

Synthesis of Biodegradable Hydrogel Microparticles for Vaccine Protein Delivery

by

Kendall Werts

Submitted to the Department of Materials Science and
Engineering in Partial Fulfillment of the Requirements For the Degree of

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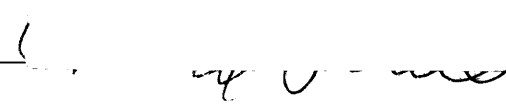
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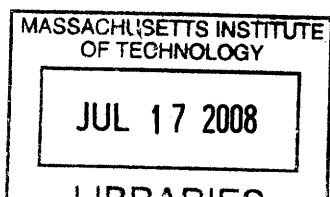
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Abstract

Soluble protein antigens used in vaccines have shown lower immune responses when compared with certain particulate forms of these same antigens. For example, it has been shown that micro- and nano-particle mediated delivery of protein antigen can use up to 100 times less protein and still produce an effective immune response [1]. In order to use this phenomenon to make vaccines more efficient, we need a biodegradable delivery particle. This thesis modifies a particle created by Jain et al., which consists of a polymer network surrounding and trapping a protein, by removing the non-degradable crosslinker used in the original particle design and replacing it with a poly (ethylene glycol) acrylate molecule attached to ovalbumin protein. When a dendritic cell degrades the particle, the ovalbumin protein will be degraded, as will the connections between the polymer network that holds the particle together [2]. The particles degraded to 56% of their original size in 3 days, while the non-degradable particle degraded to only 80% of its original size.

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I. Introduction

With worries caused by the lack of sufficient flu vaccine a few years ago, approaches to make vaccines more efficient to allow lower doses of antigen per patient are of great interest. Currently, prophylactic vaccines are produced in one of many ways depending on the type of disease they are being used to prevent. A few of these ways involve the dead or weakened organism or part of the organism [3]. It has also been discovered that synthetic protein carriers may help increase the efficiency for vaccines. Shen et al. has shown that a synthetic carrier for the vaccine's protein needed more than 100-fold less protein than the current procedures in order to elicit an immune response [1]. This shows that current vaccines are not as efficient as they have potential to be. A nanoparticle specified for protein delivery such as these synthetic carriers is an option to make vaccines more efficient.

Jain et al. have created protein-loaded hydrogel particles to improve the efficiency of protein delivery [2]. A limitation of these particles is that the gel network is relatively stable (non-resorbable) under physiological conditions. The side effects that can be caused from this non-resorbable polymer are not worth the risks for use in vaccines. Recipients of prophylactic vaccines are healthy patients who are looking for prevention of an illness, so it is imperative for vaccines to be as safe as possible. To accomplish this, a nanoparticle protein delivery system must be created that is degradable, and so will break apart and leave the body, thus limiting possible side effects caused by the packaging system.

To make this nanoparticle degradable, the outer hydrogel polymer network must be made degradable. There are a few ways to go about this. One way is to make the polymer network out of degradable monomers like poly (lactic acid) or poly (lactide-co-glycolide) [4]. We chose to instead incorporate protein as part of the network to make these particles biodegradable. With this method, the outer network holding the particle together will be degraded by the cell at about the same rate as the protein inside that it is delivering since it is made from that same protein.

Dendritic cells phagocytose the particles and then use enzymes to break the protein in the particles into smaller pieces. These pieces are antigens and are presented on MHC class I sites on the surface of the dendritic cells to Cd8+ T cells to trigger an immune response[5].

This paper explains the synthesis of this particle including the new crosslinker that will in the end cause the particle to more easily degrade and two different degradability tests that were run to determine the extent of the particle's degradability.

II. Approach to biodegradable protein-loaded gel nanoparticles

II.a. Current process

The synthesis that this thesis is based on was created by Jain et al. and summarized in Figure 1 [2]. This process involves creating a salt solution with dissolved pluronic, gel precursor monomers, and ovalbumin (ova) protein as the model protein. The pluronic phase separates in saturated sodium chloride solution at 37°C, leading to the formation of

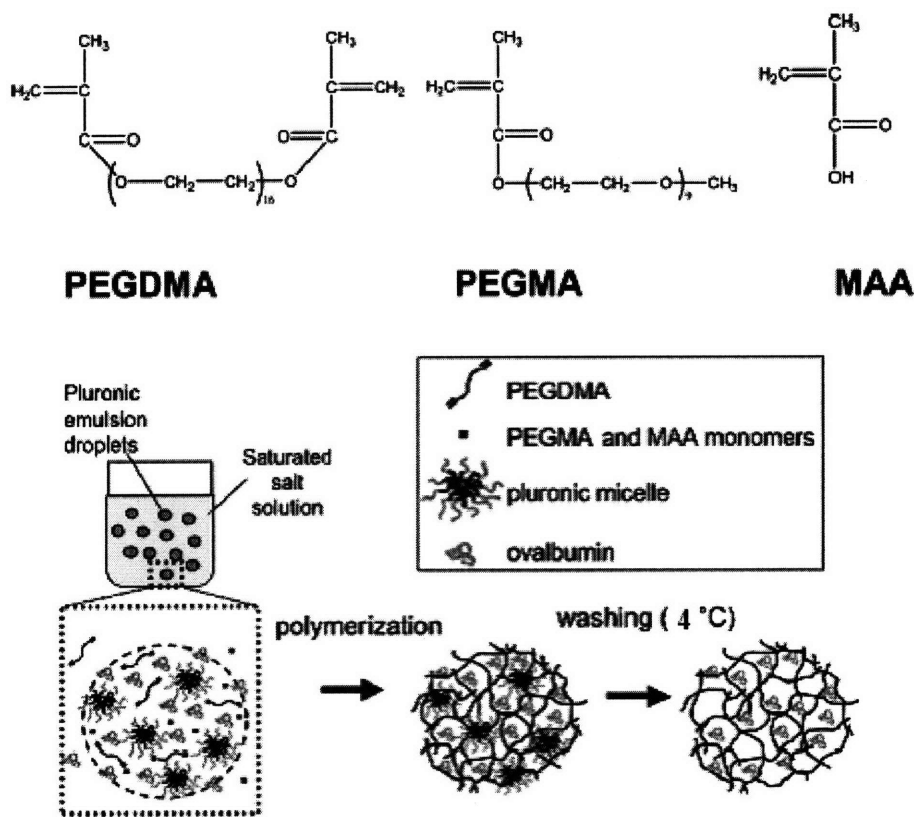


Figure 1. Schematic of Jain Particle showing the monomers used as well as the steps involved in the synthesis from the salt, pluronic, protein, and monomer solution to radical polymerization to washing the particles in order to remove excess pluronic and salts. [2]

kinetically stable pluronic/monomer/ova-rich microdroplets within the solution. The monomers, Poly (ethylene glycol) dimethacrylate (PEGDMA), Poly (ethylene glycol) methacrylate, and Methacrylic acid, surround the protein and keep it inside the particle once polymerized. PEGDMA is the crosslinker in this polymerization, and forms the connections between the PEGMA and the MAA and keeps the particle coherent after the radical polymerization. When a dendritic cell consumes this particle, the cell will break down and remove the protein from the particle, but will not be able to degrade the outer polymer casing.

II.b. Biodegradability

We hypothesized that the efficiency as well as the safety of this particle could be greatly increased by modifying it to become more biodegradable. In order to attempt this, the PEGDMA crosslinker in the original particle was replaced with succinimidyl carboxymethylated poly (ethylene glycol) acrylate (Acrylate-PEG-SCM), which, instead of binding the monomers together as in the original particle, will bind the monomers to the actual protein. This in turn will make the naturally biodegradable protein an innate part of the structure holding the particle together. This is important to the biodegradability, because once the particle is degraded by the cell, the protein will be broken down, but the polymer will not. Once the cell breaks down the protein that is keeping the particle together, the rest of the structure will disintegrate, and will be easier to remove from the body. Figure 3 demonstrates the chemistry involved in initially

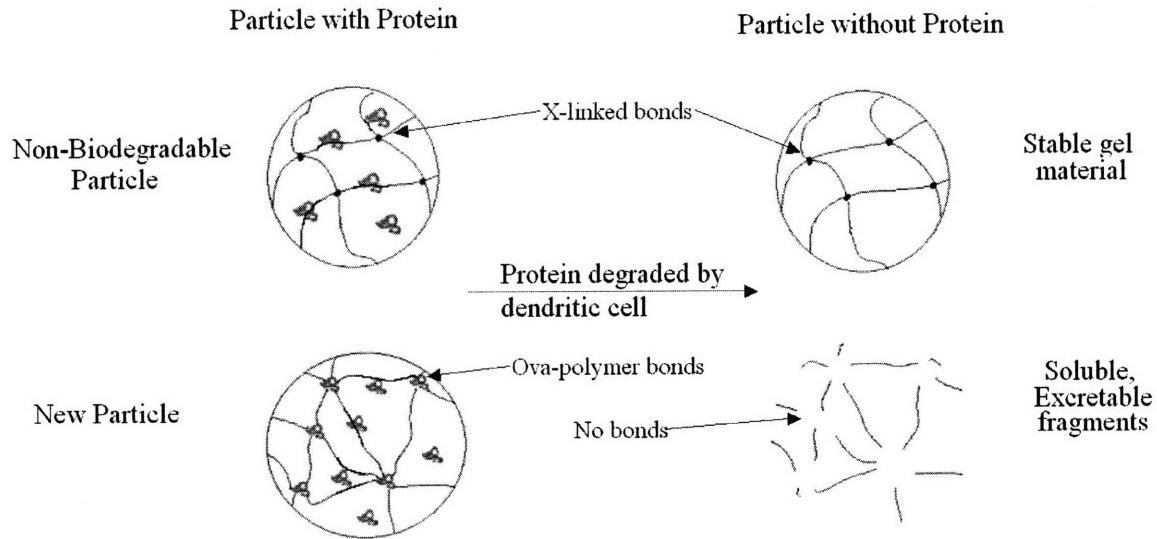


Figure 2. Close-up of how the particles should behave before and after they are degraded by the cell. The original non-degradable particle starts with ova inside a crosslinked polymer casing. After the ova is broken down by the cell, we are left with an empty polymer casing, because the cell cannot break the polymer down. However, the new particle starts with ova inside as well as ova constituting the connections in the polymer network. When the ova is degraded in this new particle, we are left with strands of free polymer that can be easily extracted from the body.

attaching the Acrylate- PEG-SCM to the protein through amine groups. Appendix I show the protein sequence of ovalbumin along with the numbers of amino acids with amine groups on their side chains.

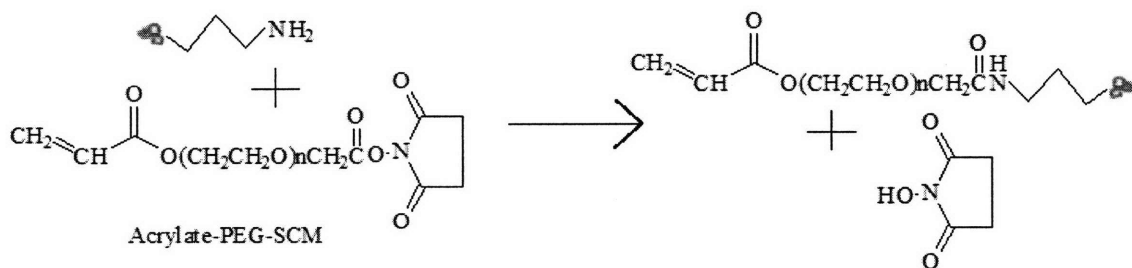


Figure 3. Chemistry of Acrylate-PEG-SCM with ova protein causing the protein to have many PEG-acrylate structures attached to it.

II.c. Fluorescamine Assay

Fluorescamine is a molecule, which when reacted with amine groups, becomes fluorescent with excitation at a wavelength of 390 nm and emission at a wavelength of 475 nm [6]. This can be measured using a fluorescence reader, and is used in this thesis to determine the amount of ova in a solution when reacted with ova's amine groups.

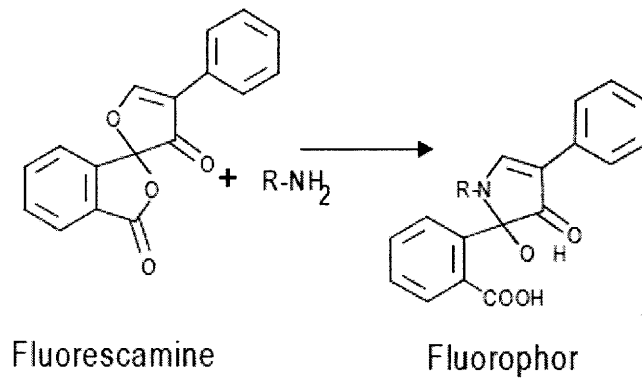


Figure 4. Fluorescamine reaction [7].

III. Materials and Methods

III.a. Ovalbumin Pegylation

Ovalbumin was pegylated with Acrylate-PEG-SCM with a molecular weight of 3.4 kDa from Laysan Bio. A process using a 1:50 mole:mole ratio of ova to Acrylate-PEG-SCM, respectively, was adapted from Abuchowski et al. and Saito et al to produce about a 25% amine group reaction [8,9]. 20mg Acrylate-PEG-SCM was added to a 1mL solution containing 50 mg ova in 0.05M PBS pH 7.4. This solution was stirred for 30 minutes at 20°C to allow PEG conjugation to occur and then filtered to remove the excess Acrylate-PEG-SCM with an Amicon Ultra 4 mL centrifugal filter unit from Millipore with a 10 kDa molecular weight cut off. Tests were then run to ensure this process was successful.

III.b. Nanoparticles

The polymer nanoparticles were created using a procedure adapted from Jain et al [2]. Two g pluronic F-68 and then 16 g Sodium Chloride both from Sigma-Aldrich were dissolved into 50 mL water. This solution was degassed with nitrogen for 15 minutes. 50 mg ova from Worthington Inc was then added and allowed to dissolve for 20 minutes while the nitrogen gas does not bubble through the liquid but instead remained out of the liquid but still in the flask. 300 mg PEGMA, and 15 mg MAA all from Sigma-Aldrich were mixed and degassed with nitrogen before being added to the protein solution which was degassed for about 5 minutes more. It was then placed in a 40°C bath where it became cloudy indicating the onset of phase separation. A 1 mL solution of water with

15 mg ammonium persulfate from Pierce Biotechnology Inc and 15 mg sodium metabisulfate from Fluka were added to the solution. The reaction was stopped after 5 minutes by removing the flask from the 40°C bath and adding 50 mL water. Particles were removed from the solution by centrifuging at 4°C and 10,000 rpm for 20 minutes. The particles were washed with water then centrifuged the same way 2-3 more times, and finally resuspended in 5 mL .4% w/vol pluronic F-68 and PBS and stored at 4°C.

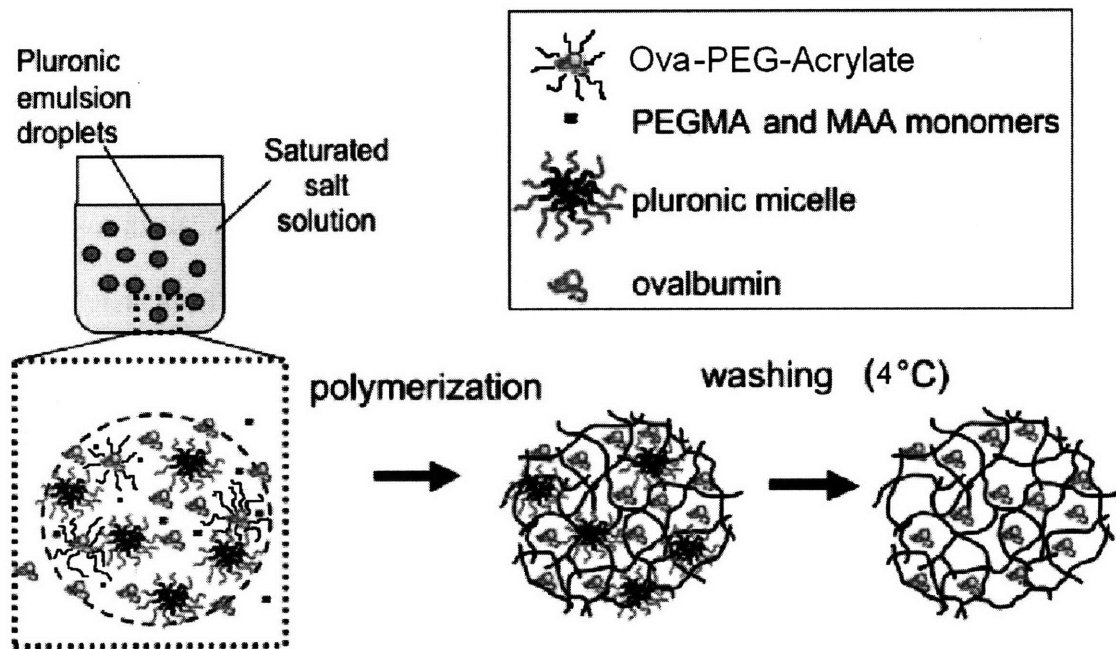


Figure 5. Modified particle synthesis. PEG-ova-Acrylate is in the place of the PEGDMA crosslinker. Figure modified from Jain et al. [*]

III.c. DLS Biodegradability test

50 ng Cathepsin S from Calbiochem was added to the particles in 200 microliters PBS pH 7.4 and incubated at 37°C for 5, 10, 22, 44, or 72 hours. After the period of

degradation, the PBS/particle/cathepsin solution was diluted with 0.8 mL water, and 0.5 mL of this solution was added to 0.5 mL of a pH 9.6 carbonate-bicarbonate buffer to a final pH of about 9.4 in order to break up polymer that may have been aggregating. Sizes of particles after the period of degradation were determined using Dynamic Light Scattering (DLS) on a 90plus particle sizer from Brookhaven Instruments, and the results from the degrading of the new nanoparticle were compared with the old non-degradable particle.

III.d. Dendritic cell tests

The dendritic cell tests were mirrored after the cell test in Jain et al. [2]. Dendritic cells were incubated with the degradable particles for 4 hours. Cd8+ T cells from an OT-I mouse (made specific for ovalbumin) were added and incubated for 72 hours. When a T cell is successfully presented with an antigen, it secretes IL-2, so after 72 hours, the IL-2 secretions and cell proliferation were measured to determine that the dendritic cells processed the particles and presented them to the T cells. Different doses of particles were given to different sets of cells. These tests were done in collaboration with Brandon Kwong.

IV. Results

IV.a. Pegylation Results

After the pegylation process was performed, 3 things needed to be determined to prove a successful test. First, we needed to confirm that the filter unit did remove excess PEG from the solution, because left over PEG in the solution could interfere with the polymerization reaction. To determine this, a solution of pure PEG in PBS was prepared and ran through the filter unit. The solution remaining in the top of the filter unit (the

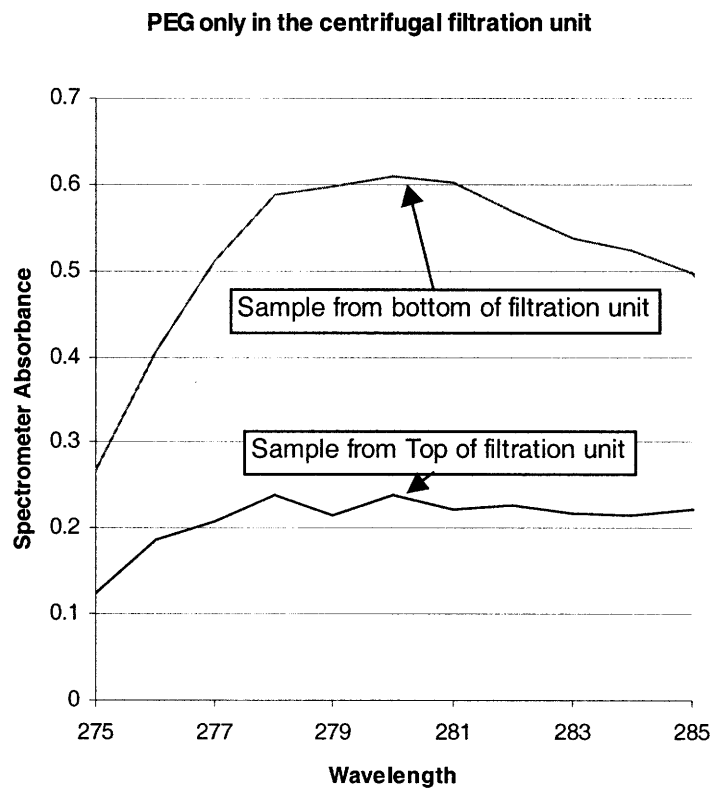


Figure 6. Graph of spectrometer data for the solution of pure PEG in PBS. The top line shows a peak around 280 nm which means there is PEG present in this solution, while there is no peak in the bottom line which means there is no PEG present in the filtered solution.

filtered solution) and the solution that finished in the bottom of the filter unit (the solution with molecules small enough to get through the filter) were both measured with a spectrophotometer. The resulting data shown in Figure 6 demonstrates a peak around 280 nm for the solution from the bottom, and no peak for the solution from the top of the filter. This means that the PEG was successfully filtered through to the bottom solution. Next, we wanted to confirm that the ova stayed in the filtered solution, and how much ova stayed in the filtered solution. For this the fluorescamine assay was used. First, all of the bottom solutions in the filter units were tested to be sure that no ova was filtered out of the solution. Figure 7 shows that the bottom filtration solutions showed no

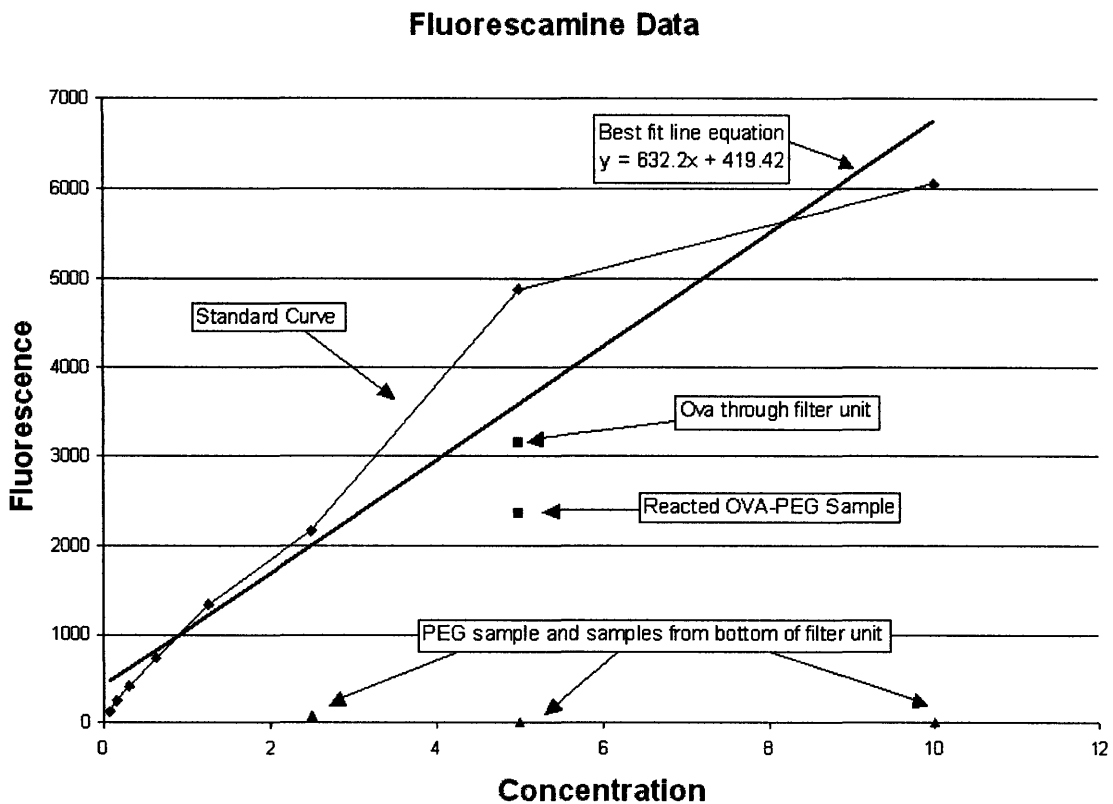


Figure 7. Fluorescamine data. All points are labeled. The best fit line of the standard curve was used to determine the concentration of the unknown ova solutions.

significant fluorescence. A solution of pure ova in PBS was prepared and run through the filter unit. The assay was used on a standard curve made of solutions of different concentrations of ova, and using this information and a best fit line, with the fluorescence from the pure ova filtered solution, an estimate of how much ova was left in the solution can be made. Figure 7 shows this data. Lastly, we needed to determine what percent of the amine groups on ova reacted with PEG. Fluorescamine was used once more for this. It was assumed that the actual amount of ova (or the total amount of amine groups) for the reacted ova-PEG sample would be the same as that of the plain filtered ova solution. Using this (what should be the total fluorescence) and the fluorescence from the reacted ova-peg solution mixed with fluorescamine which would react with any left over amine groups, the ova-peg reaction was found to produce approximately 29% reacted amine groups as Table 1 shows. So, overall, the ova pegylation seems to have worked and given us results about as predicted.

	Filtered ova solution	Reacted ova-PEG
Fluorescence	3155.7	2366.8
Calculated conc. (mg/mL)	4.328	3.08
Estimated actual conc.		4.328
Percent amine reacted	0%	29%

Table 1. Fluorescence data collected from a fluorescamine assay, which reacts with amine groups. This determines what percent of amine groups on the ovalbumin protein were reacted with PEG.

IV.b. Degradability test results

Figure 8 shows the results from the Cathepsin test using the DLS machine to determine particle size. The original size for the non-degradable particle was 939.1 ± 9.6 nm while the original size for the degradable particle was around 1082.1 ± 20 nm. In the first 2 days it seems like the degradable particle is slowly degrading while the old non-degradable particle stays about the same size. After 72 hours, the non-degradable particle seems to be degrading slightly, while the new particle is now nearly half its original size. The particle size of the original non-degradable particle is known to usually be around 500 nm, so it is a little strange that the particle size measured here seems to be around 900 nm. The solution was mixed, but if it were also vortexed or sonicated before testing, better results may have been obtained.

In the cell tests, it was determined that the particles were in fact capable of being processed by dendritic cells and activating naïve CD8+ T cells. This was determined to be true, because naïve T cells must be presented with antigen to survive and proliferate, and after 72 hours, the T cells were observed from under a microscope to be proliferating. The T cells were also secreting IL-2 and appeared to be dose-responsive—a higher concentration of particles in the dose led to a higher T Cell response.

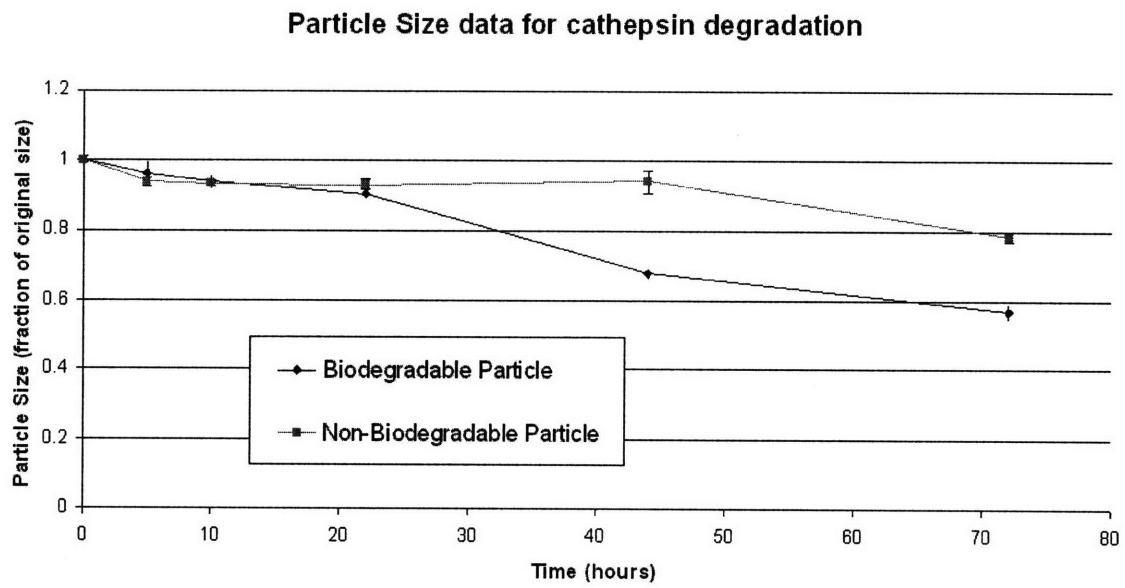


Figure 8. Particle sized data collected using DLS. Top line is biodegradable particle. This particle gets smaller over time while the non-biodegradable particle does not.

V. Conclusion

In Conclusion, the nanoparticles performed better than the non-degradable particle in degradation and also evoked a greater T cell response, but perhaps did not perform as well as expected. Some future work should be done comparing this particle to a particle made with a known biodegradable crosslinker instead of the PEGDMA in the original particle, as well as a test to determine the molecular weights of the particles left in the solution after degradation. Other cell tests can also be performed to determine safety and biodegradability.

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Appendix I.

Ovalbumin Protein Sequence [10]

1 mgsigaasme fcfdvfkkelk vhhanenify cpiaimsala mvylgakdst rtqinkvvr
61 dklpgfgdsi eaqcgtsvvn hsslrtilnq itkpndvysf slasrlyae rypilpeylq
121 cvkelyrggl epinfqtaad qarelinswv esqtngiirn vlqpsvdsq tamvlvnaiv
181 fkglwekftk dedtqampfr vtegeskpvq mmyqiglfrv asmasekmki lelpfasgtm
241 smlvllpdev sgleqlesii nfekltewts snvmeerkik vylprmkme kynltsvlma
301 mgitdvfsss anlsgissae slkisqavha ahaeineagr evvgsaeagv daasvseefr
361 adhpflfcik hiatnavlff grcvsp

Amino Acids with amine groups on side chains:

Total Lysine from above: 20 *Most likely amine group to react.

Total Glutamine from above: 15

Total Asparagine from above: 17

Total Arginine from above: 15