Preparation of Tacrolimus-loaded Biodegradable Nanoparticles for Sustained Drug Delivery

by

Reid Kinser

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirement for
the degree of

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(Honors Scholar)

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Abstract approved:

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Tacrolimus is an immunosuppressant drug approved by the FDA for the prevention of organ transplant rejection. However, the use of this drug is hindered by its narrow therapeutic window and extreme insolubility in water. Due to these properties, the currently used tacrolimus preparations must be administered once or twice daily to minimize peak and trough effects and maintain a therapeutic concentration in the subject. In this study, biodegradable polymeric nanoparticles are explored as an alternative delivery vehicle. First, a method was developed for formulating tacrolimus-loaded nanoparticles. Seven different polymers were used to prepare the nanoparticles, and their size, polydispersity index (PDI), and drug loading was quantified. The stability of the best formulations was assessed at room temperature and in a refrigerated environment. The drug release profiles of these formulations were also studied and plotted. Though these nanoparticles did not demonstrate significant extended release properties, they were successful in increasing the tacrolimus solubility in water by over a factor of 100 compared to the free drug. Furthermore, the results of this study reflect the importance of PEGylated polymers for developing tacrolimus-loaded nanoparticles.

Key words: tacrolimus, immunosuppressant, nanoparticles, drug delivery

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Introduction

Tacrolimus is an immunosuppressant drug approved by the FDA for the prevention of organ transplant rejection as well as other autoimmune disorders (Nghiem et al, 2002). It is a member of the macrolide family of compounds, extracted from the fermentation broth of soil bacteria. Since the discovery of its properties in 1984, it has shown promise as an alternative to cyclosporine, the most popular immunosuppressant for preventing transplant rejection at the time. Tacrolimus demonstrates 50-100x the potency of cyclosporine allowing for much smaller dosages (Barreiro and Martínez-Castro, 2014). Large scale studies have shown that transplant patients treated with tacrolimus have fewer instances of acute organ rejection than patients treated with cyclosporine, as well as fewer serious adverse drug reactions. Tacrolimus also shows better long term survivability and overall cost effectiveness than cyclosporine (Scott et al, 2003).

Though these benefits of tacrolimus make it a promising treatment for the prevention of organ transplant rejection, development of an easy-to-use formulation has proved difficult. Tacrolimus has a very narrow therapeutic window, 5-15 ng/mL in blood (Nghiem et al, 2002; Thervet et al, 2010). With current oral capsule and injection formulations, this requires carefully timed administration once or twice daily to maintain a therapeutic concentration in the subject (Barraclough et al, 2011). For children and elderly patients, who have the most significant risk of organ transplant rejection, it may

be difficult to remember to take a dose on time. Frequent blood analysis is required to make sure that drug concentration remains at a safe level (Levine et al, 2011). There is significant variability in tacrolimus metabolism from patient to patient (Thervet et al, 2010). Another obstacle to creating an effective tacrolimus formulation is that it has extremely low solubility in water, 4-12 μg/mL (Patel et al, 2013).

Clearly, there is a need for a better tacrolimus formulation. An important feature of this formulation would be an extended release profile. Reducing the frequency of administration would increase patient compliance, preventing rejection. The dosage of this formulation would also need to be easily adjusted to suit the individual patient's metabolism. Finally, the formulation would need to be nontoxic and soluble within the body, suitable for use in patients with compromised immune systems.

Nanoparticles are one possible way to deliver tacrolimus more efficiently. Such formulations were first developed for drug delivery purposes roughly 40 years ago (Acharya and Sahoo, 2011). The properties of a nanoparticle can be tailored to serve a specific purpose through the use of different types of polymers and manufacturing methods. Loading a drug inside a nanoparticle makes it possible to modify the properties of the drug with much more freedom than other types of formulations allow.

In order to make safe polymeric nanoparticles for drug delivery, an appropriate polymer must be chosen. Polylactic-co-glycolic acid (PLGA) is one of the most popular materials for this purpose, and is approved by the FDA for use in medical devices (Acharya and Sahoo, 2011). It is completely biodegradable, hydrolyzing into lactic acid and glycolic acid, which are easily and safely removed from the body through the Krebs

cycle (Danhier et al, 2012). Other common polymers used for this purpose include polylactic acid (PLA) and polycaprolactone (PCL).

There are many parameters by which one can judge the effectiveness of a nanoparticle formulation. Encapsulation Efficiency (EE) is a measure of the percentage of active drug taken into the nanoparticle compared to the concentration of the initial solution. A successful formulation must have a high EE, for cost-effective manufacturing and minimal waste. Loading Efficiency (LE) is the mass ratio of active drug to polymer, which also has implications in cost of production and safety. PLGA nanoparticles have notoriously low LE values, often around 1% (Danhier et al, 2012). It is typically desirable to have a consistently sized formulation, which is measured as the polydispersity index (PDI). A low PDI represents a monodispersed population, where the particles are mostly the same size. PDI is typically measured using Dynamic Light Scattering (DLS).

Not all nanoparticles made from the same type of polymer will behave the same way. Formulations made of the same polymer but with differing molecular weights will have significant differences in drug loading, stability, particle density, size, and drug release profile. In nanoparticles made of copolymers, like PLGA, the ratio of the copolymers will also have a significant impact. Studies have shown that the higher the ratio of PGA to PLA in PLGA, the faster the polymer will hydrolyze -- with the exception of 50:50 ratio, which is the fastest at degrading (Miller et al, 1977). The specific method used to prepare the nanoparticles will also result in differences in their structure, altering the properties (Javadzadeh et al, 2010).

Surface modifications to nanoparticles can be used to alter their properties while leaving the structure of the active drug unchanged. Targeting systems have been

developed, such as attached ligands that specifically bind to certain receptor sites, especially for cancer drug delivery (Guopei et al, 2010). Passive surface modification is commonplace as well. The most popular example of this is polyethylene glycol (PEG) augmented nanoparticles. PEG can be used to increase water solubility, minimizing aggregation, maximize drug loading, and alter surface charge (Danhier et al, 2012). Particles that are too hydrophobic or bear a large enough surface charge can be detected as foreign by the body, triggering their removal by the reticulo-endothelial system (Kumari et al, 2010). PEGylation can be used to increase hydrophilicity and neutralize surface charge, which extends the circulation half-life by many orders of magnitude (Acharya and Sahoo, 2011). The surface zeta potential of a nanoparticle formulation dispersed in an aqueous medium can be used to approximate the characteristic surface charge of the nanoparticles (Soppimath et al, 2001). Aggregation of nanoparticle dispersions is prevented by the electrostatic repulsion of these charges, so non-neutral formulations show better stability.

Equipment and Materials

Tacrolimus (>99%) used in the experiments was obtained from LC Laboratories. All solvents used (methanol and acetone) were of ACS Analytical Grade and purchased from VWR International, LLC. Average nanoparticle size, PDI, and zeta potential were determined using a Malvern Nano ZS Zetasizer (model #ZEN3600). Each test was conducted in triplicate, and the results averaged. Tacrolimus quantification was carried out by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) with a Shimadzu Prominence system, using a mobile phase of 84% MeOH, 16% H2O, and 0.1%

Phosphoric Acid. Tacrolimus was analyzed at 205 nm using a Zorbax C8 Column (4.6 x 75 mm, 3.5 µm, part #966967-906).

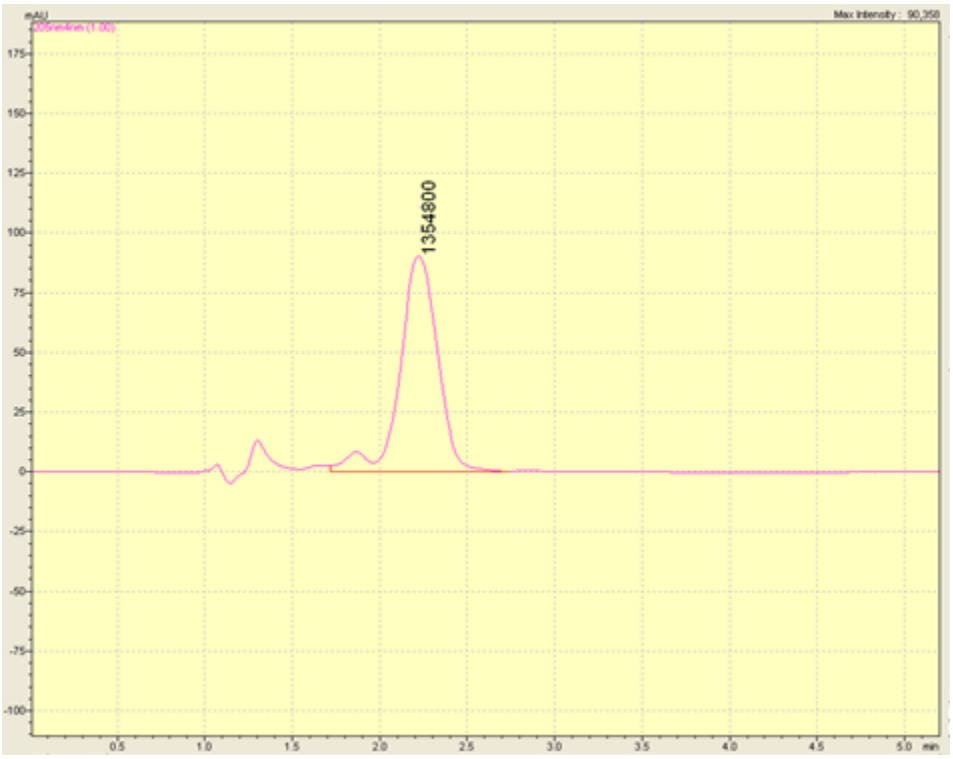

Figure 1 *RP-HPLC plot of tacrolimus (125 µg/mL.)*

Nanoparticle Production Method

Nanoparticles were produced using the nanoprecipitation method (Danhier et al, 2012). For this process, first a 2 mL acetone solution containing 1 mg/mL of tacrolimus and 3-5 mg/mL of polymer was added to a 20-mL vial. The vial was then sealed and the solution was thoroughly mixed for 2 minutes using a Vortex mixer, followed by 3 minutes in a sonication bath. This mixing process was then repeated, for a total of 10 minutes.

Next, under gentle magnetic stirring, 2 mL of DI water was quickly added to the vial. The acetone was then removed from the vial via rotary evaporation in a 40 °C water bath at 400 mBar for 10 minutes, followed by 300 mBar for 10 minutes, and ending with 200 mBar for 5 minutes. At the end of this 25 minute rotavap procedure, the volume of the solution within the vial was measured. Additional DI water was added to bring the volume to 2 mL if necessary. The solution was transferred to an Eppendorf tube for centrifugation at 10,000 RPM for 10 minutes. Finally, the supernatant fluid was removed with a syringe, and filtered using a nylon 0.2 μm filter into another Eppendorf tube.

Polymer Selection

In order to choose the polymer best suited for a tacrolimus nanoparticle formulation, seven different biodegradable and biocompatible polymers with a range of properties were tested (Table 1). These included 3 PLGA polymers, 2 PLA polymers, and 2 PCL polymers. The average molecular weights of these polymers ranged from 10,000-96,000

Name	Manufacturer	Polymer Type	PEG	Molecular Weight
RG502	Sigma	Poly(lactic-co-glycolic acid) 50:50	No	12,000
RG505	Sigma	Poly(lactic-co-glycolic acid) 50:50	No	61,500
RG756S	Sigma	Poly(lactic-co-glycolic acid) 75:25	No	96,000
PLA	Advanced Polymer Materials	Poly(lactic acid)	No	10,000
PCL	Advanced Polymer Materials	Poly(caprolactone)	No	10,000
PEG-PLA	Advanced Polymer Materials	mPEG-b-Poly(lactic acid)	Yes, 5,000	15,000
PEG-PCL	Advanced Polymer Materials	mPEG-b-Poly(caprolactone)	Yes, 5,000	15,000

Table 1 *Polymers used for nanoparticle production.*

Da. In addition, two of these polymers had PEG surface modification.

For each polymer, nanoparticles were prepared using the above method with a constant tacrolimus concentration of 1 mg/mL. Nanoparticles for each polymer were produced using polymer concentrations of 3, 4, and 5 mg/mL to test the effect of polymer concentration on the resulting particles. Each nanoparticle formulation was examined using Dynamic Light Scattering to determine particle size and polydispersity index (PDI). Next, RP-HPLC was used to determine the tacrolimus concentration of the nanoparticles. The results from these tests were compared to determine the polymers that produced the most satisfactory nanoparticles, and the concentration of polymer that was most efficient.

Drug Loading

PEG-PLA, PEG-PCL, and RG756S nanoparticles contained the highest tacrolimus concentration, respectively (Figure 2). PLA, PCL, RG502, and RG505 performed the worst, all with encapsulation efficiencies of less than 20%. Among the top three polymers, some significant differences were observed as a result of different polymer concentrations. PEG-PLA had an EE of >95% using concentrations of 4 or 5 mg/mL, with a steep drop off to approximately 83% at a concentration of 3 mg/mL. RG765S showed a sharp improvement at 4 mg/mL concentration, attaining an EE of approximately 33%.

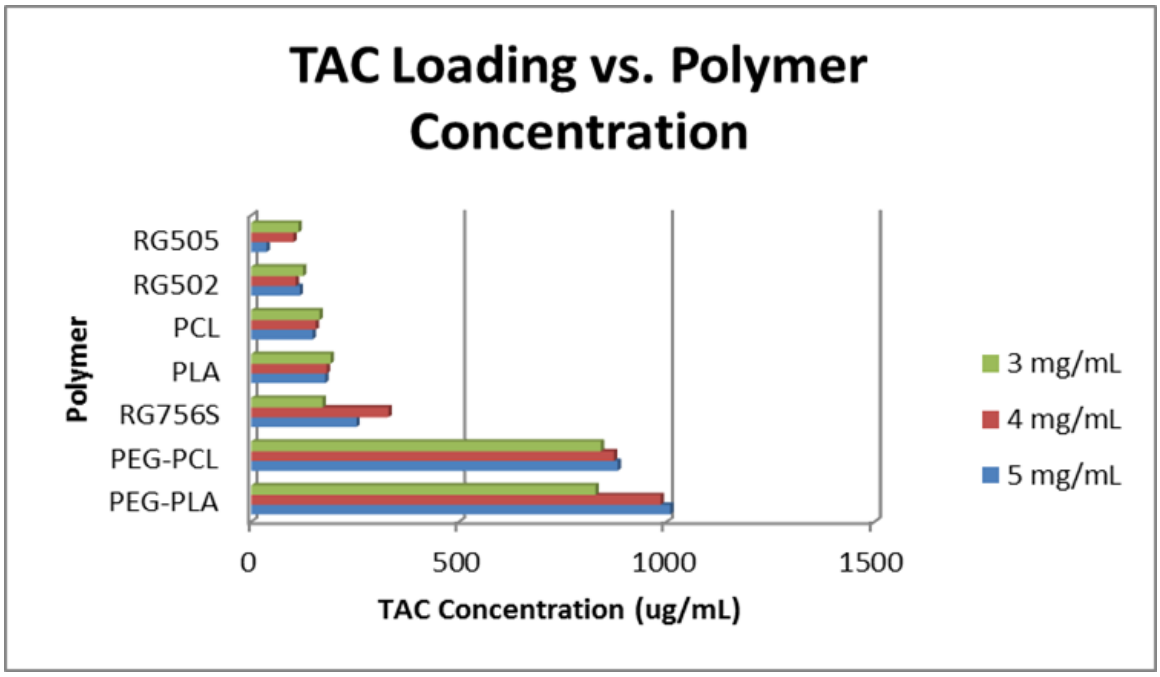

Figure 2 *Effect of polymer concentration and composition on tacrolimus loading.*

Average Size and Average PDI

To optimize the therapeutic efficacy a nanoparticle-based delivery system, it is important to maximize the time of circulation in the blood. For this purpose, particle size must be considered. The kidneys are capable of filtering small nanoparticles with a diameter less than 10 nm. Larger particles (>100 nm), however, are subject to removal from the circulatory system by the liver.

For this reason, ideal nanoparticles possess a diameter within this range (Acharya and Sahoo, 2011). To measure the size and PDI, the nanoparticle solution was diluted 50x with water and analyzed with the Zetasizer. The nanoparticles made of PEG-PLA, PEG-PCL, RG756S, and PLA all met this size criterion, and had fairly consistent sizes at each polymer concentration. The average size of some PCL and RG502 nanoparticles were unable to be determined due to the high levels of aggregation.

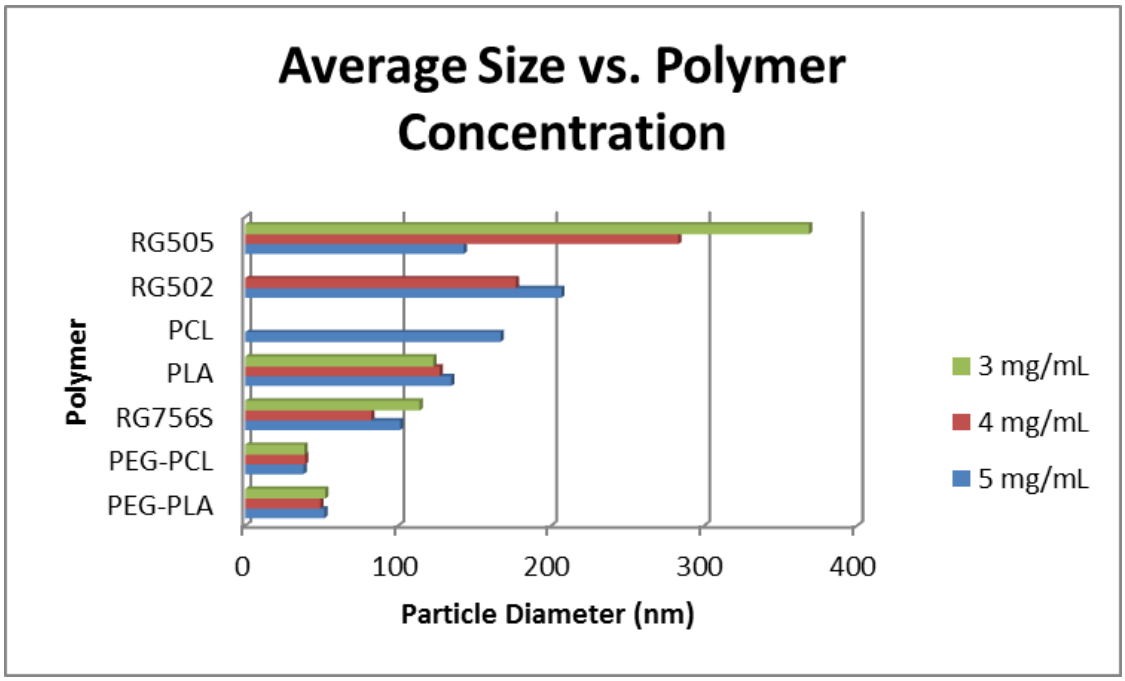

Figure 3 *Average diameter of each nanoparticle formulation. Average diameter of some RG502 and PCL formulations not determined due to extreme aggregation.*

An important feature of an ideal nanoparticle delivery system is consistent particle size, theoretically resulting in uniform degradation and drug release. A small PDI value represents a monodispersed nanoparticle solution. A very large PDI is an indicator of particle aggregation, which should be avoided. Particles with large diameter, such as an aggregate of nanoparticles, can cause occlusion or other damage to the circulatory system (Acharya and Sahoo, 2011). RG502 showed obvious signs of aggregation at 3 mg/mL, with a PDI value of 1. Aggregation might also be an issue with the PCL polymer, as there was extreme variability in the PDI values. The PDI of PEG-PLA, PEG-PCL, RG756S, and PLA all measured within an acceptable range of <0.2, indicating a fairly monodispersed system. They showed no sign of aggregation, regardless of the polymer concentration tested.

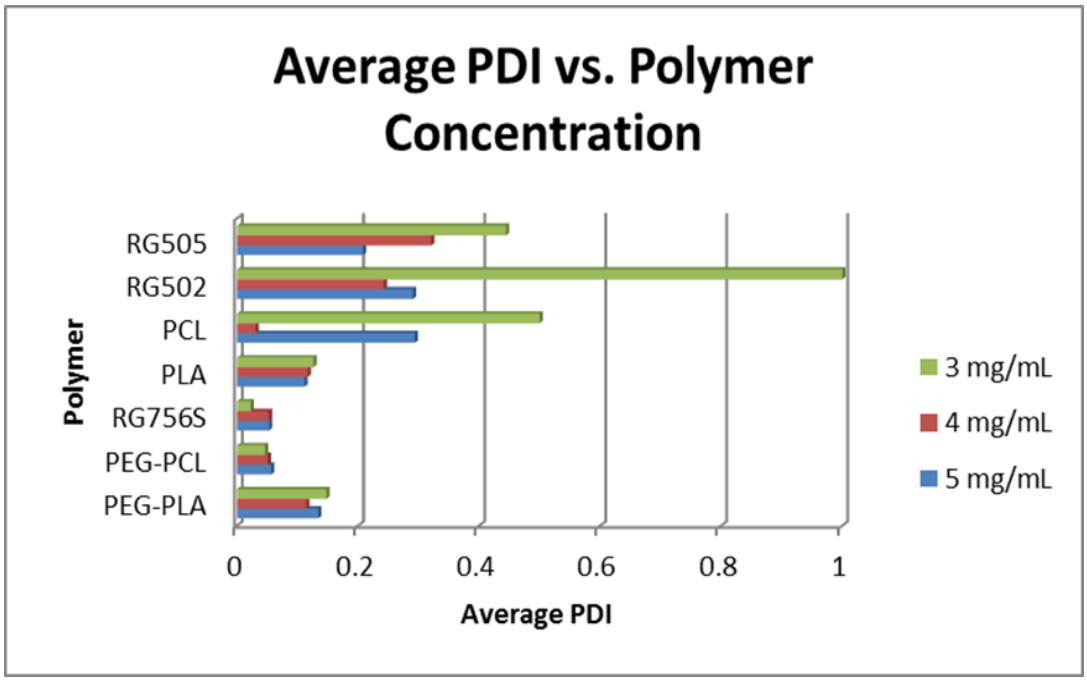

Figure 4 *Average PDI of nanoparticles produced by various concentrations of polymers.*

Room Temperature Stability Test

The three highest-performing nanoparticle formulations were selected for further testing. PEG-PLA, PEG-PCL, and RG756S all demonstrated reasonable potential for encapsulating tacrolimus, with an EE of >30%. These three formulations also met the chosen criteria for average particle diameter (10-100 nm) and PDI (<0.2). These properties are listed in Table 2 below.

Polymer (4 mg/mL)	**Tacrolimus Load (µg/mL)**	**Average Particle Diameter (nm)**	**Average PDI**
PEG-PLA	985.3	49.13	0.115
PEG-PCL	872.6	39.26	0.051
RG765S	330.5	82.39	0.053

Table 2 *Properties of the three highest performing nanoparticles.*

The nanoparticles were prepared and left at room temperature to determine their stability. The tacrolimus and polymer concentration used to prepare all three nanoparticles was 1 mg/mL and 4 mg/mL, respectively. Again, DLS was used to determine particle size and PDI, and RP-HPLC was used to quantify drug loading. These measurements were taken at day 0, 1, 2, 3, and 7. Prior to analysis, the nanoparticle solution was transferred to an Eppendorf tube for centrifugation at 10,000 RPM for 10 minutes. The supernatant fluid was removed with a syringe, and filtered with nylon 0.2 μm filter into another Eppendorf tube.

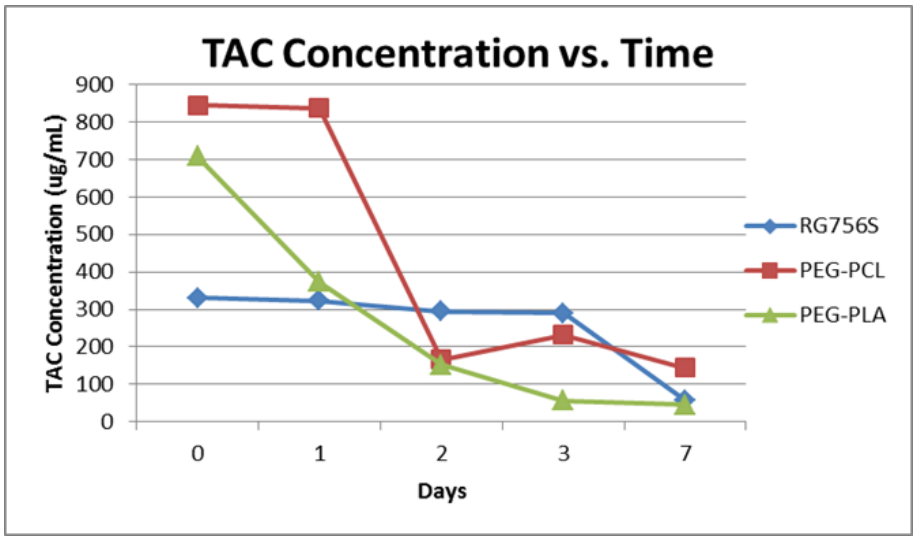

Figure 3 *Loss of tacrolimus load over time as nanoparticles degrade.*

PEG-PLA showed the most rapid decline, losing nearly half its original drug concentration after just 24 hours. PEG-PCL was able to maintain its load for 24 hours, followed by rapid polymer precipitation at subsequent time points. Of the three formulations tested, RG756S displayed the most stability, with a slow linear decline. Even 72 hours after production, only a slight reduction in tacrolimus loading was detected.

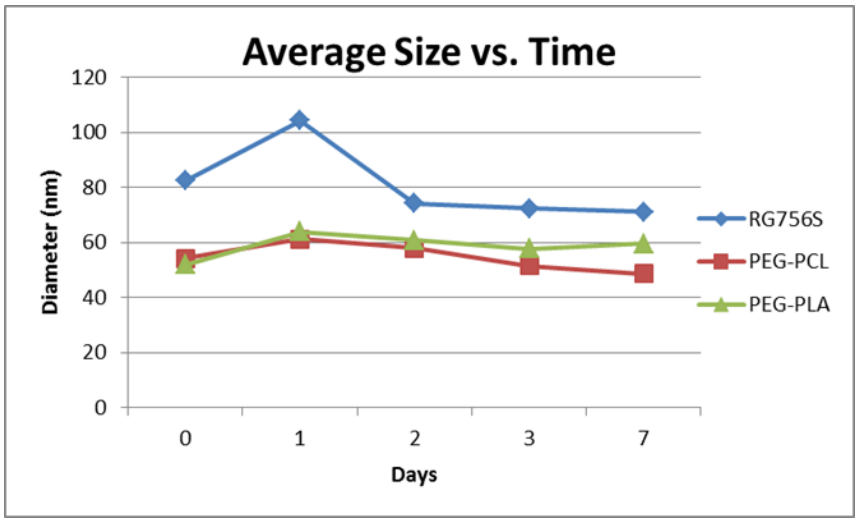

Figure 4 *Change in average nanoparticle size over time at room temperature.*

Though only a modest change in average particle size was observed for all three formulations over the course of the week, suggesting minimal degradation, the average PDI measurement provides a clearer picture. The PDI of PEG-PLA increases dramatically with an inverse relationship to its tacrolimus loading. This indicates that the particles were degrading non-uniformly, resulting in an increased disparity in particle size. The degradation of PEG-PCL appears to be more uniform, as the PDI only increases slightly over the course of the week, as average size decreases. RG756S maintained an excellent PDI of <0.1, even after a week at room temperature.

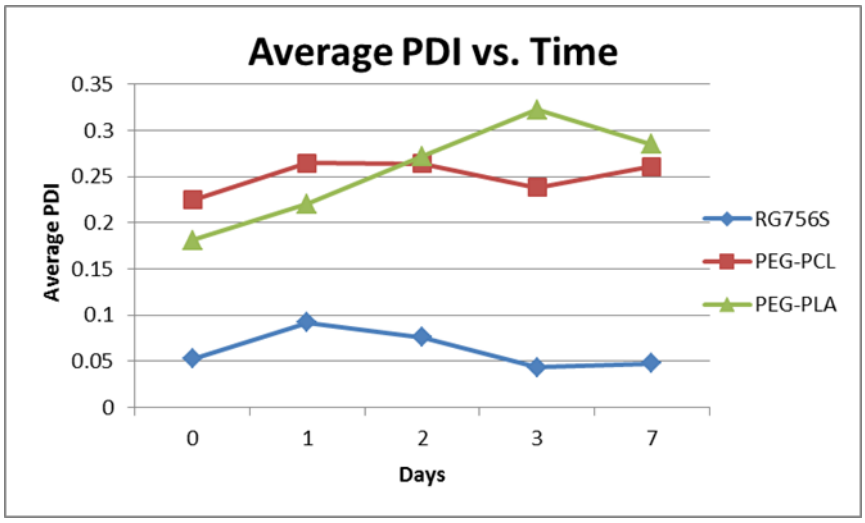

Figure 5 *Change in PDI over time at room temperature.*

Drug Release Profile Characterization

For this step of the process, only the two formulations that had the highest tacrolimus concentration (PEG-PCL and PEG-PLA) were chosen to proceed. The tacrolimus and polymer concentration used to prepare both nanoparticles was 1 mg/mL and 4 mg/mL, respectively. To test the drug release profile of the nanoparticle, a simple dialysis procedure (Figure 8) was employed, using a Thermo Scientific Slide-A-Lyzer Dialysis Cassette (MW cutoff 20,000 Da). First, the release media was prepared by diluting a 0.25 M phosphate buffer stock solution with water to yield a 10 mM buffer solution. 2.5 L of this release media was heated to 37°C, and the dialysis cassettes were then placed in the bath for an hour to hydrate.

After soaking in the release media, the cassettes were removed and filled with approximately 2 mL of nanoparticle solution. They were then placed back inside the water bath where they were kept under gentle magnetic stirring for the duration of the

experiment. At time points 0, 0.5, 1, 2, 3, 6, 12, 24, 48, and 72 hours into the experiment, 100 µL of solution was removed from inside the cassettes and replaced with the same volume of phosphate buffer. These samples were analyzed with RP-HPLC to show the percentage of tacrolimus released from the nanoparticles during the course of the experiment. At time points 3 and 12 hours, the entire contents of the water bath were replaced to maintain sink conditions for proper simulation of a living system. Each formulation was tested in triplicate, and the results averaged to obtain a general release profile of tacrolimus from the nanoparticles.

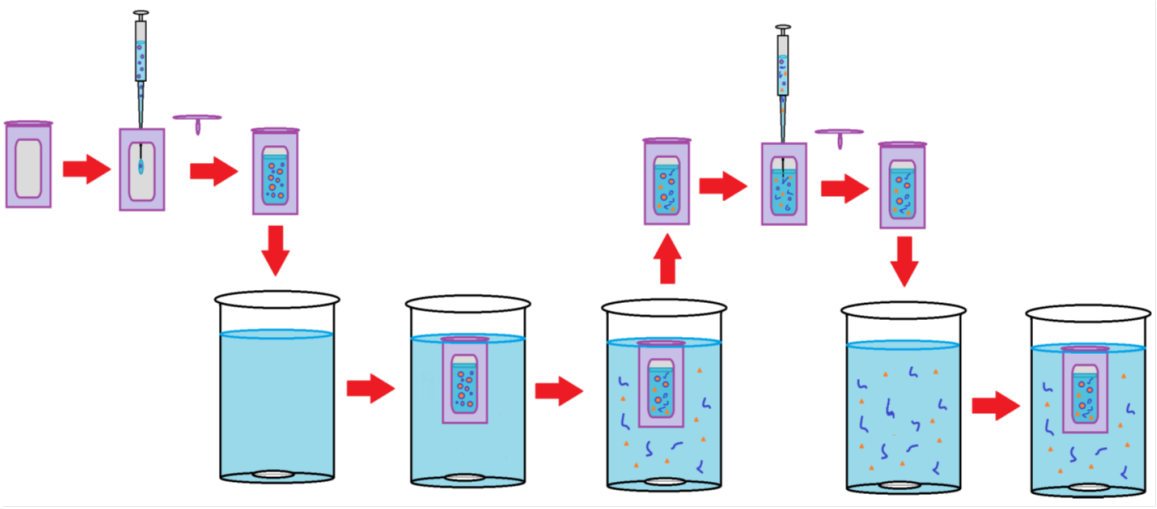

Figure 6 *Dialysis cassette is loaded with aqueous nanoparticle solution and placed in water bath. Tacrolimus concentration in the cassette is regularly quantified to plot the drug release.*

Though extended release capability is one property of the ideal tacrolimus formulation, this was not obtained (Figure 9). After 6 hours, both nanoparticles had released >50% of their drug load. PEG-PLA in particular demonstrated a burst release within the first 24 hours. PEG-PCL performed somewhat better, with a less steep slope, most noticeable in the 6-24 hour region.

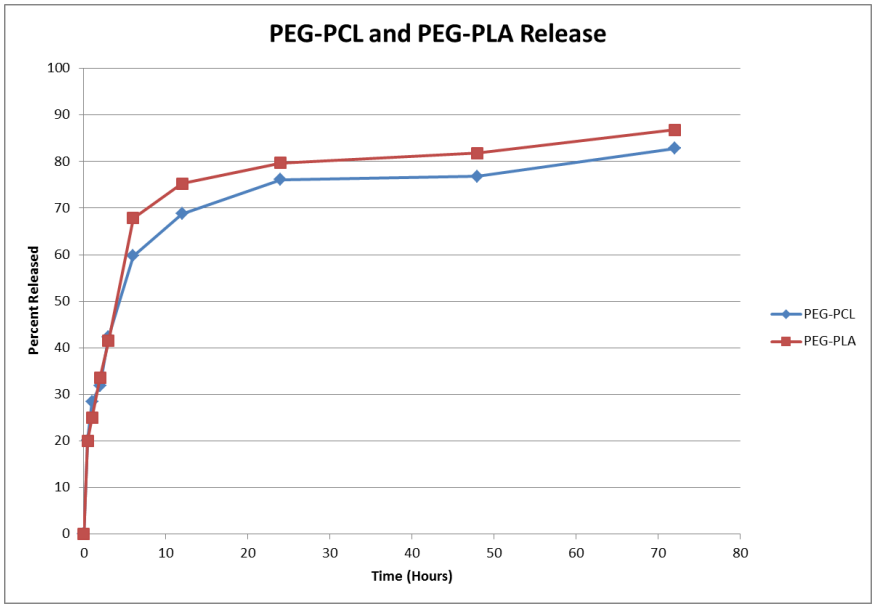

Figure 7 *Tacrolimus release profile from PEG-PCL and PEG-PLA nanoparticles.*

One potential cause of burst release is free drug adsorption to the exterior of the nanoparticle (Danhier et al, 2012). A common solution to this phenomenon, though not always effective, is an additional "rinsing" step between nanoparticle production and deployment.

Refrigerated Stability Test and Zeta Potential

The two formulations used in the release study (PEG-PLA and PEG-PCL) were tested to determine their stability in a normal refrigerated environment (4°C). This test was performed in the same way as the room temperature stability test. This time, the zeta potential of the nanoparticles was also tested on each day. To do this, a folded capillary cell was loaded with nanoparticle solution diluted 100x in water and analyzed with the Zetasizer. The measurements continued until signs of instability were noticeable (cloudiness, precipitation, etc).

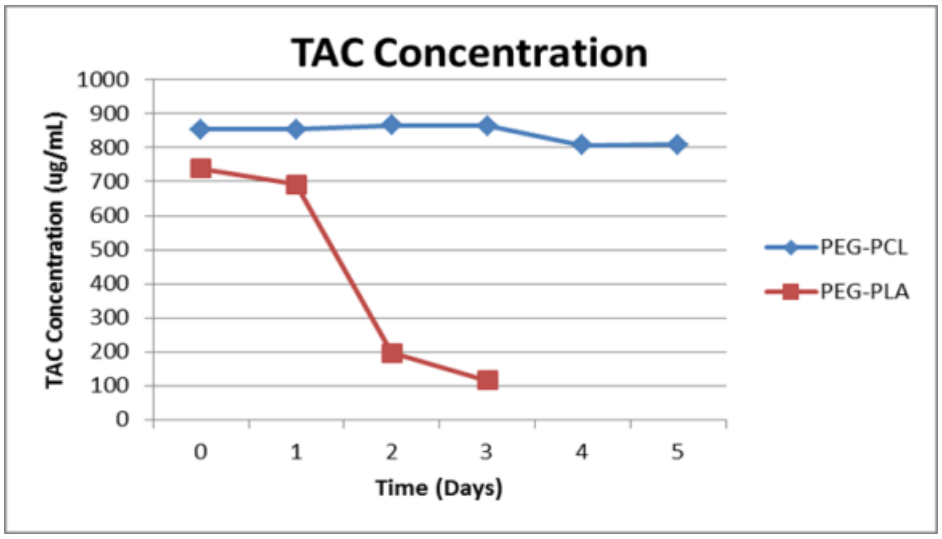

Figure 8 *Loss of tacrolimus load over time as nanoparticles degrade in a refrigerated environment.*

PEG-PLA was discontinued from the testing after three days because it showed visible signs of instability. In Figure 10, it is shown that PEG-PLA lasted about 24 hours before losing a considerable amount of its tacrolimus concentration. This is a slight improvement over its stability at room temperature. Again, its average particle size

remained consistent throughout the test, though an increase in PDI was observed. This change in PDI was significantly less pronounced than in the room temperature test.

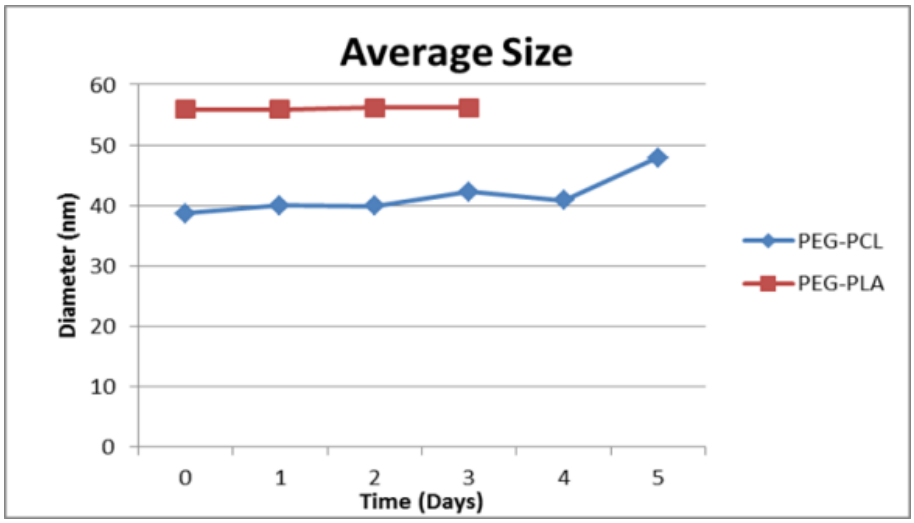

***Figure** 9 Change in average nanoparticle size over time in a refrigerated environment.*

PEG-PCL remained stable for 5 days. In that time, it retained about 95% of its tacrolimus load, a significant improvement over its stability of only 24 hours at room temperature. As before, the average size remained mostly the same. However, the PDI steeply increases with time, even more than it did at room temperature.

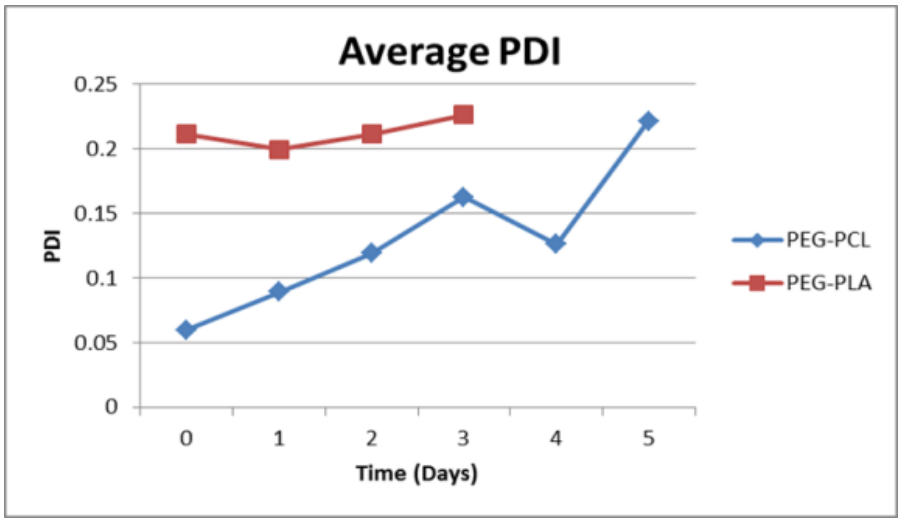

Figure 10 *Change in PDI over time in a refrigerated environment.*

Finally, Figure 13 shows the zeta surface potential of the two formulations through the duration of the stability test. Because the zeta potential is proportional to the surface charge, this value is a fair estimation of the charge of the particles (Soppimath et al, 2001), which is important for stability purposes. Particles with non-neutral surface charges of like sign repel each other, keeping the colloidal system from aggregating. Zeta potentials less than ±10 mV essentially act as neutral particles, while potentials of greater than ±30 mV are considered strongly charged (Clogston and Patri, 2011). Most cell membranes and many proteins are negatively charged, so cationic nanoparticles can be problematic – disrupting cell membranes or being removed from circulation prematurely (Clogston and Patri, 2011; Acharya and Sahoo, 2011). For this reason, a good target zeta potential range is between -10 and -30 mV for this nanoparticle application. The graph below shows some variation for PEG-PCL at days 1 and 2, but for the most part both formulations remain in the range of -15 to -20 mV.

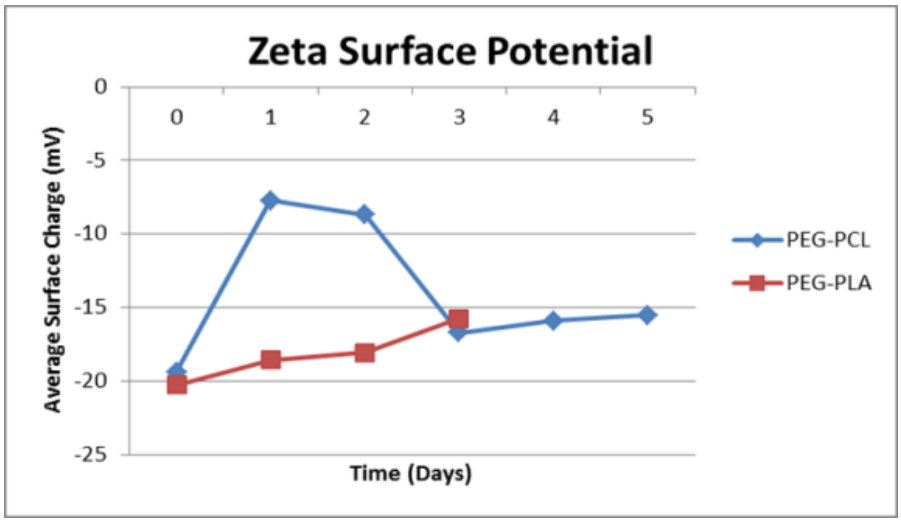

Figure 11 *Change in Zeta potential during refrigerated storage.*

Discussion

Tacrolimus is a promising immunosuppressant drug for use to prevent organ transplant rejection. Though there are many studies that indicate that it may be a better primary treatment option than other popular immunosuppressants, such as cyclosporine, issues with developing a reliable delivery method have complicated its use. Some of the most notable examples of these issues include poor water solubility, narrow therapeutic window, and the lack of an extended release delivery method. Nanoparticles have been identified as a potential method for resolving these issues.

In this study, tacrolimus nanoparticles were made of several different polymers and the most successful formulations were identified. Polymers PEG-PLA, PEG-PCL, and RG756S all showed suitable drug loading, particle size, and PDI (shown in Table 2).

The stability of the most successful formulations was tested at room temperature. The results of this test indicated that RG756S was the most stable and capable of

retaining a tacrolimus load for the longest time, showing a slow linear decline from 330.5 μg/mL to 290.7 μg/mL over the course of 3 days. It also kept a PDI of <0.1 over the whole week of testing. PEG-PLA was found to be the most fragile system, stable for less than 24 hours at room temperature, as shown in Figure 5.

The PEG-PLA and PEG-PCL formulations were chosen for release profile characterization because they had the highest tacrolimus concentration (985.3 and 872.6 μg/mL, respectively). Both formulations displayed a burst release, rather than an extended release. As suggested by the stability test, PEG-PCL slightly outperformed PEG-PLA with a slower degradation time and gentler slope. PEG-PCL further displayed its superiority over the PEG-PLA formulation in the refrigerated-environment stability test, lasting for around 5 days (5.4% loss in tacrolimus load) compared to PEG-PLA's <48 hours (over which time it lost 73.3% of its tacrolimus load).

While we were unable to create an extended release formulation, we were able to increase the water solubility of tacrolimus, normally 4-12 μg/mL. The PEG-PCL formulation has a water solubility of approximately 100x the free drug (near 1 mg/mL). Furthermore, these polymer tests demonstrate the significant impact PEGylation can have on a nanoparticle; the PLA and PCL polymers tested were outperformed in every way by their PEGylated counterparts. Both PEG nanoparticles showed more than a five-fold increase in tacrolimus load. These nanoparticles were also smaller on average. In the case of the PCL nanoparticle, PEGylation prevented an unacceptable level of aggregation, instead yielding a PDI of <0.1.

Conclusion

Nanoparticle delivery methods are a valid route to address the shortcomings of the immunosuppressant drug, tacrolimus. Three nanoparticle delivery systems (PEG-PLA, PEG-PCL, and RG756S) were identified as promising candidates for development. PEG-PCL and PEG-PLA nanoparticles demonstrated high encapsulation efficiency, which can be attributed to the PEG surface modification, however they failed to produce an extended release of tacrolimus upon hydrolysis. On the other hand, nanoparticles made from the polymer RG756S showed decent PDI and particle size, as well as exceptional stability, but the tacrolimus loading (while not insignificant) left much to be desired. The results of this study suggest that future tests with a PEGylated form of RG756S could show a substantial increase in tacrolimus concentration while retaining the polymer's stability and resilience to degradation. This indicates that such a polymer might be ideal for producing the sought after extended release quality necessary for a new, easy-to-use tacrolimus formulation.

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