapsulated in PAMAM and that PAMAM can release DEX in solution. Future studies will continue to investigate the release of DEX from the coated alginate beads. After DEX is able to be released it will be replaced with antioxidants and the antioxidant with the most appropriate loading capacity and release kinetics will be chosen for further studies. These beads will then be evaluated *in vitro* to determine protection against ROS, permeability, coating stability, and *in vitro* and *in vivo* biocompatibility.

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