Poly(ethylene glycol) Hydrogel Microspheres as a Controlled Release Device

by

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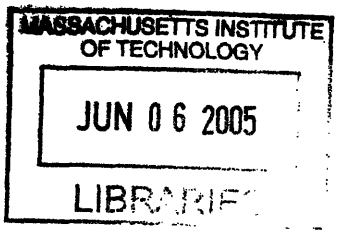

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ABSTRACT

Vaccines for infections such as measles, polio, or chicken pox contain live attenuated viruses, which can sometimes lead to infection. Our objective is to develop an improved strategy for vaccines that induces patent immune responses against persistent viral infections. Three processes must occur to successfully produce immunity; the first is the attraction of immature Dendritic Cells (DCs), loading them with particular antigens, and then maturing the DCs.

This project focuses on DC attraction to an immunization site by fabricating crosslinked polyethylene glycol hydrogel microspheres that encapsulate a chemoattractant. This study was performed to determine whether the diffusion of the chemoattractant could be controlled by varying the amount of crosslinker and by incorporating ionic groups in the polymer matrix. It was found that the crosslinker amounts successfully altered the release profiles of the protein. The ionic groups incorporated in the polymer matrix effectively altered the diffusion of both positively and negatively charged protein diffusion.

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1. Introduction

1.1 The immune system

The immune system functions as a defense against infectious microbes, but a more inclusive definition of immunity is a reaction to foreign substances including microbes as well as macromolecules such as proteins and polysaccharides [1]. Two kinds of immunities exist: innate and adaptive. Innate immunity consists of cellular and biochemical defense mechanisms. The principal components of innate immunity include: (1) physical barriers like epithelia (2) phagocytic cells like neutrophils and macrophages and natural killer cells (3) blood proteins (4) cytokines, which are proteins that regulate and coordinate many of the cell functions within the body. Adaptive immunity involves the body's ability to "remember" and respond to exposures of the same microbe. The key components of the adaptive immune response include lymphocytes and their products. Antigens are defined as any foreign substance capable of inducing an immune response.

Within the adaptive immune response, two types exist, humoral immunity and cell-mediated immunity. Antibodies that are produced by B lymphocytes/B cells conduct humoral immunity. Humoral immunity represents the principal defense mechanism against molecules against extracellular microbes.

T lymphocytes/T cells conduct cell-mediated immunity. The term "naïve" refers to individuals and lymphocytes that have not encountered a particular antigen. Generally, lymphocytes are present in the blood, but are also highly concentrated in discrete lymphoid organs where the immune responses are initiated.

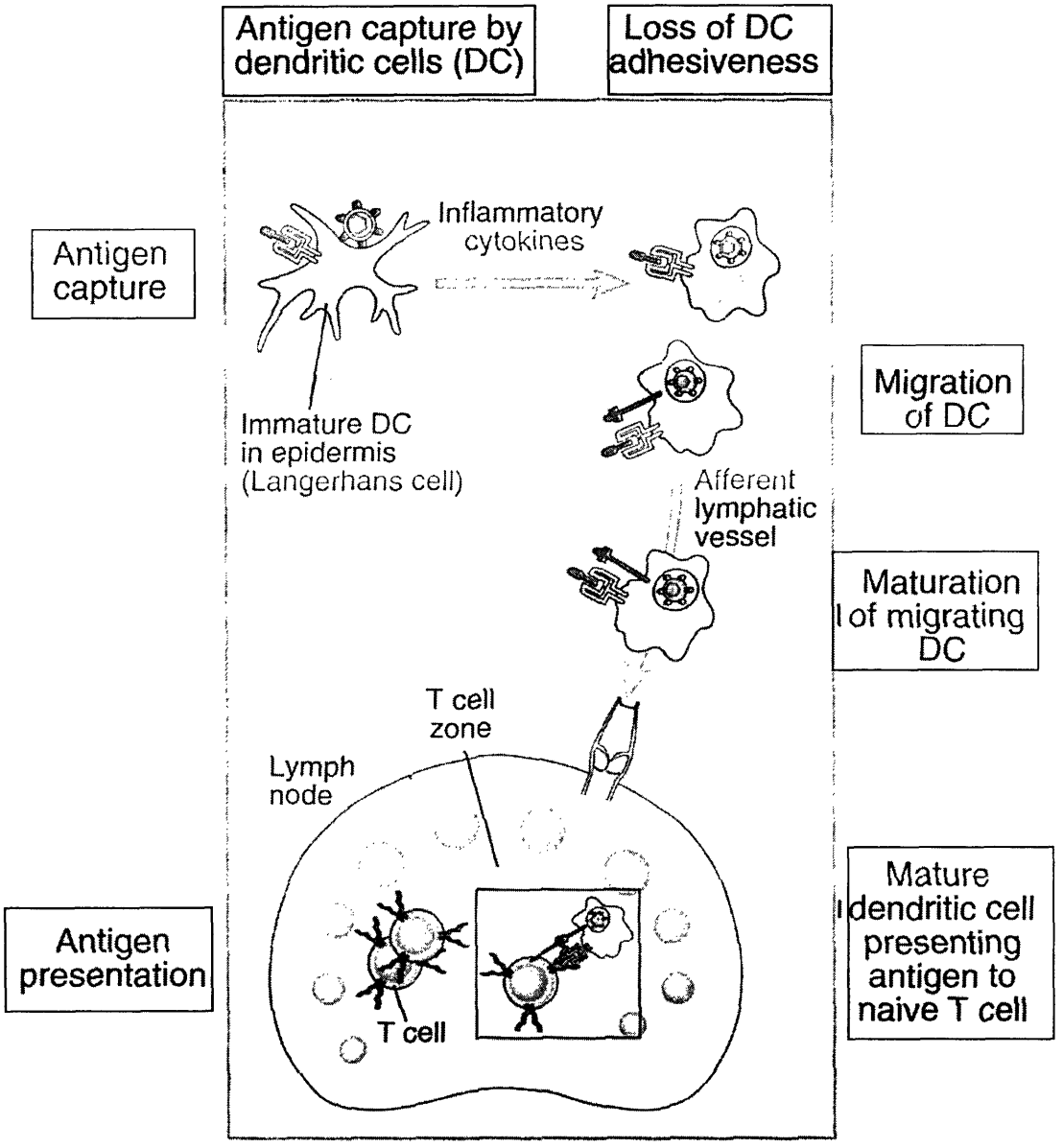


Figure 1-Role of dendritic cells in antigen capture and presentation [1].

The initiation and development of adaptive immune responses require that antigens be captured and displayed to specific lymphocytes that are known as antigen-presenting cells (APCs). Dendritic Cells (DCs) are the most specialized of APCs and capture microbial antigens, transport antigens to lymphoid organs, and

present antigens to naïve T cells to initiate the immune response. Dendritic cells play a pivotal role in antigen capture and the T cell response to protein antigens. Immature DCs exist under the epithelia, in most organs and in the gastrointestinal and respiratory systems. Upon engulfing an antigen, DCs mature during their migration to the lymph nodes. The schematic in Fig. 1 depicts the DC pathway after engulfing an antigen.

1.2 Traditional Vaccines

Traditional vaccines operate by inducing humoral immunity and are most successful on infectious agents whose antigens are relatively invariant. Attenuated and inactivated bacterial and viral vaccines contain intact nonpathogenic microbes that are created by treating the microbe such that it can no longer cause disease. Attenuated viruses are not perfect because incomplete attenuation or reversion to the pathogenic virus can occur [1].

Another type of vaccine uses purified antigen, 'subunit' vaccines, which are composed of polysaccharide antigens or protein; one example is the polysaccharide vaccine against pneumococcus. These polysaccharides ineffectively induce B cell memory, but remain in the lymph nodes for long periods most likely because the polysaccharides slowly degrade. The polysaccharide proteins are inefficient in entering the pathway of antigen presentation and cannot induce adaptive immunity with long lasting memory cells.

1.3 Progress in Controlled Release Drug Delivery

Controlled drug delivery dates back to the 1960s where silicone rubber [2] and polyethylene [3] were used as implantable drug release matrices, but the devices lacked

degradability and required removal after their useful lifetime. Biocompatibility represents an essential component in fabricating controlled drug delivery systems for the body and can be achieved by using natural polymers such as cellulose [4], chitin [5] or chitosan [6]. Alternatively, many biodegradable controlled release devices have been explored, including poly(lactic-co-glycolic acid) (PLGA) microspheres. However, the hydrophobic nature of the polymers creates issues with low encapsulation [7], and more importantly the degradation of PLGA damages the encapsulated protein [8]. To overcome these issues, hydrogels have been explored as delivery mechanisms to safely encapsulate proteins [9], [10]. The hydrophilic nature of hydrogels helps protect the protein during synthesis and also while the protein diffuses out of the polymer matrix.

1.4 Manipulation of Dendritic Cells as a Technique for Vaccination

Vaccines operate by creating an artificial immunity via inoculation. The patient is inoculated with antigenic proteins, pathogen fragments or other molecular antigens. Immunization initiates a primary immune response that generates memory T and B cells. The delivered antigen is typically an attenuated form of the original pathogen that has been chemically treated to reduce toxicity. The advantage of controlled release is that the release rate can be tailored. An application of controlled release is employing zero-order release kinetics of a drug to be used as vaccines. Controlled release solves some problems associated with vaccines.

Secondary lymphoid organs' primary function in the body involves bringing antigen-presenting cells and rare antigen-specific B and T lymphocytes into physical contact at the beginning of an immune response [11]. The exact communication process

that occurs inside the lymphoid organs reveals a complex network of chemokines as a trigger for particular cellular movements. Trafficking of lymphocyte migration is achieved by a diverse family of chemokines of approximately 10-20 kDa in size. Cells expressing appropriate chemokine receptors migrate up chemoattractant gradients toward their source, and were first characterized for their role in attracting cells to sites of inflammation. In addition, chemokines have the ability to bring B and T cells into contact with particular cells within the secondary lymphoid organs, as well as attract and guide antigen-presenting cells (APCs) to the lymph nodes [12].

The complex and specific role chemokines play in the immune system make them an appealing mechanism to exploit for immunotherapies using vaccines. These vaccines could induce specific immune responses by targeting and attracting specific cell types to a central location to engulf antigen. Dendritic Cells, a type of APC, are known to play a central role in the immune response and exist in normal tissues due to the presence of constitutively produced chemokines [13]. A primary immune response occurs when DCs engulf foreign antigens and migrate to the lymph nodes to present the capture antigens to T cells and initiate T-cell activation [14] (illustrated in Fig. 1). A number of chemoattractants are known to elicit directed migration of DCs and monocytes in this context, including monocyte chemotactic protein-1 (MCP-1), MCP-2, MCP-3, macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β, Regulated upon Activation Normal T cell Expressed and Secreted (RANTES), C5a, β-defensins, and bacterially derived formyl peptides [15], [16], [17], [18], [19] and [20].

The relatively low amount of DCs in blood and peripheral tissue (approximately 1% of the cell population [1]) make vaccines that attract DCs up a concentration gradient

more efficient and effective than traditional vaccines. Vaccines of this sort enhance the immune response because they increase the number of DCs loaded with antigen and as a result increase the number of naïve T cells activated in the lymph nodes.

Creating novel vaccines by attracting DCs with a chemoattractant has been explored and many prior studies have built on this principle by immunizing with DNA plasmids that encode the chemokines and an antigen of interest [21], [22] [23], [24]. The chemoattractant attracts DCs and elicists an increased localization of these cells at the injection site.

Kumamoto *et al* [25] implanted poly(ethylene-co-vinyl acetate) rods that released model protein antigen and MIP-3β. MIP-3β is a chemoattractant for Langerhans cells (immature DCs) and they were able to create an artificial gradient to attract LCs. LCs were loaded with antigen and protective immunity against tumors were induced efficiently. They concluded that tumor-specific immunity was inducible. Kumamoto et al's study is not quite ideal because the implanted rod is a non-degradable device that requires retrieval.

1.5 Problems with Hydrogels

Hydrogels offer an interesting vehicle as a controlled drug delivery device. They offer a couple of advantages in that they do not dissolve in water or at physiological conditions [26]. They also provide a hydrophilic environment capable of protecting the encapsulated chemoattractant [27].

1.6 Hypothesis

A controlled drug delivery release system that delivers a chemoattractant and antigen can succeed where traditional vaccines have failed by providing a non-pathogenic pathway to adaptive immunity while effectively coordinating a cell-mediated immune response to the antigen. Given the promising prior results discussed above, we sought to develop a system, which could serve as a potential platform for manipulating lymphocyte trafficking in immunotherapies. To this end, we examined controlled release hydrogel microspheres as an injectable formulation that could mimic the generation of chemoattractant gradients generated in situ in natural acute infections and load professional APCs at an immunization site. In addition, control of cellular chemotaxis via chemoattractant-releasing biomaterials could be a powerful strategy for tissue engineering, (e.g., for guided angiogenesis), where guided physical organization of multiple cell types according to wound healing or developmental principles is of interest.

Hydrogels tend to swell significantly upon immersion in aqueous medium. As a result, the encapsulated protein tends to diffuse out very quickly. We hoped to combat this problem by incorporating negatively charged groups into the polymer mesh, which is illustrated in Fig. 2. Positively-charged chemokines will temporarily bind to the negatively-charged groups, and the resulting diffusion rate will be much slower. We based our protocol on a modified version of a protocol from Podual *et al* [28].

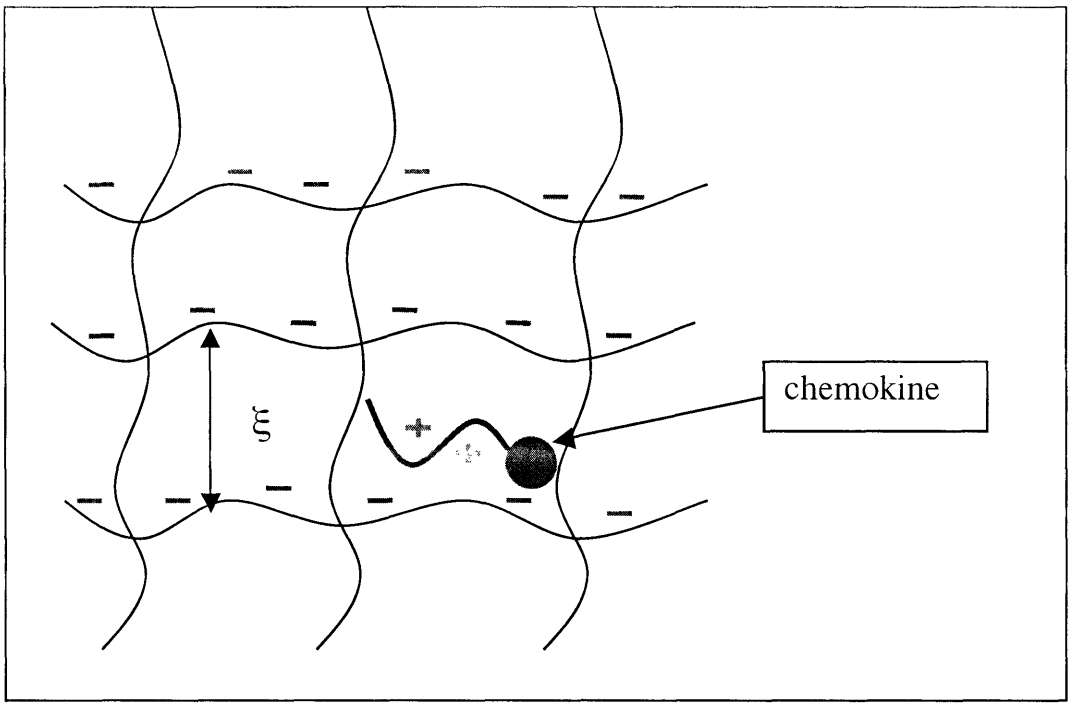


Figure 2-The hydrogel theory employs negatively charged groups where ξ=mesh size.

2 Experimental Methods

2.1 Materials

Polyethylene glycol methacrylate 526 (PEG-MA, Sigma-Aldrich Chemical Co.), polyethylene glycol dimethacrylate 875 (PEG-DIMA, Sigma-Aldrich Chemical Co.), Methacrylic Acid (Sigma-Aldrich Chemical Co.), Silicone oil (Avocado), sorbitan monooleate (Span80, Sigma-Aldrich Chemical Co., Inc.), ammonium persulfate (APS, Sigma-Aldrich Chemical Co.), sodium metabisulfite (Sigma-Aldrich Chemical Co.), Texas Red Ovalbumin (Texas Red Ova, Molecular Probes), Texas Red streptavidin (Molecular Probes), Texas Red avidin (Molecular Probes), and Hexanes (Sigma-Aldrich Chemical Co.) were used as received.

2.2 Hydrogel Synthesis

The synthesis route for the PEG hydrogels is shown in Figure 3. Nitrogen was purged for 60 minutes through 100 ml silicone oil and 100 μl Span 80 with stirring to remove the dissolved oxygen prior to the synthesis. The protein, depending on which one was used for the synthesis (2.5 mg Texas Red Ova, 500 μg Texas Red Avidin, or 100 μg Texas Red Streptavidin) was added to the monomers (2 ml PEG-MA, 3 ml methacrylic acid, 200 μl PEG-DIMA) and was purged separately for 15 minutes. The amount of crosslinker, PEG-DIMA was varied depending on the desired release rate (200 μl, 50 μl, 10 μl).

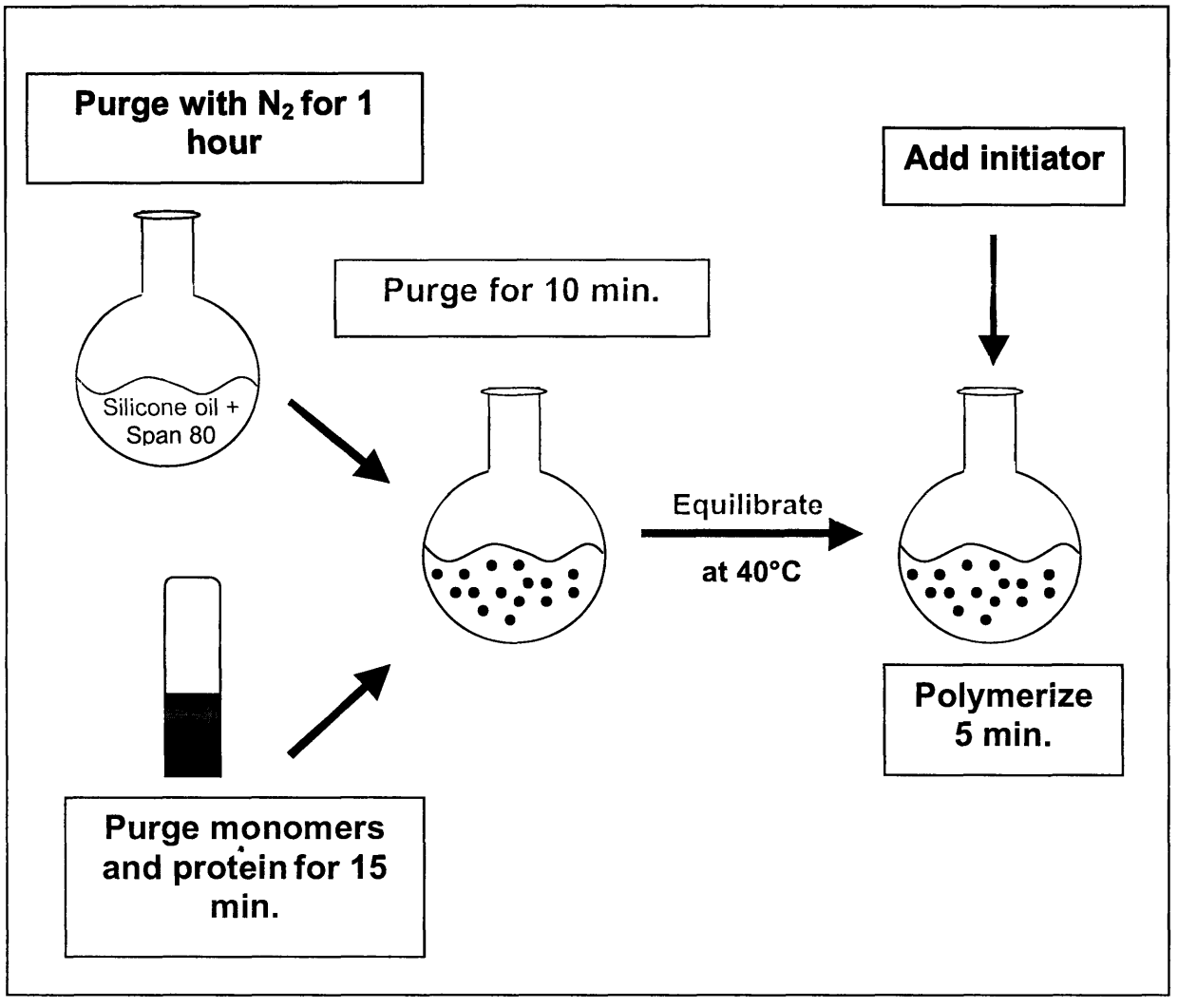


Figure 3-Synthesis schematic for PEG hydrogels.

The degassed monomers and protein were added to the silicone oil and Span 80 and were degassed for another 15 minutes. The mixture was equilibrated in a water bath at 40°C for 10 minutes at which time the redox initiators were added. The initiators were prepared by adding 10 mg APS and 10 mg sodium metabisulfite to 1ml Milli-Q water. The initiators were added using a syringe. The mixture polymerized for 5 minutes at which point it was removed from the heated water bath.

Cleaning the particles involved stirring with 100 ml Hexanes and 1 ml Span 80. The particles precipitated out to the bottom of the beaker and the silicone oil and hexanes were decanted off. This process was repeated 3 times to remove unreacted monomers and silicone oil.

Once the particles were cleaned, the hydrogel microspheres were resuspended in Phosphate Buffered Saline (PBS). A portion of these hydrogel microspheres were frozen and lyophilized using Freeze Dry System/Freezone 4.5 (Labconco).

2.3 Size distribution

All of the hydrogel microspheres contained fluorescent protein and the size and relative morphology of the particles could easily be obtained by fluorescence imaging at 40x using a Zeiss Axiovert 200 microscope equipped with a motorized stage (Ludl) and Roper Scientific CoolSnap HQ CCD camera.

2.4 Daily Sample Measurements for Release Profiles

Release profiles were created for the hydrogel microspheres to observe the amount of protein diffusing out of the particles. A known weight of the lyophilized particles were resuspended in 3 ml of the desired buffer and incubated at 37°C to mimic physiological temperature. The daily sample was taken by centrifuging the sample at 3,000 RPM for 15 minutes and drawing a 400 μl sample from the supernatant. The remaining supernatant was drawn off and discarded such that each daily sample was additive to the previous day. The buffer was replaced with new buffer each day.

2.5 Fluorescence Measurements

To quantify the amount of protein diffusing out of the hydrogel microspheres containing Texas Red Ova, Texas Red Streptavidin, and Texas Red Avidin, fluorescence measurements were made on the supernatants collected from the particles incubated in PBS for various times. A spectrafluorometer was used (SpectraMax Gemini by Molecular Devices). A series of standard curves were constructed from known concentrations of each of the proteins. The standard curves related known concentrations to the raw fluorescence numbers. The daily samples were pipetted into 96-well plates in 100 μl triplicate samples, and were then measured using a SpectraMax Gemini spectrofluorometer.

2.6 Calculating the Swelling Ratio and the Diffusion Coefficient

The mesh size for some of the varied crosslinker hydrogel microspheres was determined by measuring gel particle swelling. A known weight of lyophilized microspheres was resuspended in 1 ml Milli-Q water, and then placed on pre-weighed filter paper. The hydrogel microspheres were blotted until very little water was seen. The filter paper and blotted hydrogel microspheres were weighed, and then placed in a vacuum oven (VWR) overnight at a temperature of 70°C. Percent volume change (% volume change) of the sample is defined as the difference of the sample weight after drying and the wet weight. The water loss of the filter paper must be captured as well, and this was done by placing 3 filter papers in the vacuum oven and measuring the dry and original weight. That is also subtracted from the dry weight. Finally the number is multiplied by 100 to obtain the percent volume change.

$$\%volume\_change = 100 - \frac{(W_{dry}^{hydrogel} - W_{dry}^{filter} - W_{waterloss}^{filter})}{W_{wet}^{hydrogel}} \quad (1)$$

The diffusion coefficient was estimated by plotting the percent of protein released taken from the release profiles versus the square root of time. The slope of the linear trendline was equated to the coefficient from the following equation for diffusion from a sphere [29]. As a simplification, equation (3) was used to find the diffusion coefficient.

$$\frac{Q}{Q_\infty} = 6\left\{\frac{Dt}{a^2}\right\}^{\frac{1}{2}}\left\{\pi^{-\frac{1}{2}} + 2\sum_{n=1}^{\infty} ierfc\frac{na}{\sqrt{Dt}}\right\} - 3\frac{Dt}{a^2} \quad (2)$$

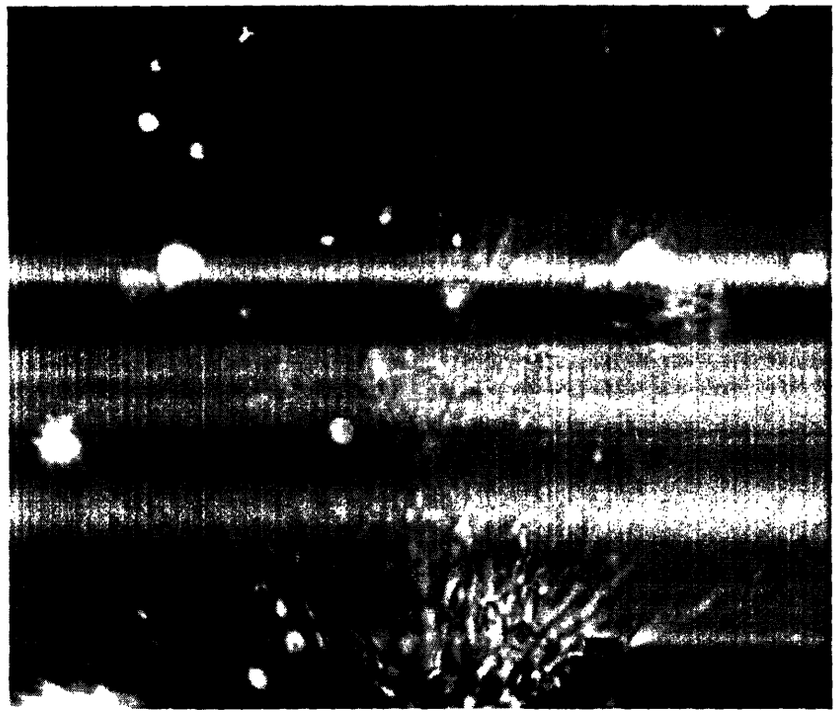
$$\frac{Q}{Q_\infty} = 4\left|\frac{Dt}{\pi l^2}\right|^{\frac{1}{2}} \quad (3)$$

3 Results and Discussion

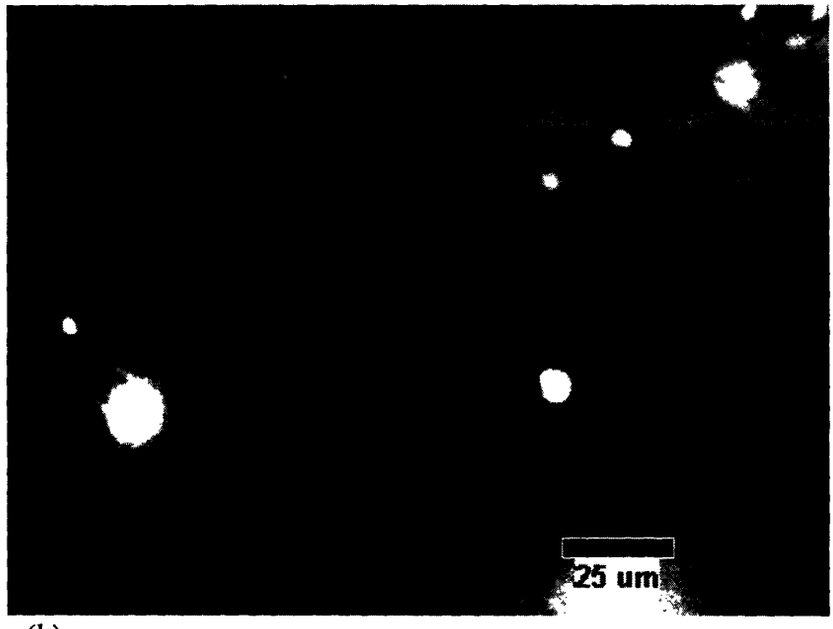
3.1 Fluorescence Images for Size

The following images were taken using a fluorescent microscope. Figure 4(a) contains Texas Red ovalbumin and 200 μl of crosslinker and represents the tightest polymer mesh of all the synthesized particles. The particles appear to be polydispersed and of at least 10 μm in size. Figure 4(b) shows the same particles as in Figure 4(a) and these particles are slightly larger (~20 μm).

Figure 5(a) contains Texas Red Ova as well, but contains less crosslinker, 50 μm. This represents the median amount of crosslinker of the synthesized particles. These hydrogel microspheres appear to be of the same size distribution as the previous microspheres.



(a)



(b)

Figure 1-Texas Red Ovalbumin hydrogel microspheres containing 200 µl crosslinker.

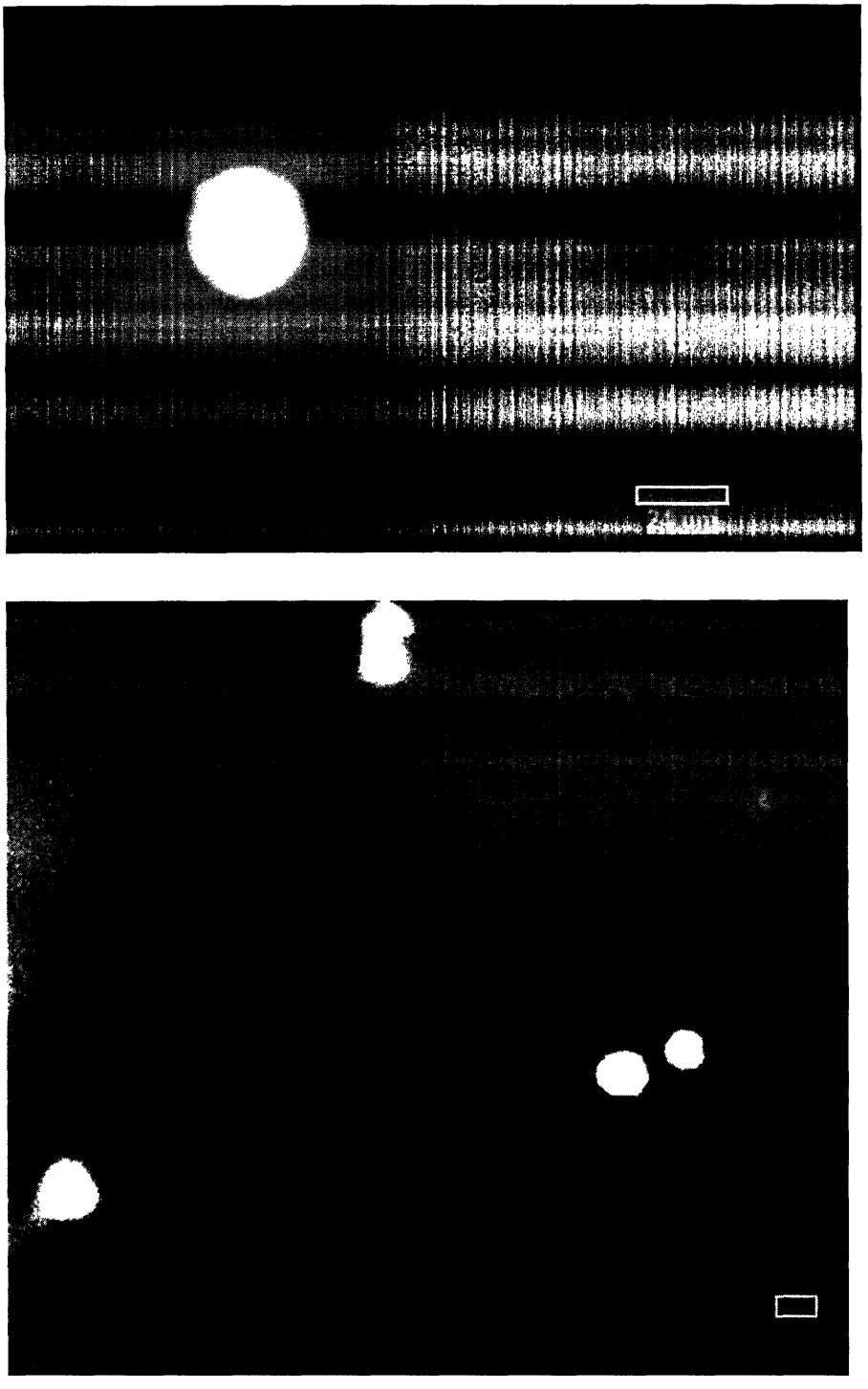


Figure 2-Texas Red Ovalbumin hydrogel microspheres containing 50 µl crosslinker.

3.2 Effect of varying crosslinker on the release of protein

The amount of crosslinker added to the synthesis determines the mesh size of the polymeric matrix. A higher amount of crosslinker produces a smaller, tighter mesh that allows for a slower diffusion of protein. A lower amount of crosslinker produces a larger mesh size that allows for faster diffusion.

As shown in Figure 6, the hydrogel microspheres containing 200 μl PEG-DIMA exhibit a baseline release rate that releases most of the Texas Red Ova within 14 days. These microspheres release protein at logarithmic release rate.

The hydrogel microspheres containing 50 μl crosslinker released over twice as fast as the 200 μl crosslinker microspheres. These microspheres release all of the Texas Red Ova within 7 days. This result confirms out expectation that a larger mesh size allows for faster diffusion.

The third set of hydrogel microspheres significantly reduced the crosslinker to a mere 10 μl. Unfortunately, these microspheres did not release along the same trends as the 200 μl crosslinker and 50 μl crosslinker microspheres. The 10 μl crosslinker microspheres should have released the fastest of the three varying crosslinker microspheres. The 10 μl corsslinker microspheres released faster than the 200 μl microspheres, but slower than the 50 μl microspheres. This problem can be attributed to difficulty in resuspending the microspheres for the release profile experiments. The 10 μl microspheres resuspended in larger pieces than the other particles and despite the mesh theoretically being larger, the particles may have behaved more like a bulk gel rather than individual microspheres. In this case, a

much larger area to diffuse through would have counteracted the larger mesh size and slowed down the protein diffusion in order to release into the surrounding medium.

3.3 Effect of pH on release

For samples with varying amounts of crosslinker, two release profiles were conducted: one at pH=7.2 in PBS and the other at pH=8 in Tris A Buffer. We hypothesized that the negatively charged methacrylic acid would have increased deprotonation at higher pH and this exhibits increased swelling. As can be noted from Figure 6, the significance of the difference in release rates is minimal. The pH difference is slight, and the observed release rates are subsequently slight as well.

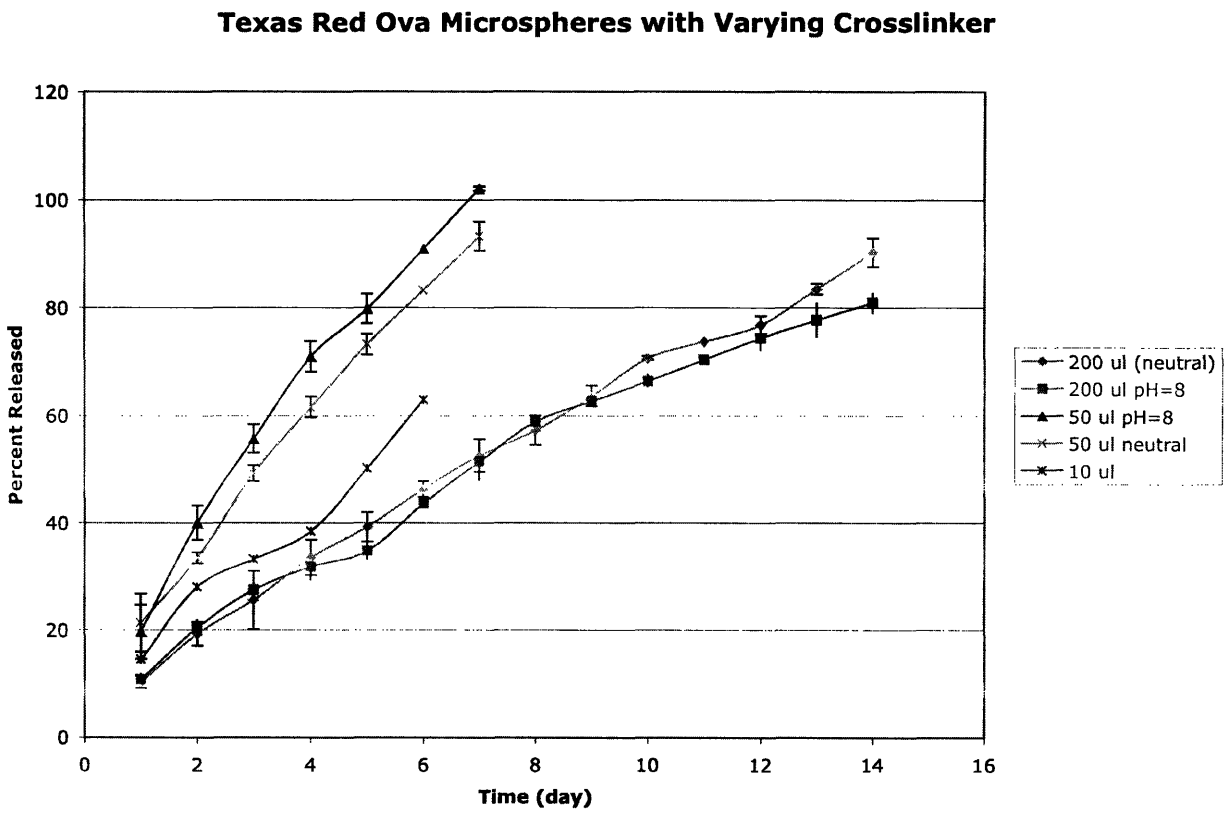


Figure 3-Texas Red Ova release profiles for varying amounts of crosslinker.

3.4 Swelling Data and Diffusion Coefficients

The swelling ratio data shown in Table 1 was used as another measure to quantify whether varying the amount of crosslinker caused an increase in the mesh size. An increase in mesh size should also exhibit an increased amount of water absorbed upon immersion in an aqueous environment and subsequent swelling. The swelling ratio should increase for decreased amounts of crosslinker. This trend is confirmed for the 200 μl and 10 μl crosslinker hydrogels, but the 50 μl crosslinker is lower than the other two. This can be attributed to a large amount of surfactant that was lyophilized with the hydrogel microspheres (the other two were not lyophilized with surfactant). As a result, a significant portion of the weight of the 50 μl crosslinker microspheres was due to the surfactant and not to the actual weight of the hydrogels. The 50 μl crosslinker likely contained a lower mass of gel particles. Fewer hydrogel particles led to an estimate of less water uptake and swelling. The surfactant in the 50 μl case accounted for 5.8% of the total weight of the hydrogels whereas it only accounted for about 1.2% in the other syntheses, based on weight. If the experiment were repeated, a significantly larger sample weight would be used in order to counteract the surfactant.

Amount of Crosslinker	Swelling Ratio (W_{wet}/W_{dry})	Diffusion Coefficient (m^2/s)
200 µl	4.17	$3.36x10^{-8}$
50 µl	2.89	$6.24 x10^{-8}$
10 µl	5.58	3.88×10^{-8}

Table 1-Swelling Ratio for varying amount of crosslinker.

3.5 *Effect of different isoelectric points on release* ***(avidin/streptavidin)***

Avidin (pI=10.5) and Streptavidin (pI=5) were examined because they both have very different isoelectric points. Release studies were performed at neutral pH, but the ionic strength was varied with one set of experiments with 500mM NaCl concentration and the other set with 1 M NaCl concentration. Since the studies were performed at neutral pH, avidin exhibited a net positive charge and streptavidin exhibited a net negative charge.

The release profiles in Figure 7 exhibit the trend that we expected in that, the negatively charged streptavidin diffused out of the polymer matrix extremely fast. It was likely repelled by the negatively charged methacrylic acid incorporated in the polymer matrix. The streptavidin sample performed at 500 mM and 1 M exhibit significant differences because the increased concentration of NaCl shielded the negatively charged streptavidin and negatively charged methacrylic acid. The charge repelling was less strong in the 1 M experiment due to the ionic shielding and allowed for a slower diffusion of streptavidin.

The avidin release profiles in Figure 7 exhibit a much slower diffusion rate, which was expected. The positively charged avidin temporarily binds to the negatively charged methacrylic acid and results in a much slower diffusion rate of avidin. The ionic shielding that occurred for the streptavidin samples did not exist as strongly for the avidin samples because the ionic shielding for the streptavidin samples was shielding a very strong repelling action while the avidin samples exhibited an attraction that was unaffected by charge shielding.

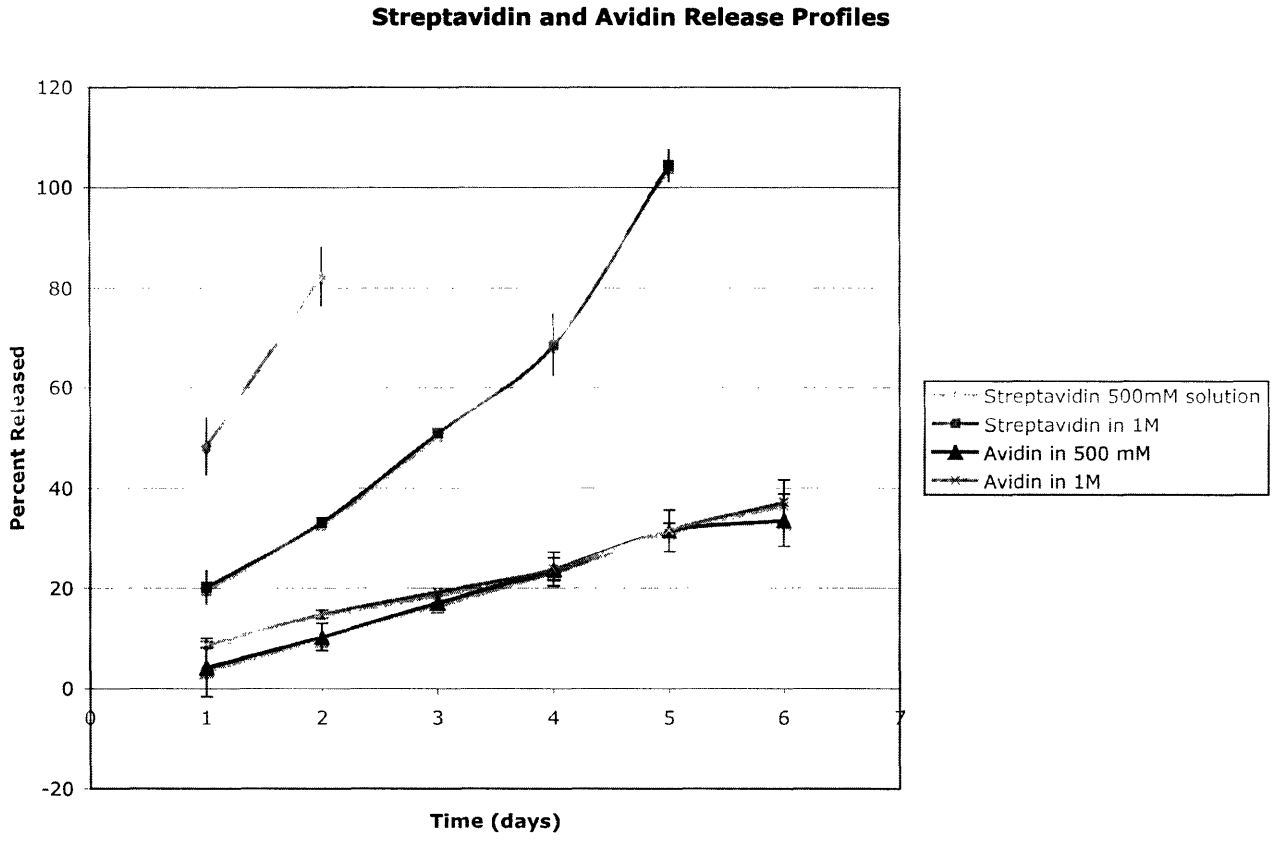


Figure 4-Texas Red Streptavidin and Avidin Release Profiles for varying ionic strength buffers.

4. Summary and Future Work

The charge sensitivity of the PEG hydrogel microspheres gives it interesting properties for vaccine applications. The crosslinker amounts can be easily varied depending on the desired release rates.

The proteins used in these studies (Texas Red Ova, Texas Red streptavidin and avidin) were used as model proteins for chemokines because chemokines can only be used in small amounts, and it was logical to experiment with the fabrication protocol using a less expensive protein in larger amounts until the fabrication protocol was perfected. The next step in this process would involve using a chemokine and eventually attaching antigen particles to the outside of the microspheres and complete the vaccine. Subsequent *in vitro* and *in vivo* experiments would follow to observe the completed device experimentally and in a native, physiological environment.

Another potential application of these hydrogel microspheres involves general delivery of drugs that require a continuous and controlled release rate.

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