High loading poly (lactic acid) nanoparticles as a vehicle for sustained insulin release

By
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B.S., Worcester Polytechnic Institute, 2015

Thesis

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in the Department of Molecular Pharmacology, Physiology and Biotechnology, and the Center of Biomedical Engineering at Brown University

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Vita

Dominick Calvao was born on September 27th, 1993 to Luis and Jennifer Calvao in Flushing, NY. He attended Worcester Polytechnic Institute where he majored in biomedical engineering and minored in materials science. He graduated in 2015 with his Bachelor of Science and then entered the Master's of Science program at Brown University. While at Brown he researched the principles of drug delivery as they pertain to the delivery of insulin. He also participated in the co-op program, working for Bard Davol in Warwick, RI for eight months on the development of powdered hemostats for controlling blood during surgical procedures. After graduation, he will for MilliporeSigma as a senior specialist in their strategic marketing and innovations division.

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Table of Contents

Table of Figures

Table of Tables

1. Background

1.1 The Role of Insulin

Insulin is a hormone in the body secreted by the pancreas which is important for controlling the body's blood glucose or blood sugar level. In a healthy person, the body's blood sugar spikes shortly after eating a meal as the food is digested and nutrients are absorbed by the body into the blood stream. The glucose in their blood is used by their cells to produce energy through glycolysis, the first step in cellular respiration. Excess glucose is converted into glycogen by the liver for storage, which is facilitated by the presence of the hormone insulin. If the body's blood sugar ever runs too low and the cells need more glucose for cellular respiration, the pancreas will instead secrete glucagon, a hormone that functions as the counterpart to insulin by facilitating the conversion of glycogen back into glucose in the liver, thus raising the blood sugar level an appropriate level (International Diabetes Federation, 2015). As seen in Figure 1, this process represents a careful regulatory process largely dependent on the proper functioning of the pancreas and the liver.

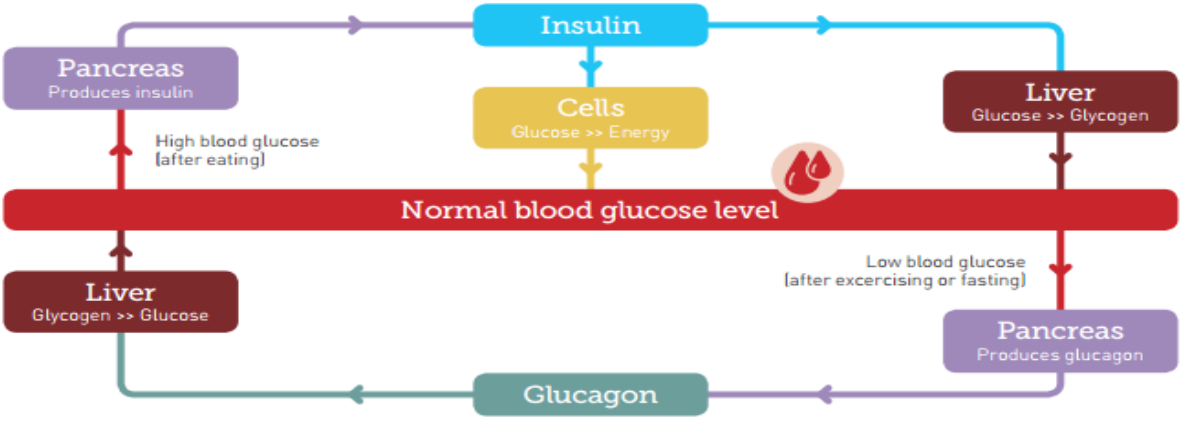

Figure 1: The hormone cycle associated with maintaining normal blood glucose levels *(International Diabetes Federation, 2015)*

1.2 Diabetes

There are several types of diabetes although the two most common afflictions are denoted as type I and type II diabetes with type II patients making up approximately 90% of all diabetes patients (International Diabetes Federation, 2015). In type I diabetes, the body attacks the pancreatic islet beta cells, which are responsible for the production of insulin. Without these cells, the body cannot produce enough insulin to successfully convert the excess glucose present in the blood into glycogen (Daneman, 2006). In type II diabetes, the appropriate amount of insulin is being produced but the insulin receptors in the liver are not functioning thus preventing the conversion of glucose into glycogen. In both cases, however, the end result is the same: the patient's blood sugar level remains elevated.

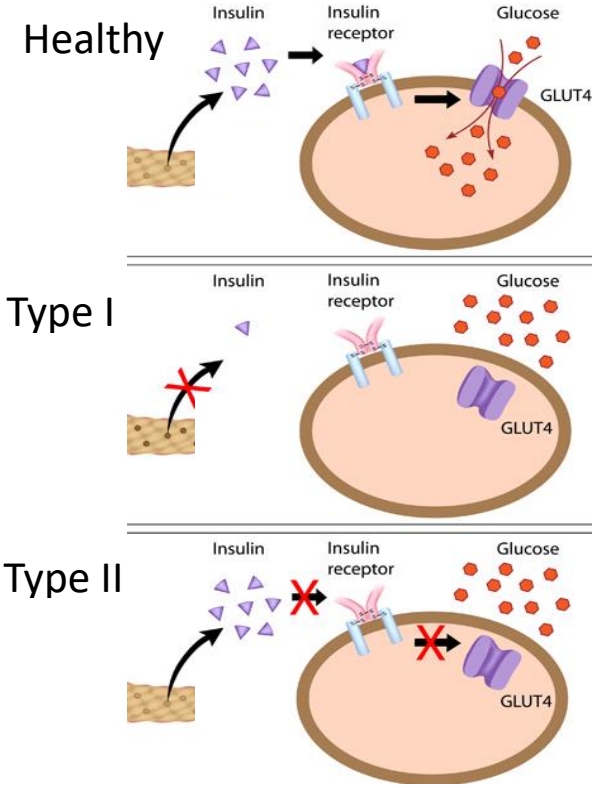

Figure 2: Graphic of differences in insulin pathways for the healthy, type I, and type II patients *(Johnson, 2016)*

This prolonged high blood sugar level can lead to several serious health complications including kidney failure, blindness, heart disease, stroke, and death. In 2015, there were 415 million adults living with diabetes and as many as 5 million deaths due diabetes (International Diabetes Federation, 2015). Currently there are several treatment options available for both type I and type II diabetes. Patients with type I diabetes always need to introduce more insulin into their system several times per day and must regularly measure their blood sugar level with a

glucometer. Patients with type II diabetes will typically have to focus more on leading a healthier life style and strict diet control, but over time they may too need to introduce additional insulin into their system as their body increases its insulin resistance, necessitating much higher insulin levels than a healthy person's body to achieve the same result (National Diabetes Data Group, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 1995).

1.3 Insulin Delivery

Patients with both type I and type II diabetes who require insulin will commonly use a basal-bolus insulin regimen. This consists of using a long acting insulin, called basal insulin to maintain the presence of insulin in the body throughout the day. This will usually be a relatively low dose and is used to ensure that the body is never completely without insulin. Patients also use a fast acting insulin, usually immediately prior to meals as eating will cause their blood sugar to spike. This fast acting insulin will convert the excess glucose from the meal into glycogen so that the body does not have elevated blood sugar levels for a prolonged period of time (NovoLog, 2017).

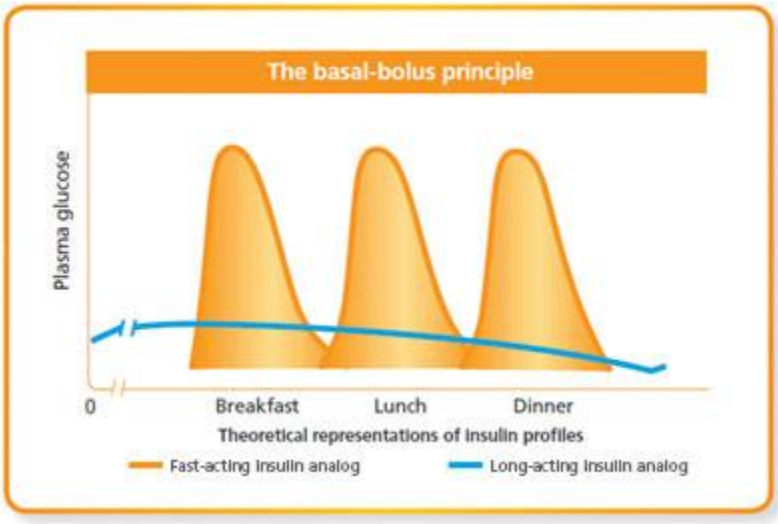

Figure 3: Basal-bolus insulin regimen *(NovoLog, 2017)*

Currently, there are a variety of insulin delivery methods that diabetes patients utilize, the most common of which is syringe injections (American Diabetes Association, 2016). With this method,

assuming a basal-bolus insulin regimen, patients give themselves an injection of basal insulin once per day, usually in the morning or evening. This dose will typically remain constant as it is based on the size of the patient. Then, at meal times, patients measure their blood sugar level with a glucometer, which usually entails pricking a finger to draw a small blood sample, and then they calculate how much insulin they need to take based on what they are eating. They will then give themselves an injection with the appropriate dose for the particular meal they are eating. Because some patients are embarrassed about using the syringes, particularly in public, there are also pen injectors, which function exactly the same as a syringe, but are disguised to look like a pen. Similar to syringes are jet injectors, which function by pushing the insulin dose through the skin so there is no need to puncture the skin. These methods can require 4 – 6 injections per day and as such have relatively low user compliance with 28% of type I patients and close to 50% of type II patients taking less insulin than prescribed (Farsaei, Radfar, Heydari, Abbasi, & Qorbani, 2014). Beyond the low user compliance, there are a number of other draw backs associated with these methods; regarding syringes and pen injectors, they can be painful, have a high potential for user error, and can be time consuming. Jet injectors are usually extremely painful, much more so than using a syringe, and due to the transdermal nature of the drug delivery, are culpable for delivering inconsistent and inaccurate doses (Dansinger, 2015).

The current gold standard for insulin delivery is through the use of an insulin pump. Insulin pumps are small electronic devices that will typically fasten to the patient's belt and will constantly inject the patient with insulin. A small needle attached to a plastic catheter is inserted into the patient and left in place. They can be programmed to continuously release a low dose of fast acting insulin to serve as the patient's basal insulin, and then to release a surge of insulin at meal time,

with the patient inputting how much insulin should be released based on the same calculations described previously (Dansinger, 2015). Despite the ease of use associated with pumps, there are several significant drawbacks for patients who use them.

Using an insulin pump requires an even greater life style adjustment for a patient who is already adjusting many facets of their life due to their diabetes because patients are constantly hooked up to them. Using an insulin pump can make playing sports or simple activities like swimming in a pool much more difficult and risky for patients. Because they can be clipped on the belt and forgotten about, there is also the risk of the pump's battery dying or of the catheter falling out without the patient noticing, particularly while sleeping. This can lead to prolonged levels of heightened blood sugar and associated complications. However, the most common complication with insulin pump use and the reason most often patients switch to a different treatment method is the heightened risk of infection at the catheter's entry point. It is not uncommon for insulin pump users to experience a staph infection at the injection site. This requires a course of antibiotics for the patient to overcome the infection and the replacement of several components of the pump with fresh parts (Mecklenburg, Benson, & Benson Jr., 1984). Due to these drawbacks, new methods of insulin delivery are being sought after.

One such method of delivery that is relatively new is the use of inhaled insulin. This functions in a similar manner to an asthma inhaler where the patient inserts the opening of the inhaler into their mouth and draws two deep breaths. However, this method is very limited in its application as much more insulin must be used for every dose due to the inefficient delivery system of the lungs. More often, this method is used to supplement one of the other delivery methods already described (Dansinger, 2015). It also may not be used by patients who also smoke or who suffer

from chronic lung diseases, a condition that is more likely to occur in diabetes patients than in otherwise healthy adults (Stachnik-Rushlow, 2017). Therefore, other methods of insulin delivery must be explored.

1.4 Principles of Drug Delivery

In general, the goal of drug delivery is to make a therapeutic agent accessible to the cells in the patient's body. Depending on the application, more targeted delivery is desired, for example when working with destructive chemotherapeutics, but in many cases the goal is to get the therapeutic agent into the patient's blood stream. This allows the drug to be transported throughout the body and the delivered to cells. Bioavailability is a term to describe how much drug is present in the patient's blood relative to the total dose that administered. There are a variety of potential routes for drug delivery, each of them impacting the bioavailability the drug. In terms of bioavailability, intravenous (IV) drug delivery is the most efficient as the drug solution is injected directly into the blood stream resulting in bioavailability near 100% as seen in Figure 4. Other commons routes of administration are intramuscular (IM), subcutaneous (SC), and oral (PO), each of which results in lower bioavailability, respectively (Harnett, 2016). IM delivery has a relatively high bioavailability due to the extra-vascular nature of muscle tissue, which allows the drug to quickly make its way into the blood stream. SC delivery consists of depositing the drug

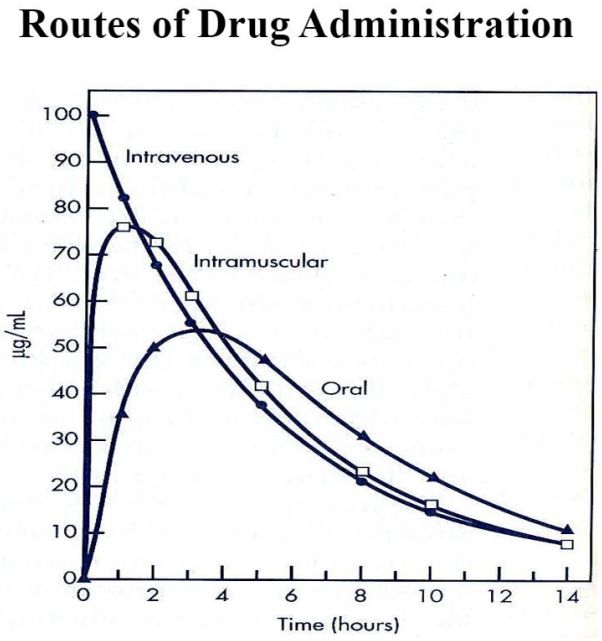

Figure 4: Relative bioavailability of different routes of drug administration *(Harnett, 2016)*

immediately underneath the skin, typically in the fatty tissue which results in a lower bioavailability as more drug is lost on the way to the vasculature (Harnett, 2016). Despite this, SC is the type of delivery occurring with insulin syringe injections and insulin pumps (Farsaei, Radfar, Heydari, Abbasi, & Qorbani, 2014). The advantages of oral delivery lie in the inherent ease of self-administration and the lack of pain upon delivery. While drugs delivered orally typically enjoy higher user compliance than other modes of delivery, effective oral delivery is often more difficult to achieve than other routes due to the path the drug must take (Wilson & Crowley, 2011).

1.5 Oral Drug Delivery

Of the methods discussed, drugs delivered orally have the most difficult path to reach the blood stream. They must travel from the mouth, through the esophagus, survive the harsh pH in the stomach, and finally arrive in the small intestine where the drug is absorbed. Throughout this process there is typically significant drug loss, necessitating an oral dose much larger than the actual therapeutic dose necessary to account for this loss (Wilson & Crowley, 2011) (Harnett, 2016).

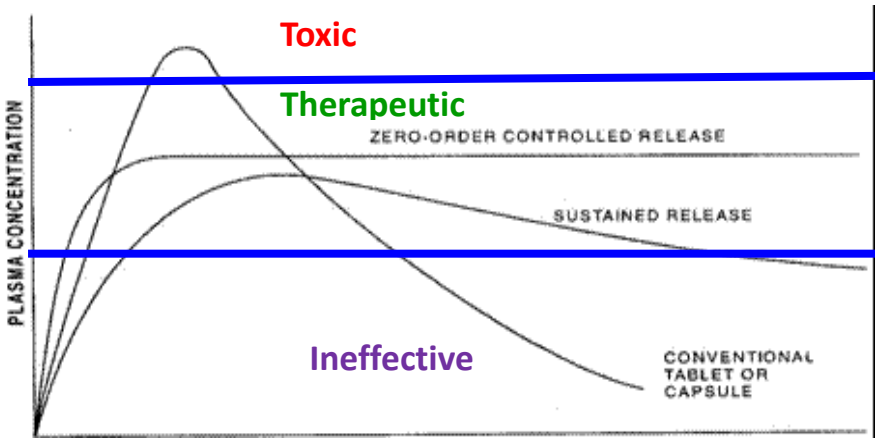

Figure 5: Common drug release profiles *(Mathiowitz, 2016)*

Regarding oral delivery there are two main drug release profiles and an often sought after “ideal” release profile called zero-order release. Each of these profiles are depicted in Figure 5. The most

common profile for drugs delivered orally is the conventional tablet or capsule profile wherein the drug concentration in the blood will quickly increase into an effective therapeutic dose and typically overshoot into a somewhat toxic dose shortly before being eliminated by the renal system. As the drug is eliminated and the dose becomes ineffective, patients typically take a second pill which will repeat the profile, except the profile will be shifted upwards as the drug is often not completely eliminated from the patient's system. This results in the patient having a toxic dose of the drug in their system for an even longer period of time before the drug is cleared by the renal system. This is why many over the counter drugs have a daily maximum number of tablets that may be taken. This conventional release profile is far from ideal as an ideal release profile would include a rapid increase in blood drug concentration until an effective therapeutic dose is achieved, and a steady state release thereafter, maintaining the effective therapeutic dose. This theoretical ideal release is modelled as zero-order release in Figure 5. However, what is achieved more often is sustained release, which can be preferable to the conventional pill profile. With sustained release the blood drug concentration typically increases gradually to the effective therapeutic dose and then very slowly decreases until the dose becomes ineffective. While not exactly ideal, the longer lasting release of the sustained profile provides patients with relief for a longer period of time, with less risk of the dose becoming toxic, and with a lower overall dose than necessary with the conventional release profile. To achieve this sustained release, a variety of techniques are employed. Of these techniques, the use of polymeric nanoparticles has become one of the more popular and widely researched methods (Mathiowitz, 2016) (Lowman, Morishita, Kajita, Nagai, & Peppas, 1998).

1.6 Polymeric Nanoparticles for Drug Release

By encapsulating drugs in polymeric nanoparticles, the drug can be protected from the harsh environment of the gastrointestinal (GI) tract and, by tuning the properties of the drug and the polymer, the rate of release can be controlled, thus achieving sustained release (Wilson & Crowley, 2011). As seen in Figure 6, drug loaded nanoparticles are commonly divided into two main systems, reservoir systems and monolithic, or matrix, systems (Mathiowitz, 2016). Each system has its own advantages and disadvantages depending on the application it is being used for.

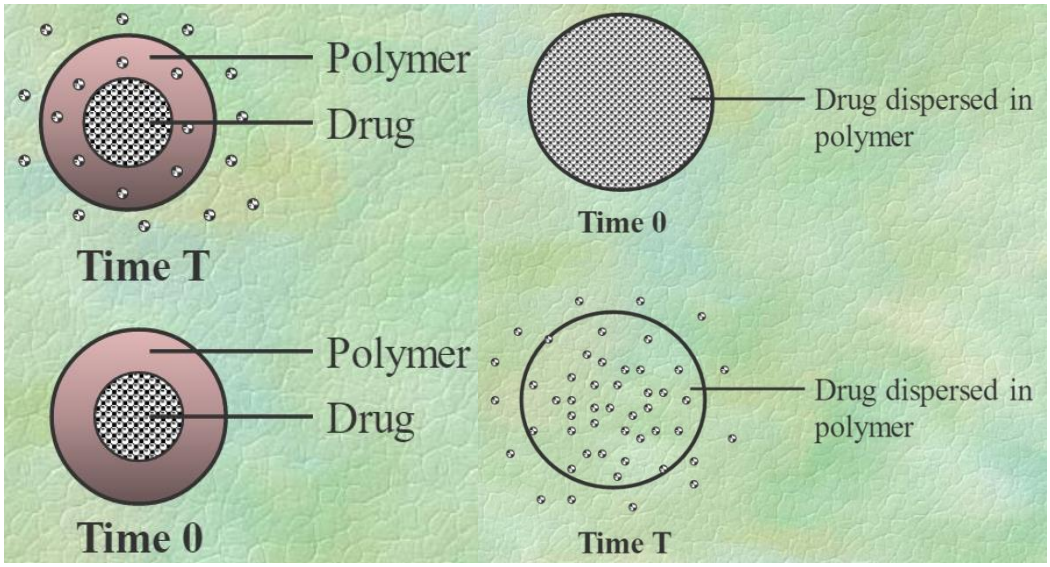

Figure 6: Reservoir (left) and monolithic (right) drug loaded nanoparticle systems. *(Mathiowitz, 2016)*

In a reservoir system, the drug is encased by a polymer shell through which the drug must travel to be released. The polymer shell will typically have a controlled pore size to control the rate of drug release. The drug release kinetics are relatively easy to control with this system and zero-order release is achievable. However, these systems must typically be removed once the reservoir of drug has emptied and they are not suitable to be used with high molecular weight drugs. They are also fairly expensive and there is a risk of a leak forming which could be dangerous

as large quantities of the drug would be released all at once, potentially becoming toxic (Mathiowitz, 2016) (Langer, 1981).

Monolithic systems are more commonly used for oral drug delivery. In these systems, the drug is dispersed evenly throughout a polymer matrix or network. Drug particles at or near the surface of the matrix system will typically release immediately while drug that is located deeper into the matrix will take longer to release. This is called a burst effect and can lead to a rapid increase of blood drug concentration. It is more difficult to achieve sustained release in a monolithic system as the distance that the drug must travel through the polymer increases as time goes on, preventing steady state release. However, monolithic systems are also safer as there is no threat of leaks occurring and they do not have to be removed from the body as reservoir systems do (Mathiowitz, 2016). What these systems have in common, however, is the protection of the drug being carried from the acidic environment in the GI tract, thus promoting enhanced uptake in the intestines. It is for this reason that polymeric nanoparticles are being researched for the application of oral insulin delivery.

2 Materials and Methods

2.1 Insulin Micronization

Before insulin can be successfully encapsulated it must be micronized or broken down into smaller polymeric units. While several micronization methods were utilized and tested all samples were prepared in the same manner. The bottle of bovine zinc insulin (Life Technologies, Cat # 18125-039) was removed from storage in the -20°C freezer and allowed to warm up to room temperature before opening to reduce condensation of water vapor in the air. The samples were weighed to approximately 32 mg into 20 mL glass scintillation vials on the analytical balance (Mettler Toledo, Model XS-105).

2.1.1 Uncontrolled Acidity

1 mL of 2% HCl (prepared by serial dilution from 37% HCl stock solution) was pipetted into the 20 mL scintillation vial with the weighed out insulin. The solution was vortexed and sonicated for 45 seconds to fully dissolve the insulin. Some crystals were still visible so 50% HCl was added dropwise to the solution until all crystals were dissolved. 10 mL of Tert-butyl alcohol (TBA) (Alfa Aesar, Cat# 33278) were added to the solution which was then vortexed and sonicated for 45 seconds again. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer (VirTis, Model 4KBTZL-105) until dry.

2.1.2 Controlled Acidity – 2% HCl

1 mL of 2% HCl was added to the 20 mL scintillation vial with the weighed out insulin. The solution was vortexed and sonicated for 45 seconds. 10 mL of TBA were added to the solution which was then vortexed and sonicated for 45 seconds. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer until dry.

2.1.3 Controlled Acidity – 50% HCl

1 mL of 50% HCl was added to the 20 mL scintillation vial with the weighed out insulin. The solution was vortexed and sonicated for 45 seconds. 10 mL of TBA were added to the solution which was then vortexed and sonicated for 45 seconds. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer until dry.

2.1.4 Acidity Experimentation

Four different acidity formulations were tested simultaneously. In the first formulation 1 mL of 2% HCl was pipetted into the 20 mL scintillation vial with insulin. 200 µL of 50% HCl was pipetted into the solution and the vial was stirred manually to dissolve remaining crystals. This process was repeated until a total of 1 mL of 50% HCl was added. Then, 10 mL of TBA was added to the solution which was subsequently vortexed and sonicated for 30 seconds. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim

wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer until dry.

The second formulation was a repeat of the Controlled Acidity – 50% HCl procedure outlined above. The third formulation was a repeat of the Controlled Acidity – 2% HCl procedure outlined above. In the fourth formulation 1 mL of 2% HCl was pipetted into the 20 mL scintillation vial with insulin. The vial was manually stirred until most of the insulin was dissolved. The vial was then vortexed and sonicated for 45 seconds. Then 200 µL of 50% HCl was pipetted into the vial which was subsequently vortexed and sonicated for an additional 45 seconds. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer until dry.

2.1.5 Acidity Experimentation – Corrected Ratios

Further experimentation was conducted with the HCl concentrations outlined above in Acidity Experimentation. The total volumes used in formulations one and four were changed to create a 1:10 ratio of HCl solution to TBA. Therefore, in formulation one 0.5 mL of 2% HCl was used with 0.5 mL of 50% HCl to create a 1:1 ratio of 2% HCl:50% HCl for a total volume of 1 mL. In formulation four 833 µL of 2% HCl was used with 167 µL of 50% HCl to create a 1:50 ratio of 2% HCl:50 HCl with a total volume of 1 mL. All samples were lyophilized in the same manner as the procedures outlined above.

2.1.6 PLA Addition

First 833 µL of of 2% HCl was pipetted into the 20 mL scintillation vial with insulin. The vial was then vortexed and sonicated for 45 seconds. Then, 167 µL of 50% HCl was pipetted into the solution which was subsequently vortexed and sonicated for 45 seconds. In a separate scintillation vial 10 mL of TBA was added to about 10 mg of polylactic acid (PLA) (Polysciences, Inc., Cat #18580) to create a 1% PLA solution. This solution was vortexed for 30 seconds to ensure proper mixing. The insulin solution was then added dropwise to the PLA solution while rotating the pipette tip around the surface to distribute the drops around the PLA solution. The mixture was then vortexed and sonicated for 45 seconds. Immediately after removal from the sonication bath the 20 mL scintillation vial was dried and covered with a kim wipe before immersing in liquid nitrogen to flash freeze. Once completely frozen the vial was transferred to a lyophilization vial and placed on the lyophilizer until dry.

2.2 Phase Inversion Nanoencapsulation

To encapsulate the micronized insulin Phase Inversion Nanoencapsulation (PIN) was used. Four different formulations were fabricated with varying degrees of loading. Particles with 10%, 15%, 25% and 40% loading were created by weighing insulin into a 20 mL scintillation vial followed by 2 kDa PLA (Polysciences, Inc., Cat #18580) so that the insulin made up the appropriate percentage of the total weight. Next, dichloromethane (DCM) (Fisher Chemical, Cat #D143-4) was pipetted into the 20 mL glass scintillation vial to create a 1.5% weight per volume solution. This solution was then vortexed and sonicated for 45 seconds and pipetted dropwise into a stirring glass beaker of petroleum ether (PE) (Fisher Chemical, Cat #E139-4) to create a DCM:PE ratio of 1:125. The beaker was allowed to stir for approximately 10 minutes and was then poured into a stainless

steel filtration column. The column was sealed and then air pressure was applied to force the solution through a 0.2 µm filter (MilliporeSigma, Ref #FLGP04700) to remove the polymeric nanoparticles from the PE solution which was captured in a glass beaker below the filtration column. Air flow was allowed to continue for an additional 10 minutes to completely dry the filter out. Then the filter was scraped with a razor blade and the powder collected into a pre-weighed 50 mL conical tube (Corning, Ref #430828). The filter was then placed into a separate 50 mL conical tube for storage. Both conical tubes were covered with kim wipes and immersed in liquid nitrogen to flash freeze. Once completely frozen the tubes were transferred to a lyophilization vial and placed on the lyophilizer until dry. Once completely dry, the tube containing the powder was reweighed and the percent yield was calculated using the equation: $\% \, Yield = \frac{Final\ weight - Initial\ weight}{Insulin\ weight + PLA\ weight} \times 100$. All productions were stored with parafilm on the tube caps and inside a Ziploc bag with desiccant inside of a -20°C freezer.

2.3 Release Studies

2.3.1 Regular Sonication

To model the insulin release *in-vitro* release studies were conducted. Powder was weighed into 1.5 mL eppendorf tubes on the analytical balance. The eppendorf tubes were then filled with 1.2 mL of 0.01M phosphate buffered saline (PBS) (MP Biomedicals, LLC, Cat #2810305). The samples were vortexed and sonicated until the powder were completely dispersed in the liquid. They were then immediately centrifuged (ThermoForma Microcentrifuge, Model 120) at 10,000G for 10 min at room temperature. The samples were removed from the centrifuge and 1 mL of the supernatant was extracted with a micropipette and transferred to a fresh 1.5 mL eppendorf tube.

The supernatant was stored in the -20°C freezer until analysis. Once the supernatant was removed, fresh 1 mL of fresh PBS was added to the eppendorf which was then vortexed and sonicated until the particle pellet was totally dispersed in the PBS again and then placed onto a tube rotator (Barnstead International Labquake, Model #415110; Fisher Scientific, Model #88861051). After the collection of the 0 time point, this process was repeated and supernatant was collected after 30 minutes, 1.5 hours, 3 hours, 5 hours, 24 hours, and each day until one week. In some studies supernatant was collected weekly after the first week.

2.3.2 Minimal Sonication

The above process was performed with minimal sonication. Sonication was only used when first introducing PBS to the powder to remove any air trapped within the particles. From that point on no sonication was included. Instead, the particle pellets were broken down with a combination of vortexing and physical manipulation using a thin wire or needle. Supernatant was collected at all of the time points listed above.

2.3.3 BCA Assay

Once a suitable number of time points were collected a bicinchoninic acid (BCA) assay was conducted using a Micro BCA Protein Assay Kit (Thermo Scientific, Prod #23235) according to the protocol provided. Briefly, the supernatant was thawed and 150 µL aliquots were placed into black clear flat bottom 96-well plates (Corning, Ref #3720) in duplicate. A standard curve was created by serially diluting the provided albumin standard in 0.01M PBS and pipetting 150 µL aliquots into the plate in duplicate. Then the three reagents provided (reagents A, B, and C) were mixed at a ratio of 25:24:1 respectively. The mixed solution was vortexed for approximately 10

seconds to ensure proper mixing and then 150 µL of the BCA solution was aliquoted into each well. The plate was then covered and sealed with parafilm and allowed to incubate at 37°C for 2 hours. After 2 hours, the plate was removed from the incubator and allowed to cool to room temperature before being placed into a UV spectrophotometer (Molecular Devices SPECTRAmax Plus, Model 384). The UV absorbance was measured using SOFTmax Pro (Molecular Device, Version 4.3.1 – Life Sciences Edition) software. The default BCA protocol provided by the software was used with an endpoint reading. Data generated by SOFTmax Pro was exported to Microsoft Excel were it was analyzed and graphed.

2.4 Scanning Electron Microscopy Analysis

To visualize the micronized insulin and the PLA nanoparticles scanning electron microscopy (SEM) was used. First, SEM stubs (Electron Microscopy Sciences, Cat #75600) were applied with carbon adhesive tabs (Electron Microscopy Sciences, Cat #77825-12). Then, the sample was applied in one of two ways. Either a small amount of powder would be smeared across the carbon tab with a metal spatula, or a small section of the filter used during the PIN process would be cut out with a razor and placed on the tab. The tabs were then sputter coated with gold (Emitech, Model K560) for 3.5 minutes at 20 mA. They were then imaged using the SEM (Hitachi, Model S-2700) at 8.0 kV and 80 second photo exposure.

2.5 Particle Size Analysis

A brief particle size analysis was conducted using ImageJ (National Institute of Health) software. Using the images collected with SEM and the scale bar in the images, the scale of each image was

set. Then, several particles in the image of varying size were measured in diameter to determine a general range of particles sizes.

3 Results and Discussion

3.1 Insulin Micronization

To assess the success of the micronization techniques, SEM and ImageJ analysis was conducted non-micronized insulin to serve as a reference. Representative images of each micronization method are depicted in Figure 7. The non-micronized insulin consisted of particles as large as 31 µm and as small as 1 µm. This large size distribution and the large size of the particles makes non-

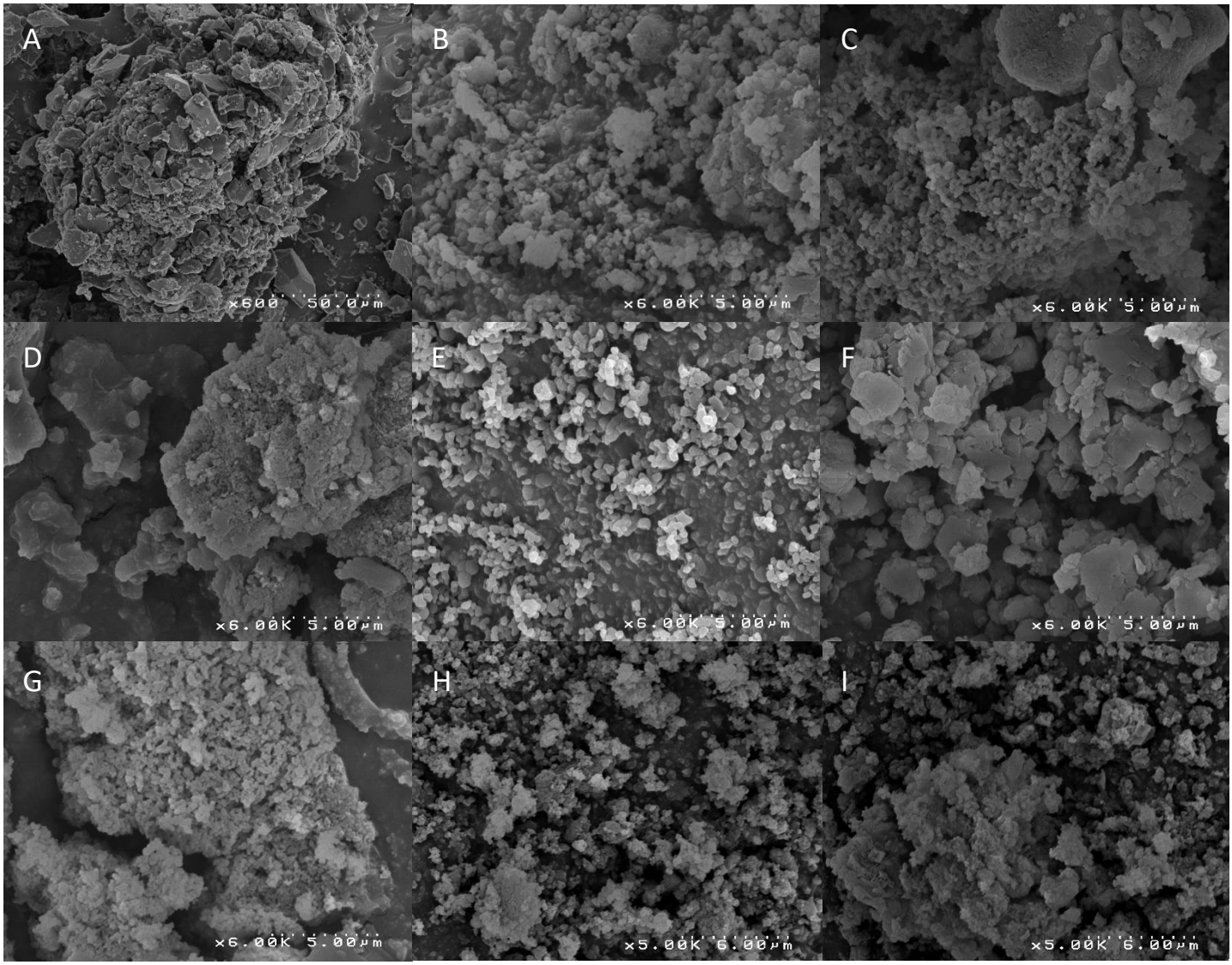

Figure 7: SEM images of bovine zinc insulin. A) Non-micronized insulin B) Uncontrolled acidity C) Controlled acidity - 2% w/v HCl D) Controlled acidity - 50% w/v HCl E) 1:1 ratio 2%:50% HCl F) 1:5 ratio 2%:50% HCl G) 1:1 corrected ratio 2%:50% HCl H) 1:5 corrected ratio 2%:50% HCl I) PLA addition

micronized insulin unsuitable for encapsulation. Conversely, insulin that was micronized with the uncontrolled acidity method ranged in size from approximately 180 nm to 420 nm, a size much more suited to successful drug encapsulation. Likewise, the insulin micronized using the controlled acidity – 2% HCl method consisted of particles ranging in size from about 100 nm to 500 nm. However, this micronization method resulted in large conglomerates consisting of many small particles and other large structures which may be only partially micronized. These large structures may be an obstacle to efficient encapsulation. The insulin micronized with the controlled acidity – 50% HCl method yielded an interesting result as there were some discrete particles as small as 60 nm as well as large conglomerate structures as large as 6 µm which appear to be the result of the smaller particles fusing together. This suggested that there is an ideal acidity at which insulin can be micronized somewhere between the 2% w/v and the 50% w/v HCl used. This was further supported by the results of the uncontrolled acidity experiment where a few drops of 50% w/v HCl was added 1 mL of 2% HCl resulting in a well micronized final product.

In an attempt to mimic the uncontrolled acidity micronization method, while controlling the acidity, the acidity experimentation in section 2.1.4 was conducted. The results of the new conditions tested in this experimentation is represented in Figure 7 sections E and F. The insulin that was micronized with a 1:1 ratio of 2%:50% HCl yield small particles at approximately 120 nm and much larger particles at approximately 900 nm. This method did not produce, however, larger conglomerate structures like the 2% HCl and 50% HCl methods did. Oddly, the 1:5 ratio of 2%:50% HCl yielded poor results with particles ranging in size from about 250 nm to 3 µm with the majority of the particles being large. Upon review the method followed for these micronization techniques, it was noticed that the 1:10 ratio of HCL solution to TBA used in all

previous micronizations was not maintained. To rectify this, the same experiment was repeated with adjusted volumes to maintain the 1:10 ratio.

The results of this adjusted experimentation can be seen in Figure 7 sections G and H. With the corrected ratio, the 1:1 micronization method yielded particles ranging in size from about 140 nm to about 400 nm, a much small size distribution than what was seen when the TBA ratio was incorrect. This time, however, there were some conglomerate structures present in the image which appeared similar to the fused structures present in the insulin micronized using the 50% HCl. The 1:5 micronization method yielded vastly different results when the TBA ratio was corrected with discrete nanoparticles ranging from about 150 nm to 500 nm. There were still some conglomerations of particles but, unlike in the 1:1 method, the particles did not appear to be fused together, but were instead stuck together while maintaining distinct separations. Finally, the insulin micronized using the PLA addition method were measured to create particles ranging from 225 nm to 2 µm. There were also large structures present similar to the partially micronized insulin seen in the 2% HCl method. It should also be noted that with SEM there is no way to be sure the particles seen and measured are insulin or particles of PLA that have formed their own nanoparticles separate from the insulin.

3.2 Phase Inversion Nanoencapsulation

The metric used to gauge the success of the PIN method is the percent yield. The yield of various fabrications using the insulin micronized in different ways can be found in Table 1. For the most part, percent yield hovered around 50% reaching as high as 73% and as low as 37%. The PIN

method is subject to a lot of variation, particularly between users. In the future, experimentation isolating the different variables associated with performing PIN.

Table 1: Fabrication parameters for insulin loaded nanoparticles

Micronization Method	Loading	Insulin (mg)	PLA (mg)	DCM (mL)	PE (mL)	Theoretical weight (mg)	Measured weight (mg)	% Yield
Uncontrolled Acidity	10%	30	270	20	2500	300	124	59.14
	15%	26.2	152.1	11.89	1487	178.3	98.5	55.24
	25%	60	180	16	2000	240	155.4	52.09
	40%	120	180	20	2500	300	177.2	48.28
Controlled Acidity - 2% HCl	40%	50	75	8.3	1042	125	89.78	71.82
	40%	50	75	8.3	1042	125	85.28	68.22
Controlled Acidity - 50% HCl	40%	50	75	8.3	1042	125	91.5	73.2
	40%	50	75	8.3	1042	125	91.4	73.12
1:5 Corrected Ratio	40%	30.8	46.3	5.14	642.5	77.1	34.54	44.8
	40%	33.2	49.8	3.3	415	83	47.09	56.73
	40%	33.2	50.19	3.3	415	83.39	41.98	50.34
PLA Addition	40%	33	51.7	3.3	413	84.7	31.68	37.4

3.3 Release Studies

A release study consisting of the first four productions, all using the uncontrolled acidity micronization method was conducted to see what the release profile would look like using various drug loading concentrations. Release curves were plotted to show the total amount of insulin release in µg as well the percent from the total loading that was released. Plotting the percentage release allows a normalized view of the release as slightly different masses of nanoparticle powder were used for each sample. As seen in Figure 8, the 40% loading powder released the greatest amount of drug, as well as the highest percentage of drug. Interestingly,

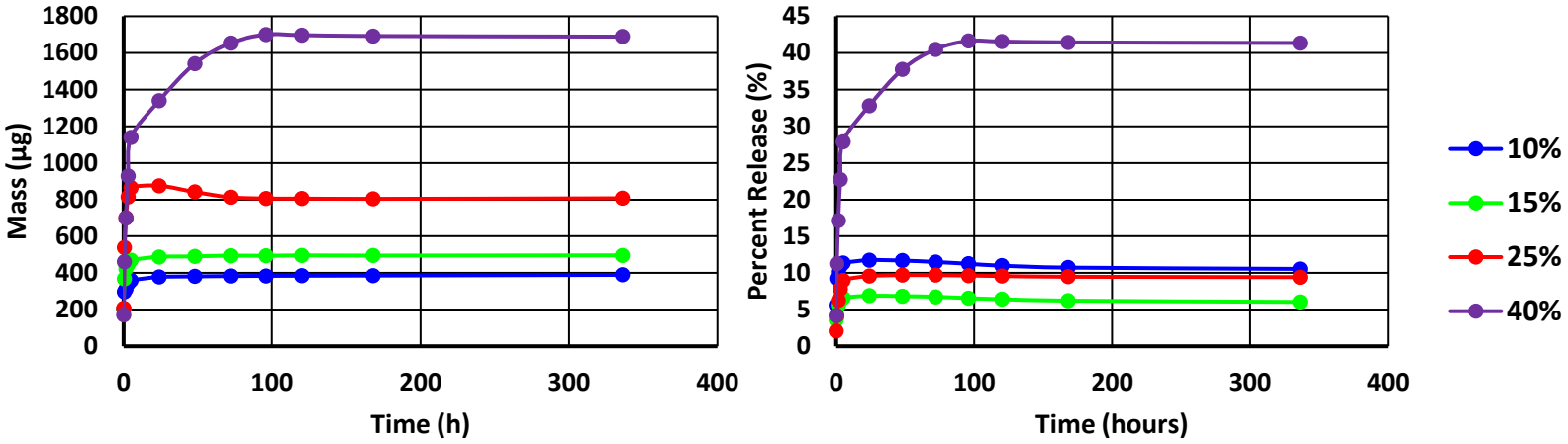

Figure 8: Insulin uncontrolled acidity micronization using four different loadings. Total mass released (left) and percent of total loading released (right)

the 40% loading also exhibited a burst release with close to 1000 µg releasing in the first 24 hours, as well as a sustained release with another 1000 µg releasing over the course of the following week. Because all four nanoparticle productions were made using the same batch of micronized insulin, it is likely that the cause of this increase in release is due to the higher loading.

To test the repeatability of these release results, new batches of the 10%, 25% and 40% loading formulations were fabricated using the same parameters as the first batches. A release study was conducted on these samples in triplicate as represented in Figure 9. While there was very little variation between the samples within each set, the release exhibited by the 40% loading samples did not mimic the release seen previously. Upon review of the fabrication protocols, a small

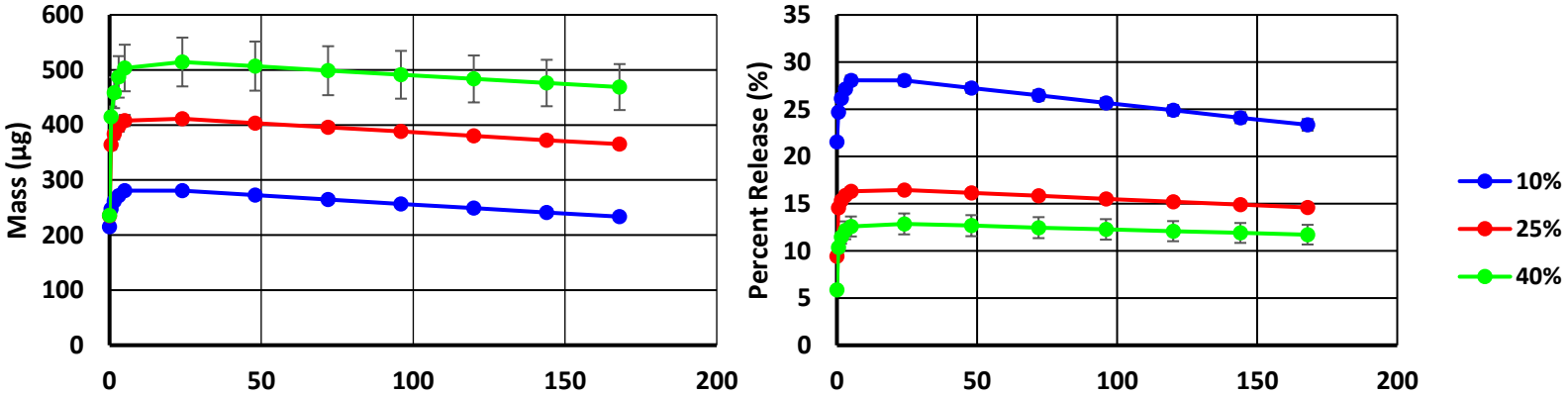

Figure 9: New batches of uncontrolled acidity insulin with varying loading formulations tested for release repeatability (n=3)

discrepancy in the insulin micronization methods was discovered, namely, the acidity of the aqueous HCl solution used to solubilize the insulin. This discovery led to the testing of the different micronization methods.

When the insulin that was micronized with the 2% HCl and the insulin micronized with the 50% HCl underwent release testing, all of the drug that was released came out in the first few hours of study, as depicted in Figure 10. There was also a relatively low percent release, much less so than the percent of release that occurred with the uncontrolled acidity micronization method. This may be due to the large conglomerate structures that were observed with the SEM. While the theoretical loading of these particles may be 40%, it is possible that the conglomerates were never actually encapsulated and thus never made it to the release experiment. Upon learning the importance of the acidity of the micronization solution, further experimentation was conducted to try to recreate the conditions of the uncontrolled acidity micronization method and thus the sustained release achieved with those nanoparticles.

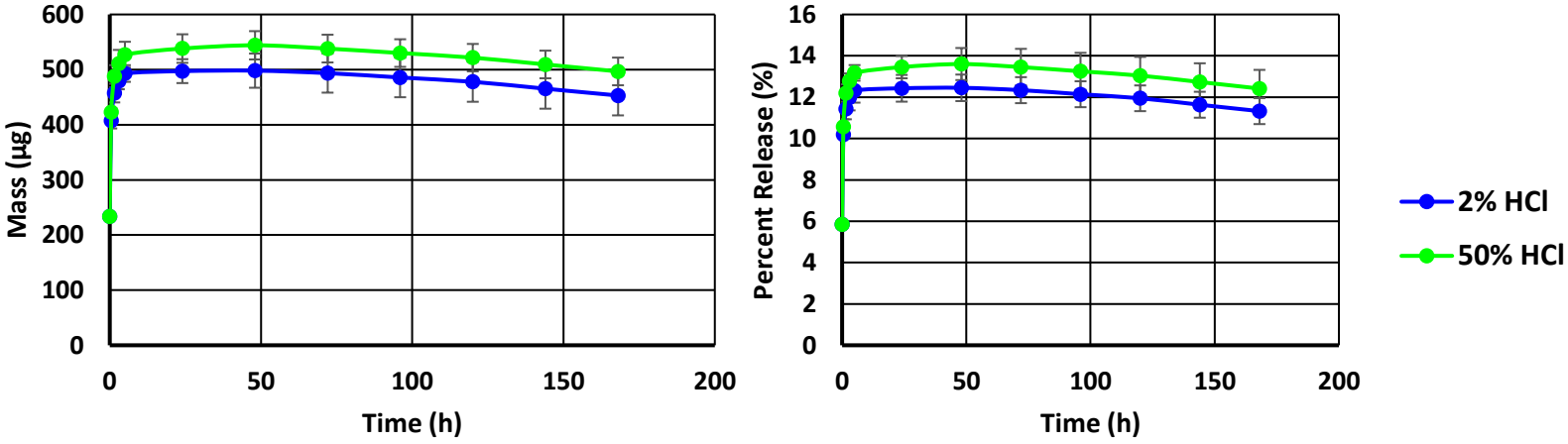

Figure 10: Insulin controlled acidity micronization with 2% and 50% HCL. Total mass released (left) and percent of total loading released (right) (n=6)

The corrected 1:5 HCl concentration seemed the most promising based on the SEM images collected so a release experiment was conducted on it. With this release, the impact of regular sonication was also tested, so a sample that was sonicated at every time point was run as well as

a sample that only received sonication initially to remove any air pockets and get the powder dispersed in the release solution. Successful drug release was achieved for a period of 4 days in the sonicated sample and a period of 3 days in the non-sonicated sample. A very high percent release occurred as well, suggesting nearly all of the drug encapsulated was able to escape from the PLA nanoparticle. Surprisingly, the non-sonicated sample performed better than the sonicated sample, as it was expected that the sonic waves from the sonicator would force the insulin out in higher quantities and more quickly than it would release on its own.

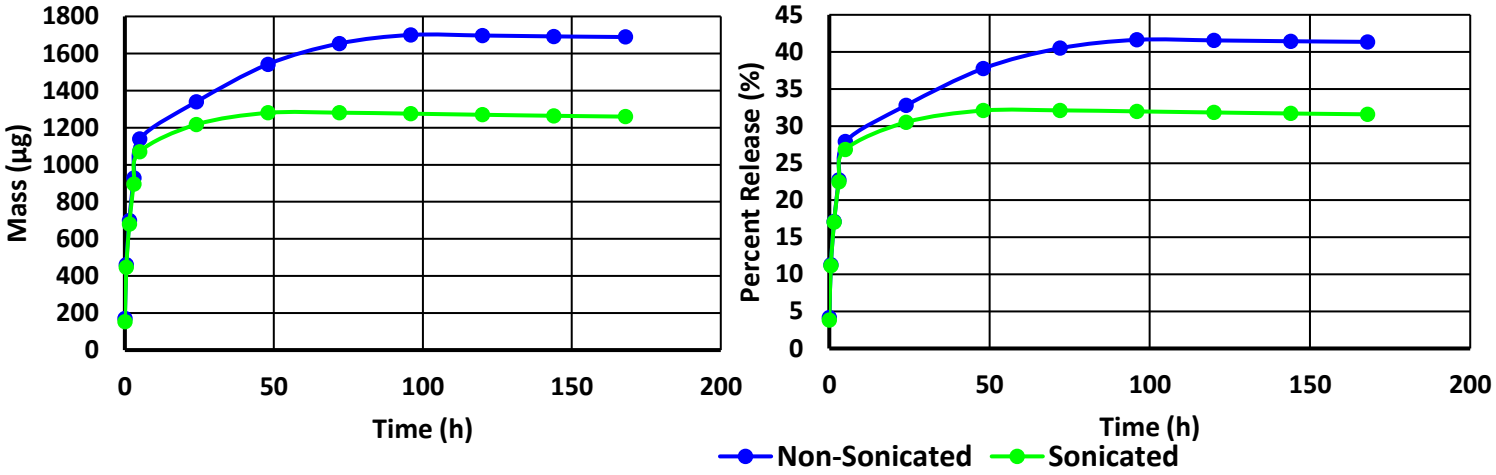

Figure 11: Insulin micronized using the ratio of 1:5 of 2%:50% HCl with the correct 1:10 TBA ratio. The total mass released (left) and percentage of drug released from the total (right)

To test the repeatability of the release achieved with the new micronization method, two batches of insulin were micronized separately using the same protocol and were then fabricated into two separate batches of 40% loading nanoparticles. A release experiment was conducted on both batches in triplicate as seen in Figure 12. These particles did not generate the same release profile that was seen previously with particles produced with insulin micronized in the same way. There was a marked decrease in overall release as well as the percent release. There was a small amount of sustained release occurring up until the one week mark at which point the study was terminated. While this release was not similar to what was achieved previously, the small amount

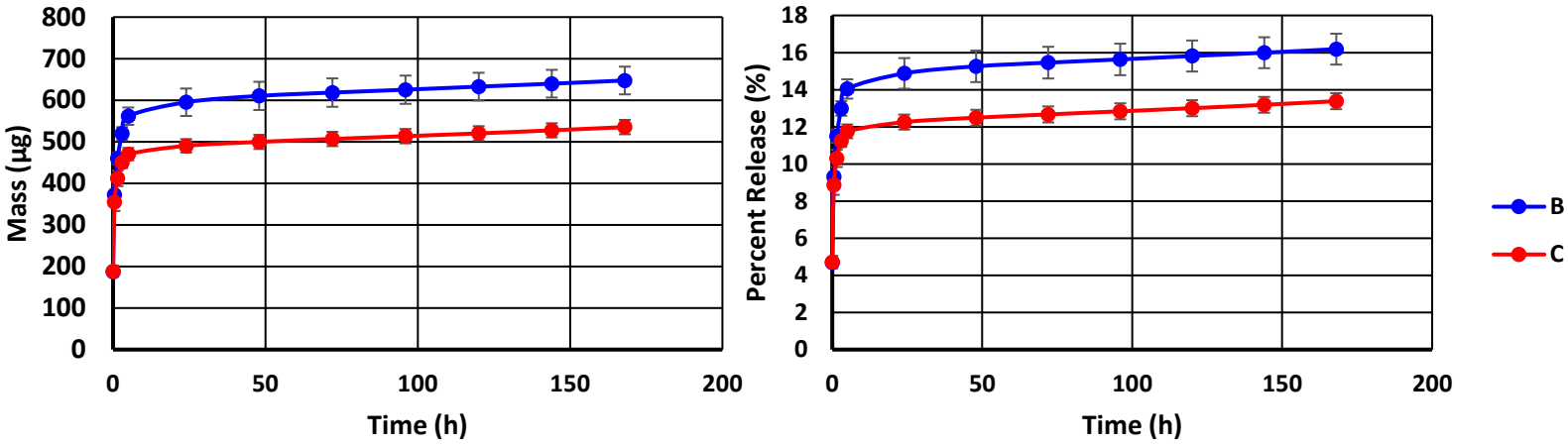

Figure 12: Repeatability of the release profile generated from particles using the corrected 1:5 HCl micronization method. Batches (B) and (C) were two distinct batches created using the same protocol.

of sustained release is promising and merits further research. The sustained release indicates that the insulin trapped inside of the PLA is well micronized and capable of escape, however in lower quantities than previously seen. This could be a result of the actual phase inversion nanoencapsulation process, as batches B and C were encapsulated on the same day under the same conditions, while more variability existed in the initial encapsulation process conducted weeks earlier. This is further supported by small disparity in the release profiles of batches B and C.

When the insulin was micronized using the PLA addition method, the release was as depicted in Figure 13. While the percent release achieved was higher than in some other methods, the variability was also higher, suggesting that this method is not easily controlled compared to other methods with much lower deviation between data points. Also, while the release may have been favorable, the nanoparticle fabrication resulted in a much lower yield than other methods, around 37% while the yield with other methods averaged around 60%. This indicates that while the method may have some potential for achieving appropriate release, the parameters when

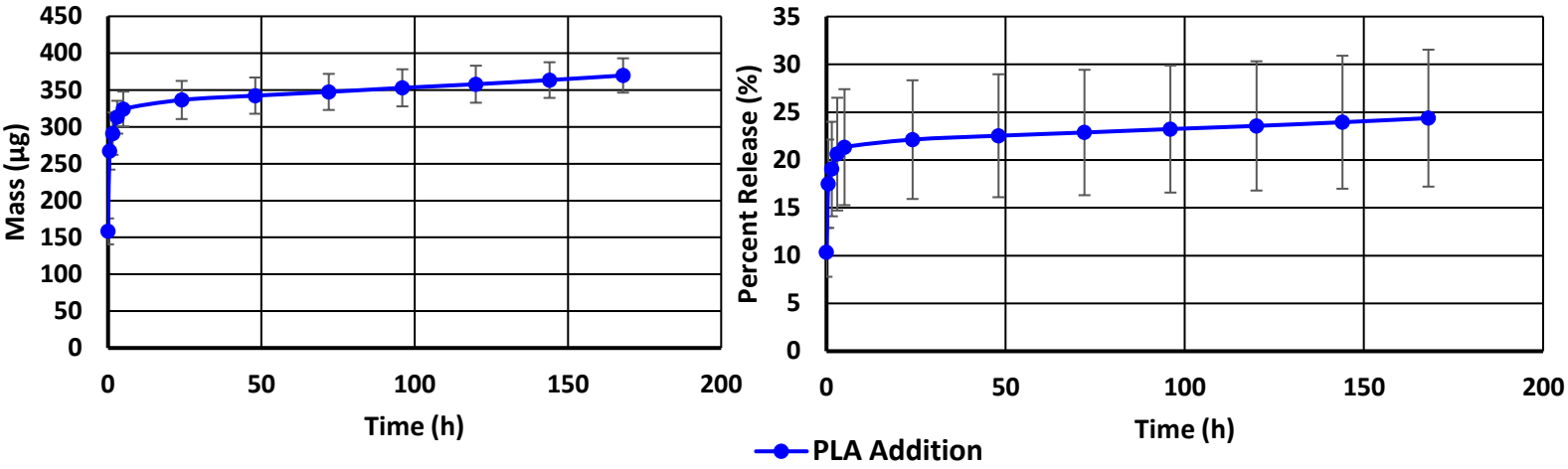

Figure 13: Release profile of nanoparticles fabricated with insulin micronized using the PLA addition method. (n=2)

utilizing the phase inversion nanoencapsulation method must be revised to produce a suitable yield.

4 Conclusion

Oral insulin delivery has long been a goal of the scientific community. There are still many obstacles to be overcome before such a technology will be feasible. The use of polymeric nanoparticles to protect and aid in the delivery of the insulin may be one such method to overcome some of these obstacles. As sustained release of insulin has been demonstrated to be possible, this technology could be applied to developing a pill to replace the basal insulin injections patients currently give themselves. If this cuts out 1-2 injections per day, user compliance could be increased, resulting in more patients receiving the full amount of insulin they are prescribed.

While the micronization of the insulin was found to be extremely important, future research should also be conducted into better regulating the phase inversion nanoencapsulation process as there is currently a lot of room for user and equipment introduced variability. If a consistent process is achieved, work testing different polymers and molecular weights of those polymers should also be conducted to better control the release profile. Finally, *in vivo* experimentation must be conducted to test the insulin for activity after processing through the micronization and encapsulation process. If this future work is carried out and successful, this high potential technology may one day improve the quality of life for diabetics and make their insulin regimens safer.

References

American Diabetes Association. (2016). *Insulin & Other Injectables*. Retrieved from Diabetes.org: http://www.diabetes.org/living-with-diabetes/treatment-and-care/medication/insulin/?referrer=http://www.diabetes.org/living-with-diabetes/treatment-and-care/medication/.

Atkinson, M. A., Eisenbarth, G. S., & Michels, A. W. (2014). Type 1 Diabetes. *The Lancet*, 69-82.

Daneman, D. (2006). Type 1 diabetes. *The Lancet*, 847-858.

Dansinger, M. (2015, May 14). *Types of Insulin Delivery Systems*. Retrieved from WebMD: http://www.webmd.com/diabetes/features/insulin-delivery#3

Farsaei, S., Radfar, M., Heydari, Z., Abbasi, F., & Qorbani, M. (2014). Insulin adherence in patients with diabetes: risk factors for injection omission. *Primary Care Diabetes*, 338-345.

Harnett, K. M. (2016). Pharmacokinetics.

International Diabetes Federation. (2015). *IDF Diabetes Atlas, 7 ed. .* Retrieved from International Diabetes Federation.

Johnson, H. B. (2016, October 26). *Prediabetes: Are You At Risk?* Retrieved from University Health News: http://universityhealthnews.com/daily/diabetes/prediabetes/

Landreh, M., Alvelius, G., Willander, H., Stukenborg, J. B., Soder, O., Johansson, J., & Jornvall, H. (2012). Insulin solubility transitions by pH-dependent interactions with proinsulin C-peptide. *Federation of European Biochemical Societies* , 4589-4597.

Lowman, A. M., Morishita, M., Kajita, M., Nagai, T., & Peppas, N. A. (1998). Oral Delivery of Insulin Using pH-Responsive Complexation Gels. *Journal of Pharmaceutical Sciences*, 933-937.

Mathiowitz, E. (2016). Introduction to Drug Delivery.

Mecklenburg, R. S., Benson, E. A., & Benson Jr., J. W. (1984). Acute Complications Associated With Insulin Infusion Pump Therapy. *Journal of American Medical Association*, 3265-3269.

National Diabetes Data Group, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. (1995). *Diabetes in America.*

NovoLog. (2017). *Basal Bolus Insulin Therapy*. Retrieved from Novolog.com: https://www.novolog.com/type-2-diabetes/general-type-2/diabetes-and-mealtime-insulin/basal-bolus-therapy.html

Stachnik-Rushlow, A. (2017). *How Diabetes Affects Your Lungs*. Retrieved from Healthgrades: http://healthguides.healthgrades.com/tips-for-managing-diabetes/how-diabetes-affects-your-lungs

Wilson, C. G., & Crowley, P. J. (2011). The Organization of the Gut and the Oral Absorption of Drugs: Anatomical, Biological and Physiological Considertions in Oral Formulation Development. *Controlled Release in Oral Drug Delivery*, 27-48.