ABSTRACT

Title of Document: FABRICATION OF POLY(D,L-LACTIC-CO-GLYCOLIC ACID) MICROPARTICLES FOR IMPROVED HUMAN PAPILLOMAVIRUS VACCINE DELIVERY

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Human papillomavirus (HPV) is the leading cause of cervical cancer and the most prevalent sexually transmitted disease worldwide. HPV vaccines require a multi-dose regimen to provide immunity, contributing to low patient compliance. We addressed this problem by formulating biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles and assessing their viability for use in controlled-release vaccines. We hypothesized that we could alter fabrication parameters to produce 1-10 μm microparticles in order to encapsulate ovalbumin (OVA) and HPV virus-like particles (VLPs). Microparticles were fabricated using a double emulsion method and used to elicit an immune response in JAWSII cells. Our results contribute to knowledge of vaccine delivery mechanisms and controlled-release technology, and could contribute to the creation of a viable controlled-release HPV vaccine.

EPIDEMICS: ERADICATING PATHOGENS THROUGH IMMUNIZATION
AGAINST DISEASES EVERYWHERE USING MICROPARTICLES IN
CONTROLLED-RELEASE SHOTS

FABRICATION OF POLY(D,L-LACTIC-CO-GLYCOLIC ACID)
MICROPARTICLES FOR IMPROVED HUMAN PAPILLOMAVIRUS VACCINE
DELIVERY

By

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Chapter 1: Introduction

1.1 Overview of Cervical Cancer

Cervical cancer was once the most common cancer in American women.[1] Cervical cancer occurs when malignant cancer cells develop within the tissue of the cervix.[2] Infection of the cervix can lead to cervical cancer when women are exposed to human papillomavirus (HPV). Other lifestyle risk factors for cervical cancer include giving birth to many children, smoking cigarettes, and taking oral contraceptives.[2] Symptoms of cervical cancer can range from vaginal bleeding, unusual vaginal discharge, pelvic pain, to pain during sexual intercourse.[2]

The four stages of cervical cancer depend on how far the cancer has spread from the initial site; stage one is characterized as being localized to the cervix and upon migration of cancerous cells to the bladder, rectum, or other parts of the body, the cancer progresses to stage four.[2] By stage four, the life expectancy of a cervical cancer patient is decreased to merely 16%.[1] For this reason, diagnosing cervical cancer in its early stages is critical for survival. Pap smears and Human Papillomavirus (HPV) vaccines are effective measures for screening and preventing cervical cancer, respectively.

1.2 Human Papillomavirus

Human papillomavirus, or HPV, is the main cause of cervical cancer. HPV was chosen as the ideal candidate for this research project for multiple reasons. First, HPV is a vaccine-preventable disease. Second, the current HPV vaccines require multiple doses over a period of six months. Lastly, studies have reported low patient compliance in completing the required HPV dosage regimen, leading to incomplete immunizations.[3,4]

Human papillomaviruses are double-stranded DNA viruses that infect the basal layer of the epithelium primarily through genital contact.[5] HPV is the most common sexually transmitted infection in the United States; however, the infected state is difficult to identify because HPV infections often go undetected and can clear within one year.[6] If the HPV infection does not clear, it can cause cellular abnormalities that can form genital warts and eventually develop into cervical cancer.[7]

There are over 40 types of HPV with Types 16 and 18 being the two most virulent, causing over 70% of all cases of cervical and anal cancer.[5] HPV preventative vaccinations have a great potential to lower cancer rates in the US. HPV is the leading cause of cervical cancer, which is the second most common cancer in women[8]. Just in the United States, 12,000 women are diagnosed with HPV and over 4,000 women die of cervical cancer annually.[9] Over 6.2 million people aged 14-44 contract HPV annually and over 80% of sexually active women over the age of 50 have contracted HPV at least once in their lifetime.[5] Because of the high indices of HPV contraction, the Center for Disease Control and Prevention recommends that women be screened for cervical cancer within three years of becoming sexually active.

1.2.1 Current Preventative Measures for HPV

Gardasil® and Cervarix™ are the two major multi-dose vaccinations on the market that mainly target Types 16 and 18 of HPV, aiming to ultimately prevent cervical cancer.[8] These two vaccines are fairly common with over 55 million doses of Gardasil® and 12.2 million doses of Cervarix™ having been distributed worldwide.[10,11] Because of the nature of multi-dose vaccination, the full regimen of the Gardasil® or Cervarix™ is not always completed or received within the recommended time frame. Within the US,

53% of 13-17 year olds have received at least one dose of the HPV vaccine, but only 37% received all three doses of the vaccination within the recommended time frame of six months.[3] However, of those who did receive all three doses of the vaccine, 68% followed the recommended time frame for each dose of the vaccination.[3] Although one dose of Gardasil® or Cervarix™ provides some protection against HPV infection, HPV preventative vaccinations are most effective when patients receive all three doses during a specific time frame of six months. Medical providers are not only essential in informing patients about the proper way to receive the HPV vaccine, but studies also show that medical provider recommendations elicit higher vaccination rates.[4] Although 60% of the general population report receiving a healthcare provider recommendation, racial minorities were shown to be less likely to receive a recommendation from their healthcare provider.[4] In addition to information barriers to vaccination, Gardasil® and Cervarix™'s financial burden of about $130 per dose can be a deterring factor when patients are considering whether or not to vaccinate themselves, especially if they are uninsured.[7]

A follow up vaccine dosage is required after eight years of receiving the initial vaccination in order for an individual to remain protected against types 16 and 18 of HPV.[7] Despite the successes of vaccines in preventing disease and death, current methods of vaccination are not as successful as they could be, as previously discussed. In this research project, we aim to address one component of current vaccinations methods that can be improved: the multi-dose vaccine. We propose to develop a polymer vaccine delivery system that delivers all the required doses of the HPV vaccine into one single controlled-release dose requiring only one vaccine administration.

1.3 Vaccines

Vaccines have had widespread social implications since they were first introduced in the early 1900s. They have saved millions of lives from many once feared diseases. Vaccines have also been instrumental in preventing infection and the spread of diseases worldwide.

Immunization impedes the manifestation of certain diseases, called vaccine-preventable diseases. Various diseases including influenza, hepatitis A, hepatitis B, human papillomavirus (HPV), pertussis, polio, smallpox, tetanus, and tuberculosis are all vaccine-preventable. Vaccines for these diseases have been key in reducing the rate of incidence and spreading of disease. Smallpox, for example, was eradicated in the latter half of the twentieth century due to worldwide vaccination programs.[12] Similar programs have allowed for near-eradication of certain diseases, such as polio.[13]

Other vaccines, however, have not had such a widespread or significant success compared to the smallpox and polio vaccines. Nearly 3 million people die each year from preventable diseases.[13] These deaths are due to many reasons, but one contributing factor is the inability of individuals to achieve complete immunization.

A variety of factors, both logistical and social, influence whether an individual will be vaccinated. Logistical barriers encompass the manufacturing and distribution of vaccines. Underproduction of vaccines, especially during times of pandemics, decreases the ability of health providers to immunize patients.[14] Additionally, some health providers may not have adequate means to store and safely transport vaccines, many of which require refrigeration to maintain the integrity of the components within the vaccine.

This is often a problem faced by health providers in lower socioeconomic regions or developing countries, where refrigeration methods are not readily accessible.

The social barriers influencing lower immunization rates can arise from misconceptions, personal beliefs, or personal circumstances. Lack of proper vaccine knowledge can lead people to underestimate the importance of vaccination and its benefit to society. In addition, this lack of knowledge can result in people not recognizing what diseases are preventable by vaccine immunization. Parents may decline the vaccination of their children due to their concerns regarding the safety of the vaccines and their perceived repercussions of vaccinating their children. Reports of adverse, yet, rare effects that children have experienced after receiving a vaccine have deterred some parents from vaccination for fear of their child developing a similar condition. Other reasons for the failure of individuals to be immunized include long traveling distances, inaccessible transportation, inconvenient clinic hours, associated costs, and even the fear of needles.[15]

These logistical and social barriers are further exacerbated by the use of vaccines that require multiple doses. Multi-dose vaccines are vaccines in which multiple shots are administered to a patient according a specified dosage regimen. These vaccinations can occur over weeks, months, or years and are necessary to ensure complete immunological protection. These multiple or recurring doses are sometimes necessary for vaccines that use a live-attenuated virus, and are necessary for vaccines that contain an inactivated, or dead, virus. This is due to the fact that the weakened or inactivated virus may not elicit a full immune response from the patient until the immune system has been exposed to the virus multiple times. Or, as is the case with the influenza vaccine, patients may have to

receive periodic immunizations against a disease to account for new strains of a disease that arise due to mutations.[16]

1.3.1 HPV Vaccine Delivery Methods

Traditional methods of administering HPV vaccines, Gardasil® and Cervarix™, are intradermal and multi-dose, meaning that the patient is administered the vaccine multiple times by injecting the vaccine into the patient's arm. Dose one of the vaccine is given at day zero, dose two is usually given one to two months after dose one, and dose three is given six months after dose one.[17] This multi-dose delivery method is necessary to elicit stronger and prolonged protection against HPV. Current vaccinations are 93% effective in protecting against cervical cancer arising from HPV 16 and 18.[17]

1.4 Controlled Release

Controlled-release mechanisms have been commonly used in drug delivery in order to obtain a sustained biochemical response in the body. Many drugs have been developed with this method, creating a time-dependent release. Some advantages of controlled release include the ability to assist the drug in crossing physiological barriers, holding the drug longer in the body, and targeted release of the drug to a specific tissue.[18] Controlled-release drugs have been developed for oral use, subcutaneous injection, and regional delivery to organs such as the eye or lung. Our research will take advantage of sustained release over time by using microparticles as a vehicle to deliver the drug. These microparticles will degrade over time, slowly releasing our vaccine to the target cells.

1.4.1 Microparticles for Controlled Release

Biodegradable polymers, made from either natural materials or synthetic materials, allow for controlled release. One commonly used non-toxic, biodegradable, synthetic polymer is poly(D,L-lactide-co-glycolic acid) (PLGA). Since synthetic polymers are made of many subunits (or monomers) their composition can be changed by varying the ratio of the subunits, thus allowing for a wide range of chemical and behavioral properties. PLGA microparticles have been utilized previously by many other research groups for the controlled release of their selected antigen. The microparticles are spherical, porous structures that can be made of a homogenous size distribution, which is important for cellular uptake. The microparticles also degrade over time, giving us our controlled-release profile.

1.5 Research Questions

The overarching question guiding our research was: To what degree can a controlled-release, poly(D,L-lactic-co-glycolic acid) microparticle vaccine delivery system induce an immune response to HPV virus-like particles, the antigenic component of the HPV vaccine? More specifically, we divided our research into sequential studies to address the following research questions:

(1) Which parameter variations in microparticle synthesis will yield microparticles with a diameter of 1-10 micrometers in order to optimize uptake by dendritic cells?

(2) Which parameter variations in microparticle synthesis will yield protein-loaded microparticles of appropriate size, while maintaining high encapsulation efficiency?

(3) Will the degradation and release profiles of protein-loaded microparticles be comparable to that of the multi-dose HPV vaccine?

(4) Will protein-loaded PLGA microparticles induce an immune response in murine dendritic cells? How does this response compare to the response induced by protein alone?

(5) Will vaccine-loaded PLGA microparticles induce an immune response in murine dendritic cells? How does this response compare to the response induced by the Gardasil® HPV vaccine?

1.6 Study Hypotheses

We hypothesized that a PLGA microparticle vaccine delivery system can deliver the HPV vaccine in a controlled-release manner and induce an immune response comparable to that of the traditional vaccine. Specifically, we hypothesized:

(1) Higher concentrations of polyvinyl alcohol (PVA) and longer stir times would yield smaller microparticles in our desired size range, as measured by laser diffraction and microscopy.

(2) The addition of a model protein to the double emulsion microparticle fabrication process would not greatly affect the size of the microparticles. Furthermore, adding a higher concentration of protein to the double emulsion would yield a higher encapsulation rate.

(3) The release profile of the protein-loaded microparticles would consist of an initial burst release followed by a gradual release of the remaining protein.

(4) Protein-loaded microparticles would induce a greater immune response in murine dendritic cells than protein alone, as measured by an enzyme-linked immunosorbent assay (ELISA) for tumor necrosis factor-alpha (TNF-α).

(5) Gardasil®-loaded microparticles would induce a greater immune response in murine dendritic cells than Gardasil® alone, as measured by an ELISA for TNF-α.

1.7 Study Limitations

We had several limitations throughout our study. During the microparticle fabrication and characterization phase, the use of a homogenizer to create each emulsion would have minimized the range of microparticle size.[19] We were also unable to obtain pure VLPs due to financial and technical constraints, so we opted to encapsulate Gardasil®, which included AS04 adjuvant in addition to VLPs. Lastly, when measuring cellular response to proteins, microparticles, and Gardasil® we measured immunogenicity of our microparticle formulations, but not in the time-course in which release would traditionally happen.

1.8 Research Contributions to the Field

This study added to the growing body of knowledge regarding the potential of HPV vaccine delivery using microparticles. Although other diseases such as diphtheria and tetanus have been targeted using microparticle vaccine delivery systems, this approach has not previously been utilized for HPV. By exploring microparticle fabrication methods and the variables that affected microparticle size, our study was able to shed light on potential methods for future HPV vaccine delivery systems that use PLGA microparticles. First, we concluded that stir time does not affect microparticle size. However, percentage

of PVA in its aqueous phase did prove to have an effect on the size of microparticles. As PVA concentrations in the aqueous phase increased, microparticle sizes decreased. OVA concentration and microparticle size showed no significant effect on microparticle size. Our study was able to produce microparticles close to target size and with high encapsulation efficiency rates. By investigating microparticle fabrication methods of microparticles loaded with OVA and characterizing size, encapsulation, release and immunogenicity; future researchers interested in microparticle vaccine delivery for HPV will be able to base their work on these findings.

Chapter 2: Review of Literature

2.1 Cervical Cancer and HPV

Prior to the implementation of routine pap smears and the utilization of the human papillomavirus (HPV) vaccine, cervical cancer was relatively unpreventable and underdiagnosed. Even with improved technology, there are still over 12,000 women in the U.S. diagnosed with cervical cancer each year, and over 4,000 deaths.[20] Worldwide, cervical cancer results in over 270,000 deaths. The burden of cervical cancer is disproportionally large in developing countries and underserved populations.[21] Nearly 85% of cervical cancer cases worldwide occur in developing countries. In the U.S., more than 60% of cervical cancer cases occur in small, medically underserved regions. Scarinci et al. found that the age-adjusted rates of cervical cancer in these populations range from 1.5 to 4 times higher when compared with the national rates.[22]

2.1.1 Cervical Cancer Impact and Treatment

Prevention of cervical cancer primarily involves the prevention of HPV infection. Vaccines for HPV have been found to be over 90% efficacious, resulting in drastic reductions of new HPV infections over the last decade. Beyond vaccination, early detection of cervical cancer can significantly improve health outcomes. Screenings to identify precancerous lesions and early stage cervical cancer allows for early treatment.[22] The U.S. Preventive Services Task Force recommends routine Pap smears for women 21 to 65 every three years, or every five years for women 30 to 65 in combination with an HPV test.[23]

2.1.2 Human Papillomavirus

The human papillomavirus (**Figure 2.1**) is a double stranded DNA virus that infects human epithelial cells, specifically the cutaneous and mucosal epithelial cells.[24–26] HPV exhibits highly specific tissue tropism, meaning that specific types of HPV only infect epithelial cells in specific locations, such as on the hands, feet, and mucosal areas. HPV infection normally causes the formation of warts, but can lead to the development of cancer. Specifically, HPV can cause genital warts and lesions on the vocal chords in both males and females and can lead to cervical, vaginal, anal, throat, and other cancers.[24] HPV is spread by the shedding of virus particles from the infected surfaces of a person's skin and reproductive surfaces and linings. Thus, HPV can be transmitted through sexual activity.
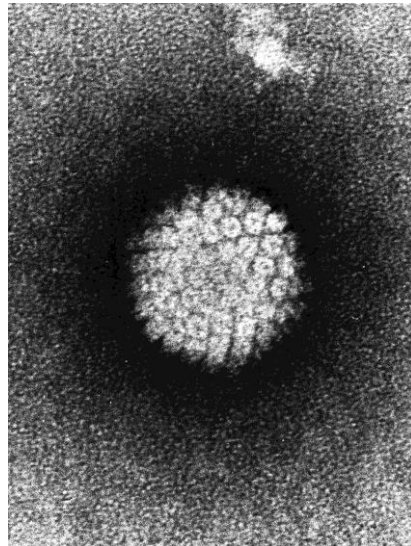
Figure 2.1. An electron micrograph of human papillomavirus. Laboratory of Tumor Virus Biology, 1986.[27]

2.1.3 Mechanism of Disease

Once the virus enters the body, it remains predominantly in the mucosal and cutaneous epithelial cells of the reproductive systems. There it is able to evade the host's immune system. Typically, when infections occur in a host, inflammation caused by cell death arises to alert the immune system and antigen presenting cells (APCs) of foreign materials in the body. However, HPV infections do not cause inflammation because cell death does not occur during HPV replication. Therefore, the infection can go undetected by the body, allowing the infection to grow and spread. This undetected and uncontrolled growth can sometimes lead to mutations of human cells, which can result in cancer.[26] Approximately 80% to 90% of HPV infections and symptoms clear within two years, but the remaining infected individuals who continue to exhibit symptoms are at a higher risk of developing cancer.[24,26]

While not all 130 types of HPV are associated with causing cancer, there are fifteen, including types 16 and 18, that are most strongly associated as being "high risk" and are likely to cause cervical and other types of cancer. Types 16 and 18 are estimated to cause 70% of the cases of cervical cancer.[24] Accordingly, the two current vaccines administered in the United States, Gardasil® and Cervarix™, are specific to types 16 and 18.

2.2 Vaccines

Vaccination utilizes parts or all of a pathogen to provide protection against future infection by that pathogen. The origin of vaccination by use of small doses of disease to prevent more severe cases can be traced back to early Chinese literature; however, the first recorded vaccination was accomplished by Edward Jenner in England in 1796.[28]

Jenner was able to successfully relate exposure to low levels of a related, less-lethal virus to rate of later infection, and thus developed the first vaccine for smallpox through his work with the cowpox virus. In 1796, Jenner inoculated an 8-year-old boy with the pus from a cowpox lesion, and noted that all subsequent exposures of the pus to the initial site did not affect the boy.[29] Jenner confirmed his hypothesis that human exposure to cowpox would provide protection from smallpox through repeated trials, and thus developed the first human vaccine.

Future breakthroughs in vaccinations include Louis Pasteur developing the first attenuated vaccine in the 1880s. Attenuated vaccines include a live, but weakened version of the targeted pathogen. During his work with chicken cholera, Pasteur had inoculated chickens with an aged culture of chicken cholera. He later inoculated the same chickens with a fresh culture of the virus, and noted that the chickens were resistant to the new culture. Pasteur consequently hypothesized that pathogens could be attenuated through environmental exposure, such as passing the virus through multiple hosts, and was able to confirm this through his later work.[28]

During the creation and growth of vaccines, populations with the more advanced medical culture were those who most benefited. Over time, great health care disparity has developed across the world. The current need for vaccines falls primarily in underdeveloped and medically underserved parts of the world. A WHO report on past accomplishments and future goals of vaccines further exemplifies the disparities in vaccine coverage, finding that the European, Western Pacific, and American regions have greater than or equal to 90 % vaccine coverage for the diphtheria vaccine (DTP3), a vaccine commonly administered to infants. Meanwhile, only 69 % in South East Asia and

66 % in African are covered by this vaccine. Since 1974, diphtheria has been included in the WHO's recommended immunization series, along with measles, polio, pertussis, and tetanus. Yellow fever and Hepatitis B were added to the list in 1988 and 1992 respectively.[30]

The global impact of each of these diseases can be most easily assessed by the mortality estimates. The WHO estimated that in 2002, the number of deaths for children under 5 was less than 1,000 due to polio, 4000 deaths due to diphtheria, 198,000 due to tetanus, 294,000 due to pertussis, 386,000 due to Hib (Haemophilus influenzae type b), and 540,000 due to measles. In addition, the WHO estimated 600,000 adult deaths due to Hepatitis B, and 240,000 deaths due to HPV.[31]

Although governments and world aid organizations have been pushing many important and effective initiatives to increase the availability of vaccines and vaccine compliance, social and logistical barriers to vaccination continue to restrict vaccination rates. Logistical barriers to vaccination include patient geographic relocation, vaccine cost and availability, and missed vaccine opportunities, either as a result of a missed visit or the health care provider being unaware of the respective vaccine's dose regimen.[15]

Notable social barriers to vaccination include willful patient rejection due to lack of information about safety and necessity, fear of injections, low self-efficacy, and participation in illicit activities.[32,33] In a 2008 study on the acceptability of a new human papillomavirus vaccine, researchers found that major objections to the vaccine were related to the lack of available safety and efficacy information. Further, the study noted that mothers whose daughters were not opposed to being administered injections were

more likely to accept vaccination, further implicating fear of needles or pain as a significant factor in willful rejection of vaccination.[32]

A study conducted in 2008 on patient compliance of multi-dose vaccine schedules examined the rates of completion of Hepatitis A, Hepatitis B, and Varicella vaccines from 1996 through 2004. The researchers found low levels of vaccine completion within one year, 55%-65% for Hepatitis B and 40%-50% for Hepatitis A and Varicella. Further, it was noted that for a significant number of patients that ultimately received the full vaccination series, the interval between commencement and completion was long enough to render them under-vaccinated and at higher risk of disease contraction for that period of time. The researchers concluded that a single dose vaccine, or vaccine options that require fewer doses, would increase the rates of compliance across all age groups.[34]

2.2.1 Immunological Response to Vaccines

Immunity is the ability of an organism to recognize and coordinate a response in order to combat a particular disease. The focus of our study is artificially acquired immunity which is the artificial introduction of antigens into an organism in order to elicit an immune response. Antigens are foreign substances, such as proteins, sugars, or lipids that can cause an immune response in an organism. Vaccines contain antigens that can be individual components from a pathogen or the actual pathogen presenting many antigens on itself. The purpose of using antigens in vaccines is to induce an artificially acquired immune response that results in the organism's stimulation of antibody production and cell-mediated responses, without causing the organism to become sick. The cell-mediated and humoral responses allow the organism to be able to recognize and fight the pathogen upon future exposure.

Cell-mediated immunity is the ability of immune cells to identify antigens and destroy the affected cells. Once a pathogen enters the body, it may be attacked by macrophages, cells that engulf foreign substances and then label the respective antigens for other more specific cells to identify and act upon. Helper T-cells recognize these marked antigens and alert cytotoxic T-cells to find and destroy infected body cells.[35] Dendritic cells, a type of antigen-presenting cell (APC), and Toll-like receptors also play a large role in cellular response. Toll-like receptors on the outside of dendritic cells are specialized to detect different features of pathogens called epitopes, such as polysaccharides or proteins present on cell membranes or walls. The dendritic cells can then transfer this information to T-cells, which seek out and destroy the pathogen.[35]

During the humoral immune response, the body produces antibodies to recognize and mark antigens. Antibodies are antigen-specific proteins that are produced in response to an organism's exposure to a pathogen. Antibodies function to identify their specific antigens and to tag the antigen for destruction in order to rid the organism of it.

Antibodies are produced through the interactions of different immune system cells. First, B-cells bind to an antigen with their B-cell receptors. The B-cell then displays the antigen on its outer surface for helper T-cells to recognize. This antigen presentation can also be completed by other professional antigen-presenting cells, such as macrophages and dendritic cells. After helper T-cells recognize the antigen, they secrete cytokines, which are cell-signaling proteins. This release of cytokines stimulates B-cells to differentiate into plasma cells. In turn, these plasma cells are now activated and can begin producing antibodies that will recognize the specific antigen responsible for activating the antibody production response. The T-cell dependent antigen activation also generates

a population of long-lived memory B-cells, which respond to the same antigen, but do not require helper T-cell stimulation for antibody production.

There are different classes of antibodies, or immunoglobulins, which are produced at different times and different amounts during the immune response as seen in **Figure 2.2**. Immunoglobulin M (IgM) and immunoglobulin G (IgG) are two different types of antibodies. IgM is produced immediately at the first exposure to the pathogen during the primary response. However, IgM does not remain in the body for long and decreases in amount. IgG is produced during the secondary response which occurs at a subsequent exposure to the antigen. The secondary response elicited by IgG is faster and more robust than the primary response elicited by IgG. IgG is responsible for the immunological memory that an organism acquires over a lifetime.
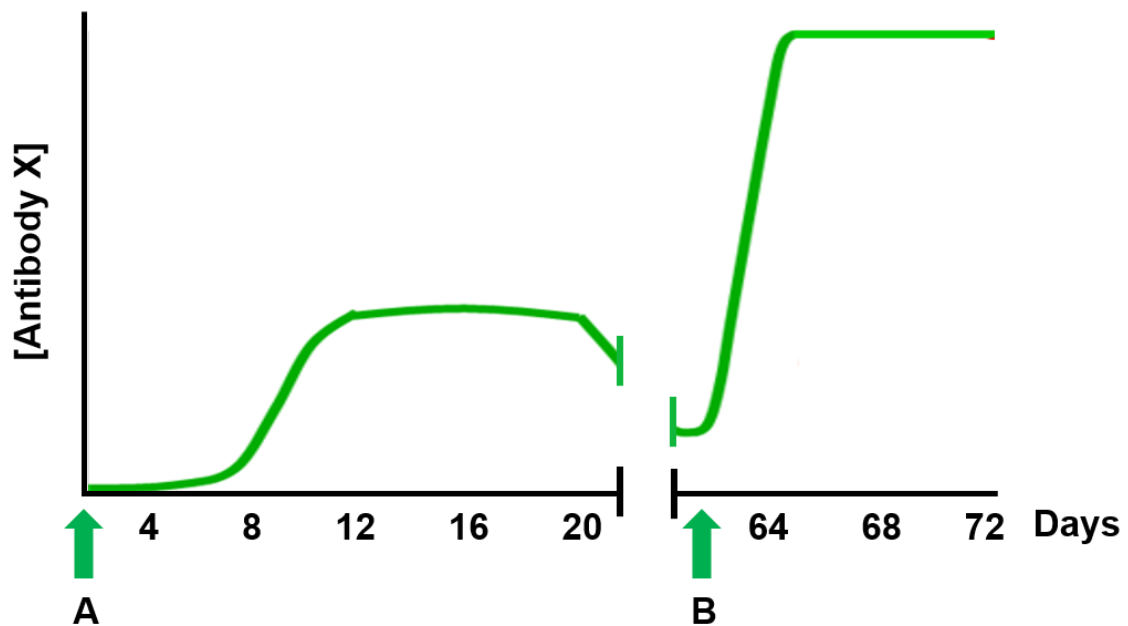
Figure 2.2. Antibody production over time. Antibody X production in response to Antigen X exposure at time A and time B. The primary response occurs due to exposure of Antigen X at time A. The secondary response occurs due to exposure of Antigen X at time B. Adapted from Owen et al. 2013.[36]

2.2.2 Traditional Vaccines

There are two major types of antigens: live attenuated and inactivated. Live attenuated antigens are viruses and bacteria that can continue to reproduce in the host but are unable to cause disease in most patients because they are less virulent. Viruses and bacteria can be made less virulent through different procedures, such as passing them through unnatural hosts, passing them through multiple hosts of the same species, selecting a strain that will only reproduce effectively at a specific temperature, or by removing the virulent gene through genetic engineering.

Live attenuated vaccines have a few characteristics that make them effective for immunization. First, because the antigenic component within the vaccine is a live pathogen, it is able to proliferate in the cell, but to a lesser degree than its wild type, virulent form. This means that the pathogen can activate both the humoral immune response and the cell-mediated immune response, which leads to both the production of antibodies and cytotoxic T-cell activation, respectively.[16] Second, due to this activation of both the humoral and cell-mediated immune responses, a stronger immune response occurs with a single dose of the vaccine, meaning fewer doses and booster shots are required for an individual to develop immunity against the pathogen.

However, there are some disadvantages to using live attenuated vaccines. First, because the pathogen is still alive, it has the potential to revert back into a virulent form through evolution. Second, individuals with weakened immune systems may not have a strong enough immune response to compete with this weakened form of the pathogen. Third, these vaccines typically have more stringent requirements for storage, such as the need to be refrigerated in order to remain potent. This need creates another difficulty for

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vaccination in regions that may not have access to such requirements.[37] Lastly, it is also very difficult to create live attenuated vaccines for bacterial pathogens as compared to viruses.[16]

For these reasons, inactivated antigen vaccines have also been developed. Most inactivated vaccines are subunit vaccines made from fractions of pathogens, including toxoids, protein subunits, or polysaccharides from the capsule of a bacterial pathogen or from the envelope of a virus. Although they are safe even in immunocompromised patients, they only effectively produce a humoral response and require three to five doses to achieve full immunization. Even after that, antibody levels decrease as time passes after immunization, and supplementary "booster" shots are required.[37] Additionally, subunit vaccines may require potent adjuvants in order to agonize the Toll-like receptors in the dendritic cells that are responsible for presenting the antigen to the rest of the immune system.[38] An example of an inactivated vaccine is that for human papillomavirus, which utilizes a conformational protein of the virus to induce an immune response. Although the vaccine is effective, full immunization requires multiple doses and potent adjuvants.

2.2.3 Current HPV Vaccines

Cervarix™ is a recombinant vaccine comprised of virus-like particles (VLPs) containing the L1 capsid protein. It protects against HPV types 16 and 18, which are thought to account for over 70% of cervical cancer cases.[39] Adjuvanted with the O4 system (ASO4), Cervarix™ requires three intramuscular doses scheduled at months 0, 1, and 6 months to impart protection, which usually lasts at least 5.5 years.[25] However, this efficacy is demonstrated to be longer, as seen in a phase II study of a cohort of Brazilian

pre-adolescent girls. In this case, the efficacy of Cervarix™ against HPV type 16 and 18-associated infection was 95.3% after 6.4 years of follow-up, and 95.1% after 8.4 years. In addition, the vaccine was found to protect people who had been previously infected with these HPV types, although it could not treat those with prevalent infection.[40]

Gardasil®, like Cervarix™, is another recombinant vaccine comprised of L1 VLPs. However, it contains protein from HPV types 6 and 11, in addition to the high-risk types 16 and 18 found in Cervarix™, enabling the vaccine to expand its protection to include genital warts and laryngeal papillomas.[39] Gardasil® is adjuvanted with aluminum hydroxyphoshate sulfate, which is shown to have a comparable, though slightly lower, antibody response than the adjuvant AS0ra4. Nonetheless, Gardasil® is highly efficacious. Like Cervarix™, it requires 3 intramuscular doses, at months 0, 2, and 6. A prophylactic efficacy study showed a 98% protection against HPV types after the dose regimen, and a 96% protection in the population for 5 years afterward.[41]

2.2.4 HPV Virus-Like Particles

As previously mentioned, HPV is a doubled stranded DNA virus. The viral genome is surrounded by a viral protein shell called the capsid. The capsid is composed of a repeating pattern of 360 L1 proteins assembled in a pentamer, as Lowy et al. showed in their work.[42] When the capsid is formed around the viral genome and other covalent modifications have occurred then the virus is known as a mature and infectious virion. The virion measures approximately 55-60 nm in diameter.[43]

The repeating nature of the viral capsid makes the L1 protein a good candidate for being the antigenic component of an HPV vaccine. When the L1 protein is expressed in yeast, it has been shown that the L1 proteins can self-assemble into an empty capsid

resembling the shape, size, and characteristics of the mature HPV virion. These virus-like particles (VLPs) are noninfectious because they do not contain the HPV genome, but when presented to a host, are able to induce an immune response similar to the immune response initiated by HPV virions.[43] For these reasons, the current two available vaccines, Cervarix™ and Gardasil®, both contain HPV VLPs composed of the L1 protein as the antigenic component.[39,41]

2.3 Microparticles for Controlled-Release Delivery

A growing body of research supports the clinical utility of polymer microparticles or nanoparticles for use in novel and application-specific vaccine delivery systems. These particles are made of a polymer matrix which can encapsulate antigens and other compounds.[44] Microparticles have the ability to release the encapsulated macromolecules over extended periods of time.[45] This ability for sustained release has suggested the possibility of reducing the number of required doses, which will increase patient comfort and compliance.[46]

2.3.1 Microparticle Design

Microparticles have been investigated in a variety of applications primarily because of the variety of release profiles that can be achieved through microparticle property manipulation. Microparticles can be formulated to provide release at a fairly constant rate, such as the release profiles shown in **Figure 2.3**, or in pulses. Pulsatile release describes a variety of release profiles that can include anywhere from one to many different peaks of high release rate at any given time point in the release time period. This can include a profile that includes a burst release of drug or protein, an affect more

thoroughly explained in **2.3.6 Release Kinetics**. The particle size, morphology, composition, and release profile are all influential factors in the resultant immune response, and are thus key factors in tuning the particles for a specific application.
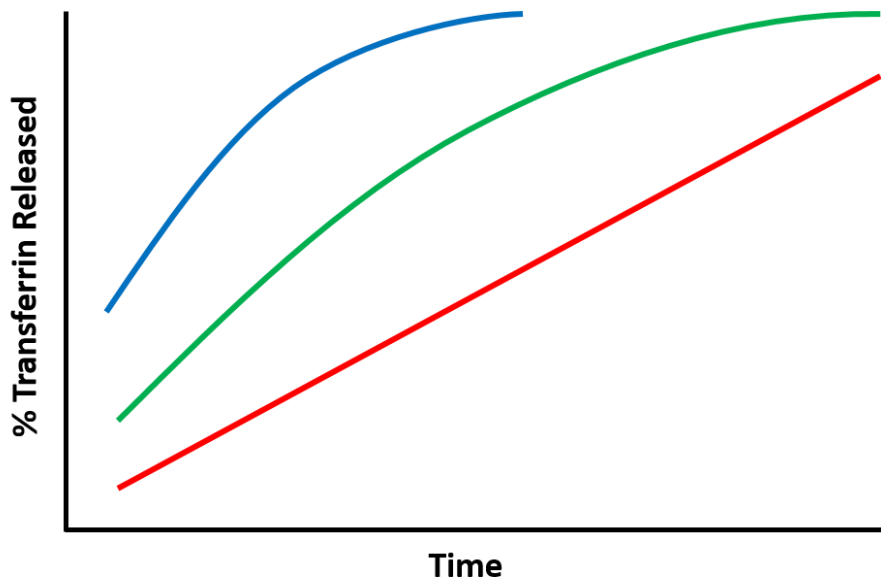
Figure 2.3. Microparticle release profiles. Release profiles showing percent encapsulated Transferrin protein released over time for varying polymer compositions. Adapted from Sah et al.[45]

Early research suggests that vaccines delivered via polymer microparticles show potential to elicit similar immune responses to multi-dose vaccine equivalents. O'Hagan et al. showed in 1998 that bonnet monkeys injected with a single dose of vaccine-loaded particles showed similar protein binding capacity to those monkeys following a three-dose mechanism.[47] This binding capacity could reflect the ability for the microparticle injection to invoke a similar response to the vaccine in comparison with the multi-dose regimen.

Microparticles have many advantages that are favorable for use in single-dose vaccine delivery systems, but there are several challenges to overcome before their widespread adoption. Microparticles can encapsulate a variety of compounds and deliver them with a variety of release profiles, while still able to be easily administered through a syringe needle.[46] Although microparticles can increase the ease of administration, the microparticles are difficult to manufacture at a large scale. Additionally, there is a risk of inactivation of the antigen or drug during fabrication

2.3.2 Polymer Composition

Biodegradable polymers used in microparticle delivery systems can either be derived from natural materials or synthetically processed. Natural polymers have several advantages which include increased biocompatibility, lower toxicity, and better biological functionality.[48] However, they are usually more complex, require more processing, and can have immunogenicity problems such as inability to permeate cell membranes and low bioavailability.[49] Commonly used non-toxic, biodegradable, natural polymers are chitosan and dextran. However, chitosan has been shown to cause blood clotting[50] and dextran is better suited for short release periods of minutes to hours for responsive drug payload drops.[51]

Alternatively, synthetic polymers are easily created, durable, and their properties can be easily manipulated. Poly (D,L-lactic-co-glycolic acid) (PLGA), seen in **Figure 2.4**, is a commonly used polymer for microparticle synthesis and biological applications. Lactic acid, a carboxylic acid found naturally in the body, exists in two optically active chiral forms: D- and L-. When the negative chiral center D- molecules and positive chiral center L- molecules form a racemic mixture and this mixture is copolymerized with the

glycolic acid (an alpha hydroxyl acid), PLGA is formed. Since PLGA is a synthetic polymer, its molecular weight and monomer ratio are easily manipulated to achieve desired degradation characteristics.[52]
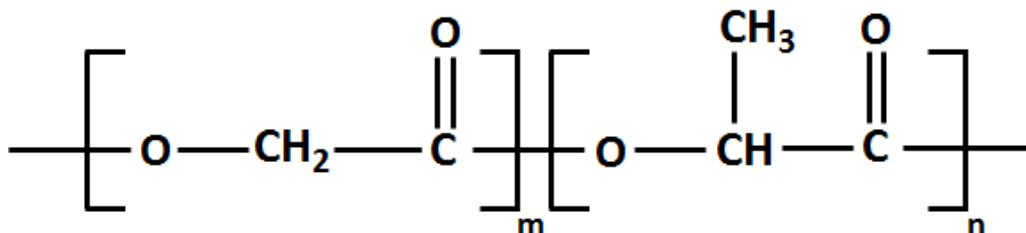
Figure 2.4. Poly(D,L-lactic-co-glycolic acid) (PLGA). PLGA copolymer where m denotes the number of glycolic acid units and n denotes the number of lactic acid units.

PLGA is a copolymer with several desirable properties for microparticles. It is biodegradable and nontoxic at low concentrations. PLGA degrades over time, allowing controlled-release of loaded matter, namely proteins and antigens in drug delivery applications, without causing adverse effects.[53] PLGA contains two monomers, lactic acid (LA) and glycolic acid (GA), which are connected by an ester bond. The ratio of the monomers determines the degradation rate due to varying hydrophilicities between the two monomers. PLGA degrades hydrolytically through the action of water (**Figure 2.5**).
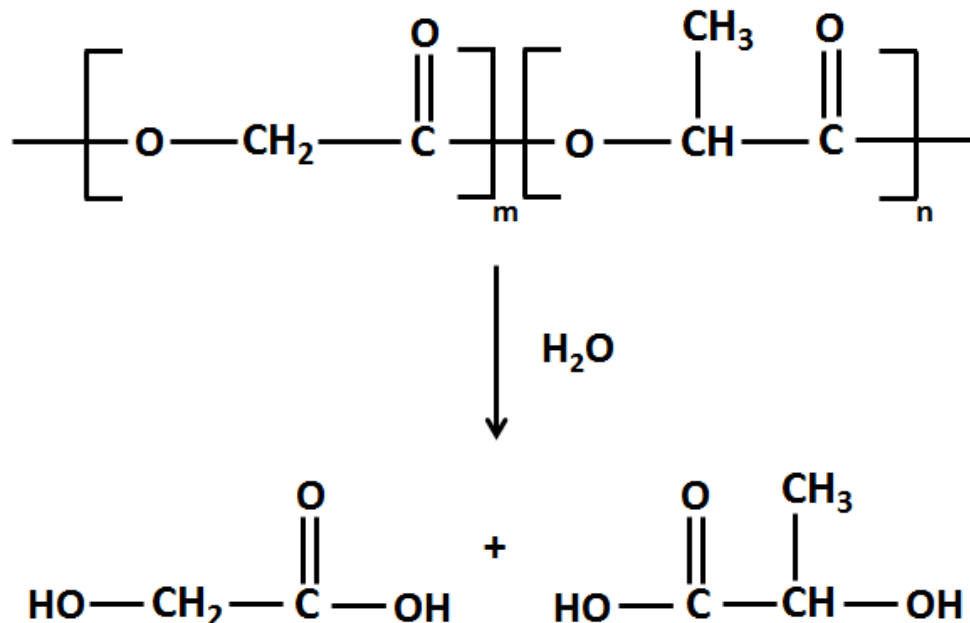
Figure 2.5. Hydrolytic degradation of PLGA. PLGA degrades into glycolic acid and lactic acid through the action of water.

The added methyl group on the lactic acid leads to a slower water penetration rate and therefore a slower degradation rate. Since the degradation of a PLGA microparticle is gradual and not catastrophic, the dispersed encapsulated antigens are also released gradually. By altering the ratio of lactic to glycolic acid and changing the degradation rate, the antigen release rate can be predicted and controlled. In addition, variation in the molecular weight can also affect degradation. PLGA of higher molecular weight has been shown to have a lower degradation rate.[54] These factors of controllability have led to the popularity of PLGA in drug delivery applications. In our study, we use PLGA of very large molecular weight, 150, in attempt to minimize degradation rate to maintain a long period of sustained release while still obtaining full delivery over the dosage period.

2.3.3 Model Proteins Used in Vaccine Research

Often, virus or virus components used in vaccines can be expensive or difficult to obtain. For this reason, researchers use alternative, easily obtainable proteins that invoke an immune response in early or developmental experiments in place of the real vaccine components. This is very commonly done in controlled-release and polymer microparticle drug-related research. One of the most common proteins used is ovalbumin (OVA), the main protein found in chicken egg white. There is a vast amount of data and literature on the use of OVA as a replacement for vaccine components in terms of encapsulation and release, so we chose to take advantage of this information and use OVA as our model protein for our controlled-release microparticles.

2.3.4 Fabrication and Synthesis Methods

Fabrication of the microparticle can greatly influence the performance of the vaccine mechanism and of the resulting biological and immune responses. Microparticle uniformity, size, polymer attachment, and other compositional and structural characteristics all affect cellular uptake, biodistribution, and release rate. The type and size of the particles, entrapment, release characteristics, and the stability of the drug in the microparticle formulations are dependent on the synthesis technique.[55]

Synthesis options include solvent-based methods, hydrodynamic methods, microfluidic systems, and fluidic nanoprecipitation. Hydrodynamic methods produce fair uniformity, but are not highly researched and documented. Microfluidic systems perform well in uniformity tests on the nanoscale, but the procedures are difficult to perform and require specialized equipment.[56] Fluidic nanoprecipitation provides the best uniformity, but the technique is a fairly recent development involving expensive equipment.[57]

Solvent-based methods of microparticle fabrication are the most tested and familiar of current methods because they were the first to be utilized and tested in research. However, these methods perform poorly in uniformity tests.[58] Since precise size uniformity is not of utmost importance in this study, we utilize solvent-based methods due to their low cost, manipulability of phase composition, and low degree of difficulty to execute.

The double emulsion technique, also known as the multiple emulsion technique, uses oil, water, and a surfactant to form particles in solution. The technique is based on principles of interfacial tension and intermolecular forces.[59,60] This method, as diagrammed in **Figure 2.6**, effectively entraps hydrophilic drugs by emulsifying an aqueous solution containing bioactive compounds (internal aqueous phase) into an organic solution containing the polymer. The entanglement of the polymer chains yields the porous microparticle. This primary emulsion is then dispersed in a second aqueous phase containing a suitable emulsifier, resulting in a transient formation of a double emulsion. The volatile organic solvent is then removed and the solid microparticles are collected.[61]
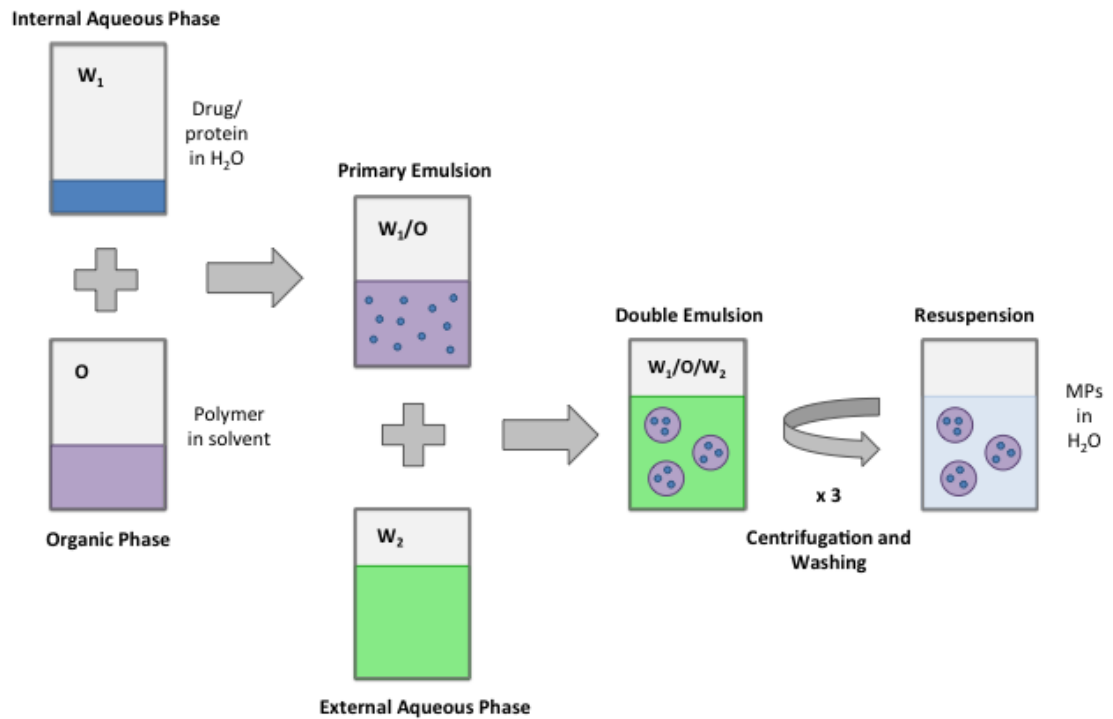
Figure 2.6. Double emulsion microparticle fabrication. Diagram of the double emulsion synthesis technique for fabricating protein-loaded polymer microparticles. Diagram is adapted from Giri et al. 2013.[62]

The double emulsion technique has many technical and user advantages. Unlike other common microparticle fabrication methods, it yields a very high encapsulation of hydrophilic drugs including proteins and peptides. The double emulsion method has been considered relatively simple and convenient in controlling process parameters. Additionally, the necessary materials are easy to obtain and are relatively inexpensive.[55] Particle size, to a certain extent, can also be controlled because the concentration of the polymer in solution influences the size and weight of the particles.[59] In addition to phase concentrations, the intensity and duration of the agitation of the emulsions can also be utilized to affect particle size. This is important because parameters such as release

characteristics are affected by the molecular weight of the PLGA. Further, this method yields large quantities of particles, which is critical because high concentrations of particles are necessary to deliver the correct dosage of the antigen. Overall, microparticles made by the double emulsion technique are excellent reservoirs for the encapsulation of a variety of pharmaceutical compounds.[55]

2.3.5 Microparticle Size

When choosing to use polymer particle delivery system for a specific application, one of the most crucial parameters to consider and design is the size of the particles. The size of particles contributes to their *in vivo* behavior in several ways, most importantly in how they can travel through the body and their release profile.

Microparticles, which can range in diameter from 1 µm to several hundreds of microns, will travel differently in the body than nanoparticles, which are sized below 1 µm. Both microparticles and nanoparticles have been studied extensively for drug delivery systems, and selecting the correct order of magnitude for our particles is crucial to the success of the delivery system.

For our application, in order to produce the strongest immune response, the microparticle size must allow for dendritic cells to uptake the microparticles. Optimal size for APCs to uptake cells was found to be 1–10 µm.[46] Additionally, decreasing the size of microparticles leads to an increase in surface area to volume ratio, which increases the rate of release of the encapsulated component from the microparticles. Additionally, we must consider the size of HPV VLPs which will be encapsulated within our microparticles. HPV VLPs are approximately 55-60 nm in diameter.

For these reasons, our research will use 1-10 μm microparticles, rather than nanoparticles as the vaccine delivery system in order to allow for the microparticles to be able to encapsulate the HPV VLPs and to be uptaken by dendritic cells.

2.3.6 Release Kinetics

There are a number of ways to control the rate at which a drug is released from the microparticle. The loading efficiency of a loaded microparticle is based off of the amount of drug encapsulated in comparison to the initial amount of drug present in the primary emulsion. The loading efficiency effects the composition and density of the microparticle as a whole, which can influence the release rate. Phase composition and size can be controlled by varying the processing parameters in the fabrication protocol. Microparticle diameter influences the release rate inversely: the smaller the microparticle is, the faster the encapsulated drug will be dispersed for a certain loading efficiency and particle composition.[57]

A major problem with controlled-release delivery systems is the effect of "burst release." During the initial activation of a polymeric drug delivery system in a release medium, a large amount of the encapsulated drug is released in a very short time compared to the entire release profile of the system.[63] This effect in terms of release is diagrammed in **Figure 2.7**.[63]
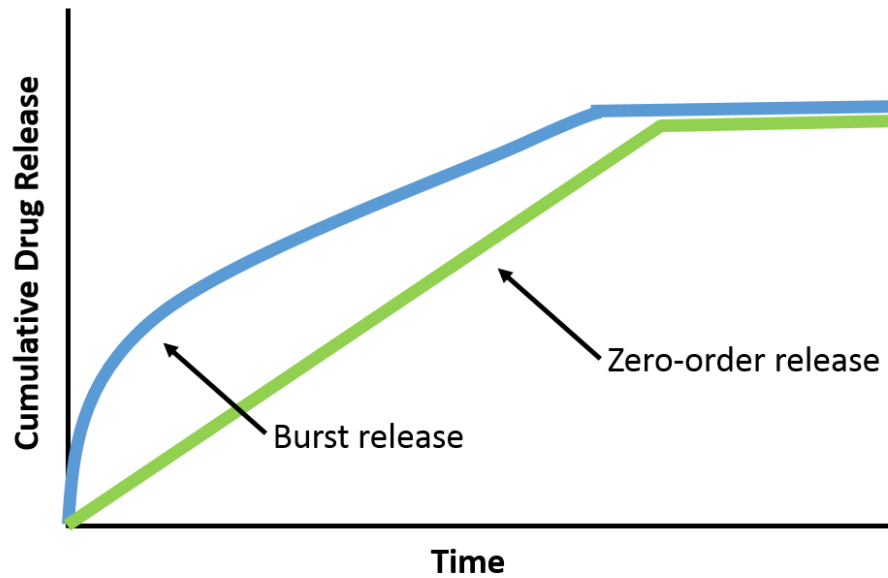
Figure 2.7. Burst effect schematic. Burst effect results in a higher initial drug delivery compared to a linear release rate. Adapted from Huang and Brazel, 2001.[63]

This effect has been attributed to drug content on the surface of the microparticles or a large fraction of the drug content close to the surface of the microparticles which is easy to access for the hydration disintegration mechanism.[64] Other possible causes for drug delivery include crack formation in the microparticles or catastrophic disintegration of the bulk particles.[63]

Recent investigation in the mechanism of burst release has discovered various ways to reduce the effect. Possible methods include forcing the drug to diffuse through at least two separate layers, encapsulation of the drug in alginate beads coated with a polycation, and adding certain chemical substances to injectable drug delivery systems.[63,65] The goal of this study is to create particles with a release profile that induces an immune response comparable to the traditional HPV vaccine. Reducing the burst effect will result in higher release rate at later time points, which in turn will help create the longer release periods needed for this investigation.

2.4 Vaccine Cell Studies

The development of vaccines and/or vaccine delivery systems require multiple *in vitro* and *in vivo* studies to determine the strength of the immune response elicited by the antigen of choice. The most common method of study is to inject the infectious agent into an *in vivo* model such as a rat or rabbit and collect the whole blood to determine the extent of the immune response by measuring the production of antibodies, cytokines, and and other proteins involved in the cell-mediated immune response.[66] Alternatively, cytokine production can also be measured in tissue cultures composed of the various cells involved in generating the immune response. Given the cost and difficulty of animal testing, cell culture based studies are often more powerful and thus more useful during the preliminary stages of vaccine development.

In vitro testing of vaccines utilizes cell types that are commonly employed by the immune system. These cells include dendritic cells, T-cells, and macrophages. Since dendritic cells are the primary cells used for antigen presentation to T-cells and macrophages, dendritic cells can be used as the primary cell line to determine the initial immune response of a system to an antigen. Although dendritic cell lines can be isolated from blood samples, this process is difficult and time consuming. Instead, many dendritic cell lines are available for purchase and the Murine dendritic cell line JAWSII is believed to most accurately match the response generated by bone-marrow derived dendritic cells.[67]

In investigating the immune response to HPV and the HPV vaccine, TNF-α and IFN-γ were identified as two of the major cytokines involved in the attempt to combat the viral attack. Both cytokines possess antiviral and apoptotic characteristics that allow them

33

to destroy infected cells.[68] Although both cytokines are excellent indicators of a cell's attempt to combat a viral infection, dendritic cells have been proven to be more sensitive to TNF-α production during a viral attack as opposed to IFN-γ.[66]

Chapter 3: Microparticle Fabrication and Characterization

3.1 Objectives

The objective of this study was to modify the steps in the double emulsion synthesis of PLGA microparticles to yield microparticles with diameters of approximately 10 μm in a reasonable time frame. In this study, we utilized two methods to achieve different microparticle characteristics. The first method used longer stir times and produced microparticles in a homogenous distribution with a mean diameter of about 11 microns. The second method used a 4 hour stir time in order to complete microparticle fabrication in a single day and produced microparticles with mean diameters ranging between 15 and 30 microns. The second method produced higher encapsulation rates, which will be discussed in Chapter 6.

3.2 Methods

3.2.1 Fabrication of Microparticles

3.2.1.1 Method 1

The first microparticle fabrication protocol consisted of a double emulsion technique to synthesize both loaded and unloaded microparticles. The double emulsion technique is one of the most tested techniques available to yield high encapsulation of hydrophilic drugs such as proteins and peptides. As described previously, the technique uses oil, water, and a surfactant to create microparticles in solution. Hydrophilic drugs can be encapsulated by emulsifying an aqueous solution containing the bioactive

compound into an organic solution with the polymer.[59] The entanglement of the polymer chains yields a porous microparticle structure.
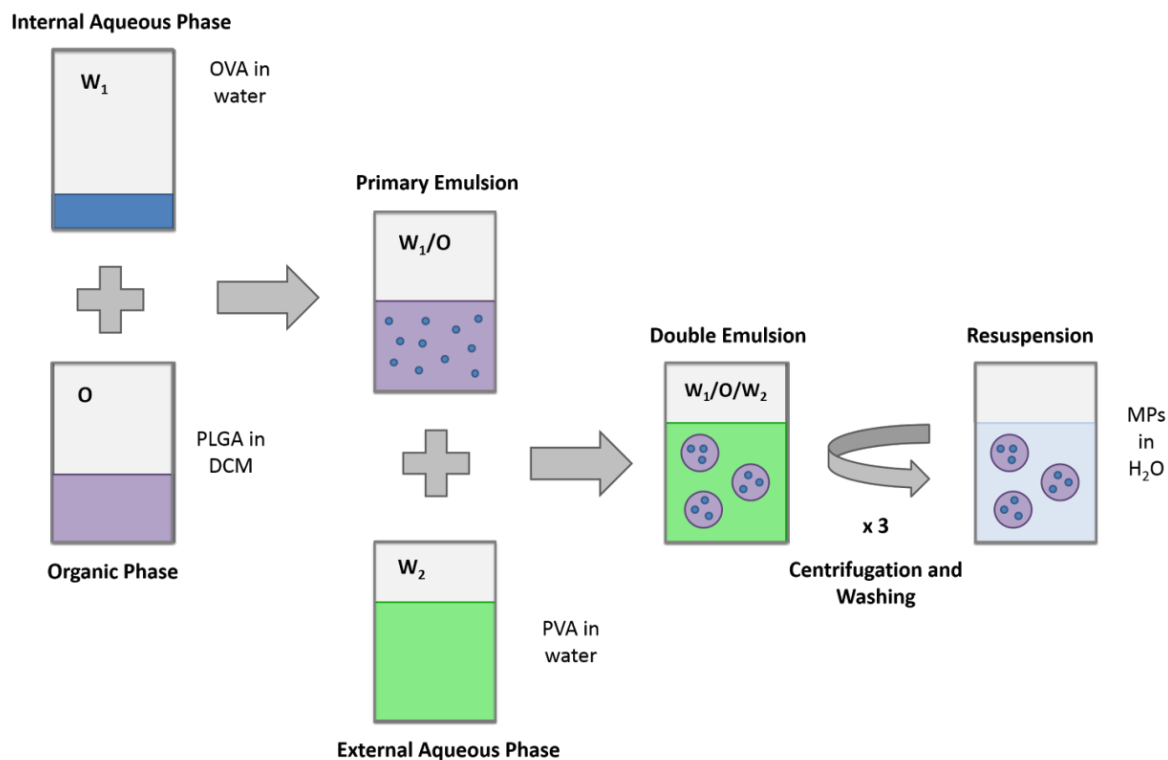
Figure 3.1. Schematic of the double emulsion synthesis technique. This method fabricates PLGA microparticles loaded with the model protein, OVA.

In this study, a modified version of the double emulsion protocol as stated by Kofler et al.[69] was followed for porous microparticle synthesis (**Figure 3.1**). To fabricate various batches of microparticles, 3 % (w/v) PLGA with varied concentrations of PVA, at 5, 10, and 15 % (w/v), were stirred for 12, 16, or 20 hours.

To fabricate unloaded microparticles, the appropriate mass of PVA was dissolved in 50 mL phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4), pH 7.4 on a hot plate under magnetic stirring at 300 RPM. The PVA was allowed to reach approximately 130 °C and cooled to room temperature when fully

dissolved. Next, 0.18 g 50:50 LA:GA PLGA with a molecular weight of 150 was dissolved completely in 6 mL 3 % (w/v) dichloromethane (DCM) contained in a beaker using a bath sonicator. In order to avoid the evaporation of DCM during this period, the beaker was covered by aluminum foil. Finally, the PLGA solution was slowly poured into the PVA solution under magnetic stirring at 300 RPM. The solution was left to stir until the desired number of hours.

To fabricate the OVA-loaded microparticles, a similar procedure was followed in a sterile environment. All batches were done in quadruplicate. First, 10 mg/mL OVA in PBS was shaken until the OVA fully dissolved and sterile filtered using a 0.22 μm filter. Then, 0.18 g PLGA was dissolved in 6 mL DCM as stated above. To form the primary emulsion, 0.3125 mL 10 mg/mL sterile-filtered OVA was added to the 3 % (w/v) PLGA solution and sonicated for 45 seconds in a bath sonicator. To form the secondary emulsion, this solution was added to 12.5 mL 5, 10, or 15 % (w/v) sterile-filtered PVA in PBS. This emulsion was sonicated for approximately 1 minute in a bath sonicator. This combination was finally added to 50 mL sterile-filtered PBS containing the same concentration of PVA under magnetic stirring at 300 RPM. The final solution was left to stir for 12, 16, or 20 hours.

Both the unloaded and loaded microparticles followed identical washing and collecting procedures. After stirring was complete, each beaker of microparticles in solution was poured into 50 mL Falcon tubes and centrifuged for 10 minutes at 3200 RPM. The supernatants were removed, and approximately 1 mL was saved for encapsulation analysis. The microparticles in the pellet were washed with approximately 40 mL PBS and centrifuged for 10 minutes at 3200 RPM. This washing step was repeated

two more times. After the microparticles were washed three times, the supernatant was removed and the remaining microparticles were collected in an eppendorf tube and resuspended in approximately 1 mL distilled water.

3.2.1.2 Method 2

First, two solutions of 10% PLGA (100 mg 50:50 LA:GA PLGA with a molecular weight of 150 in 1 mL DCM) were prepared in glass test tubes, covered with aluminum foil and parafilm to prevent evaporation, and left to dissolve overnight (**Appendix A – Microparticle Fabrication: Method 2 Protocol**). These solutions were then UV-sterilized for 15 minutes. Second, 1, 5, or 8 % (w/v) PVA in Millipore water was prepared by heating the water to approximately 80 °C and slowly adding the PVA under magnetic stirring. Then, the temperature of the stir plate was increased to approximately 180 °C and the bottle covered with aluminum foil. Once the PVA dissolved, it was autoclaved.

For the loaded microparticles, 10, 50, or 100 mg/mL OVA in Millipore water (in duplicate) was dissolved by vortexing and sterilized using a 0.22 μm syringe filter. To form the primary emulsion, 400 μL OVA solution were then added into the previously prepared PLGA solution and vortexed 2 to 3 times for approximately 5 seconds each until the solution became opaque. Afterward, the solution was sonicated in ice water in a sonicator for 2 minutes. To form the secondary emulsion, 3 mL PVA at the desired concentration was added to the primary emulsion, vortexed, and sonicated again in ice water for 2 minutes. Next, the solution was vortexed again for 10 seconds, and another 3 mL PVA was added. Finally, two test tubes containing the double emulsion were poured

into 200 mL PVA under magnetic stirring at 360 RPM. The microparticles were allowed

to stir for four hours uncovered before collecting as seen in **Figure 3.2**.
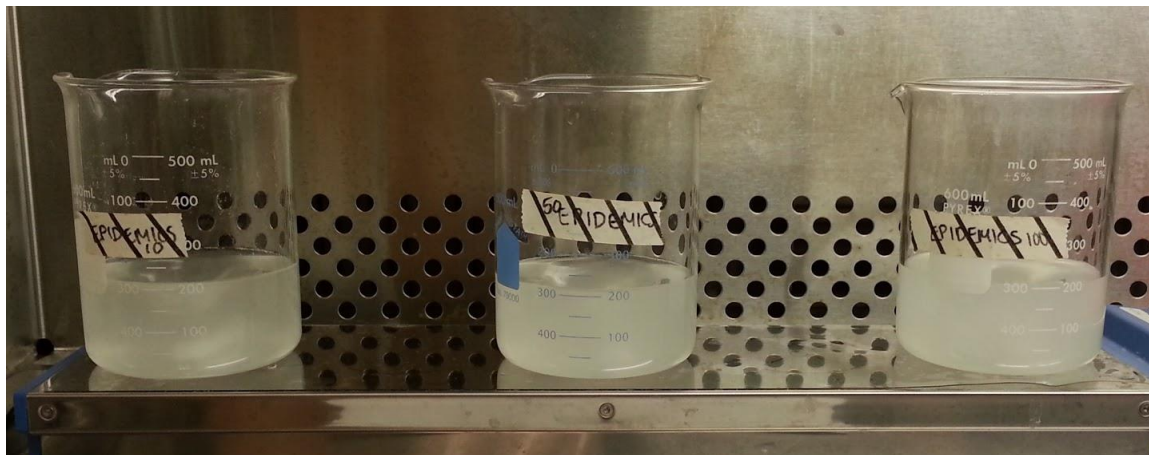
Figure 3.2. Stirring OVA-loaded microparticles. Three batches of OVA-loaded microparticles fabricated using Method 2 with 10% (w/v) PLGA, 5% (w/v) PVA, and varying concentrations of OVA (10, 50, or 100 mg/mL), stirring at 360 RPM in a sterile hood.

For the unloaded microparticles, the same protocol was followed, except no OVA

was dissolved in the PLGA. The same double emulsion synthesis technique was also used

to make one batch of microparticles encapsulating the HPV vaccine, Gardasil®, in which

400 µL vaccine was directly added to the PLGA in DCM (**Figure 3.3**).

The unloaded and loaded microparticles were washed and collected using the

same protocol. The microparticle solution was distributed centrifuged in 50 mL Falcon

tubes at 1000 x *g* for 15 minutes. Again, 1 mL supernatant was removed from each

Falcon tube and saved for encapsulation studies at -20 °C. The rest of the supernatant was

aspirated and discarded. Sterile water was added to each Falcon tube and gently stirred to

break up the microparticle pellets. Then, the same protocol of centrifuging, aspirating,

and washing the supernatant was repeated three times. After the last wash, the

microparticles were resuspended in a small volume of water (**Figure 3.3**), flash-frozen in liquid nitrogen, and lyophilized.
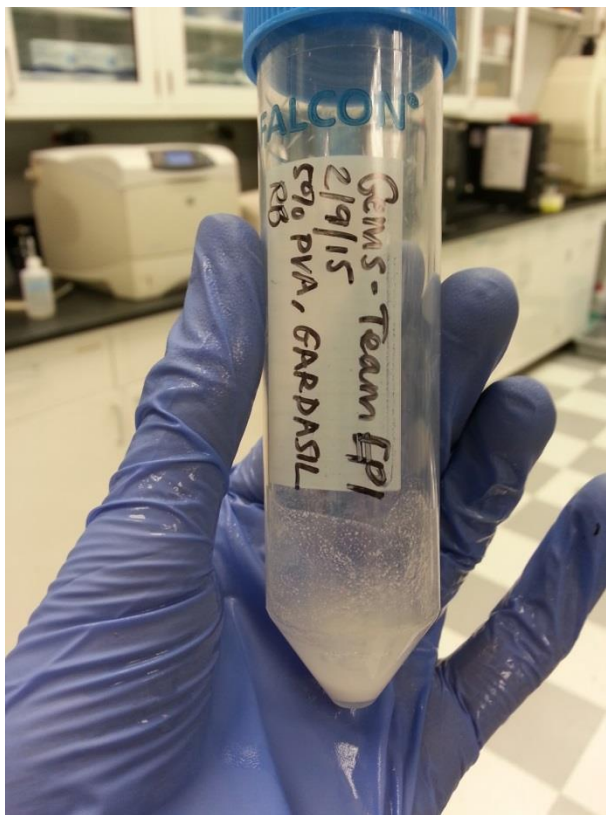
Figure 3.3. Washed and collected microparticles. Washed and collected Gardasil®-loaded microparticles fabricated with 10% (w/v) PLGA and 5% (w/v) PVA.

3.2.2 Size Determination

3.2.2.1 Light Microscopy

Aliquots of microparticles were taken from each batch, suspended in various dilutions with PBS in a 96-well plate and imaged with a Zeiss Axiovert 40 CFL inverted light microscope to obtain clear images of separated microparticles. The microparticles were imaged at 40X magnification. The images were uploaded into MATLAB, and a program (**Appendix B – MATLAB Sizing Program**) was run to a black and white filter to encode the pixels of the image as binary microparticle and non-microparticle space.

Using the pixel-to-micrometer settings from the microscope software and the Hough transform algorithm, the microparticles were measured and distributions were created for each time point as seen in **Figure 3.4** and **Figure 3.5**.

3.2.2.2 Laser Diffraction

A Horiba LA-960 laser microparticle size analyzer capable of measuring microparticle sizes between 10 nm and 5 mm with the complement software was used to determine the average diameter of microparticles fabricated using each method described above. The following protocol as described in Andorko et al. was utilized for the determination of microparticle size.[19] First, the supplied cuvette-style glass fraction cell was cleaned with deionized water and wiped with a cotton-tipped swab. Approximately 10 mL distilled water was transferred to the fraction cell. A magnetic micro stir bar was added, and then the fraction cell was loaded into the cell mount of the microparticle size analyzer. After alignment and blanking of the system using distilled water, a small volume (50 to 100 µL depending on if the volume of the microparticle sample added was sufficient to generate appropriate signal strength) of the microparticle suspension was taken and inserted into the fraction cell. A magnetic stirring mechanism was employed to disperse the microparticles in the cell. Average microparticle diameters were calculated using a number basis with the complement software.

3.3 Results

3.3.1 Light Microscopy

Light microscopy was used to visualize microparticles fabricated using Method 1 and collected after 17.5, 19.5, 21.5, and 23.5 hours under magnetic stirring **(Figure 3.4).**
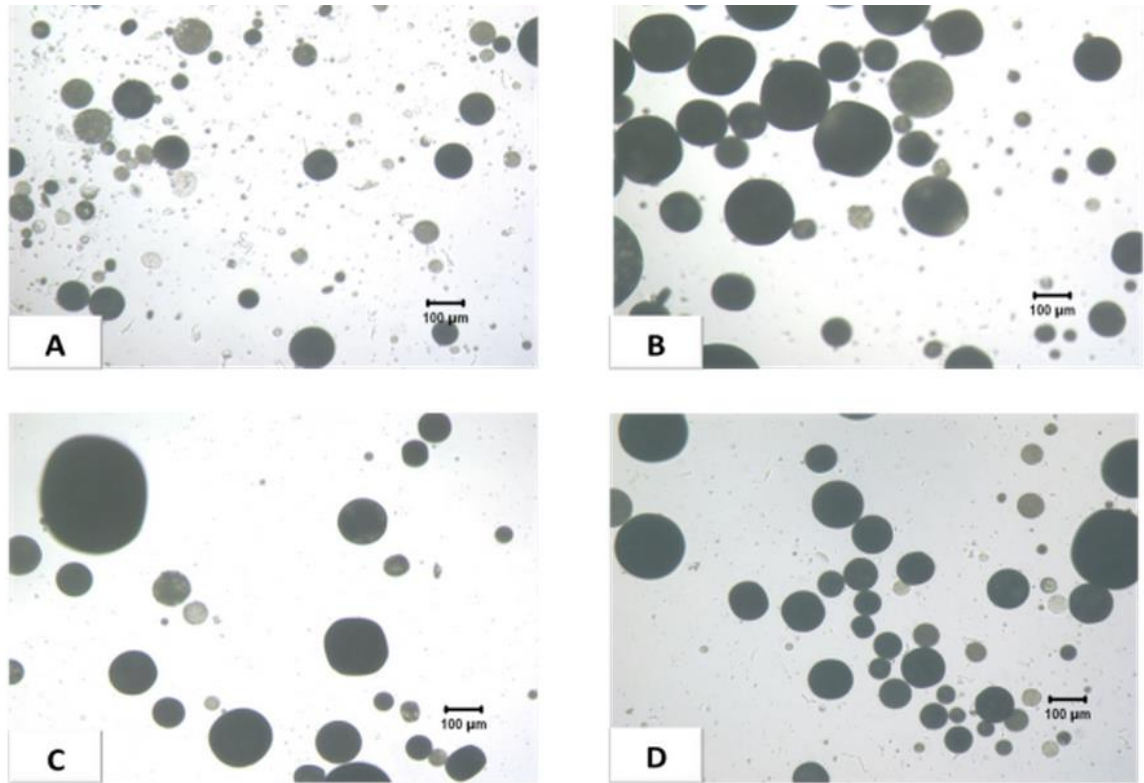
Figure 3.4. Light microscopy images of microparticles. Microparticles fabricated with 3% (w/v) PLGA and 10% (w/v) PVA, sampled, and stirred for (A) 17.5 hours, (B) 19.5 hours, (C) 21.5 hours, and (D) 23.5 hours.

In **Figure 3.5**, all four stirring times resulted in an overall similar shape with most of the microparticles having a diameter of 20 μm or less and fewer microparticles having a diameter greater than 20 μm. However, as seen with the 19.5 hour stirring time, as much as 70% of the microparticles exceeded a diameter of over 20 μm.
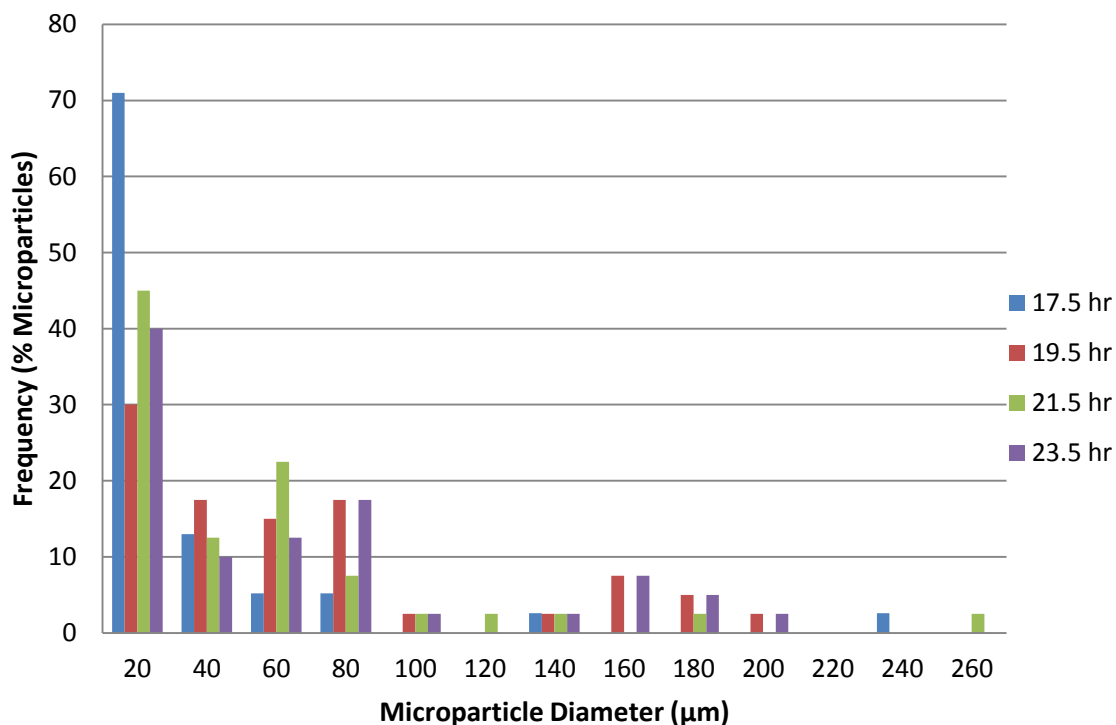
Figure 3.5. Frequency of microparticle diameters measured by the MATLAB program. Microparticles were fabricated with 3% (w/v) PLGA and 10% (w/v) PVA and stirred for 17.5 hours, 19.5 hours, 21.5 hours, and 23.5 hours. The average diameters of microparticles stirred for 17.5 hours, 19.5 hours, 21.5 hours, and 23.5 hours are 3.66 ± 1.64, 2.37 ± 1.87, 1.62 ± 1.28, and 1.35 ± 1.35, respectively.

Additionally, microscopic images were taken in order to visualize whether microparticles fabricated using Gardasil® had encapsulated the vaccine and to estimate the microparticle diameter (**Figure 3.6**). Although this image was not used to calculate a microparticle size distribution, we observed that opaque, large (approximately 80 µm in diameter) microparticles were formed, as shown in this sample image of a sample of the Gardasil®-loaded microparticles.
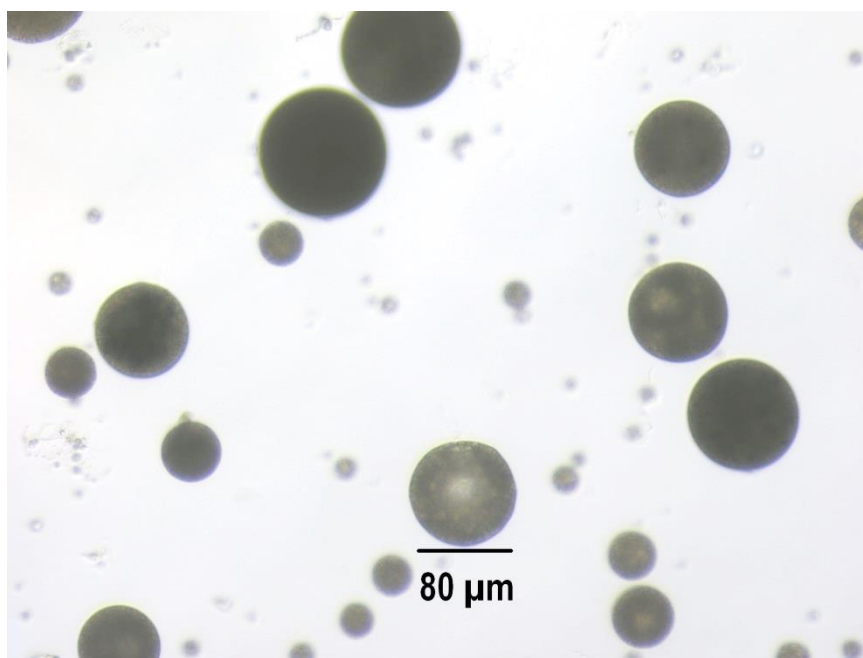
Figure 3.6. Gardasil®-loaded microparticles. Fabricated using Method 2 with 10% (w/v) PLGA, 5% (w/v) PVA, and the Gardasil® vaccine.

3.3.2 Laser Diffraction

Laser diffraction was used as a complementary method for determining microparticle size distribution. **Figures 3.7** and **3.8** illustrate how various stir times affect the mean size of OVA-loaded microparticles fabricated with 3% (w/v) PLGA and 10% (w/v) PVA using Method 1.
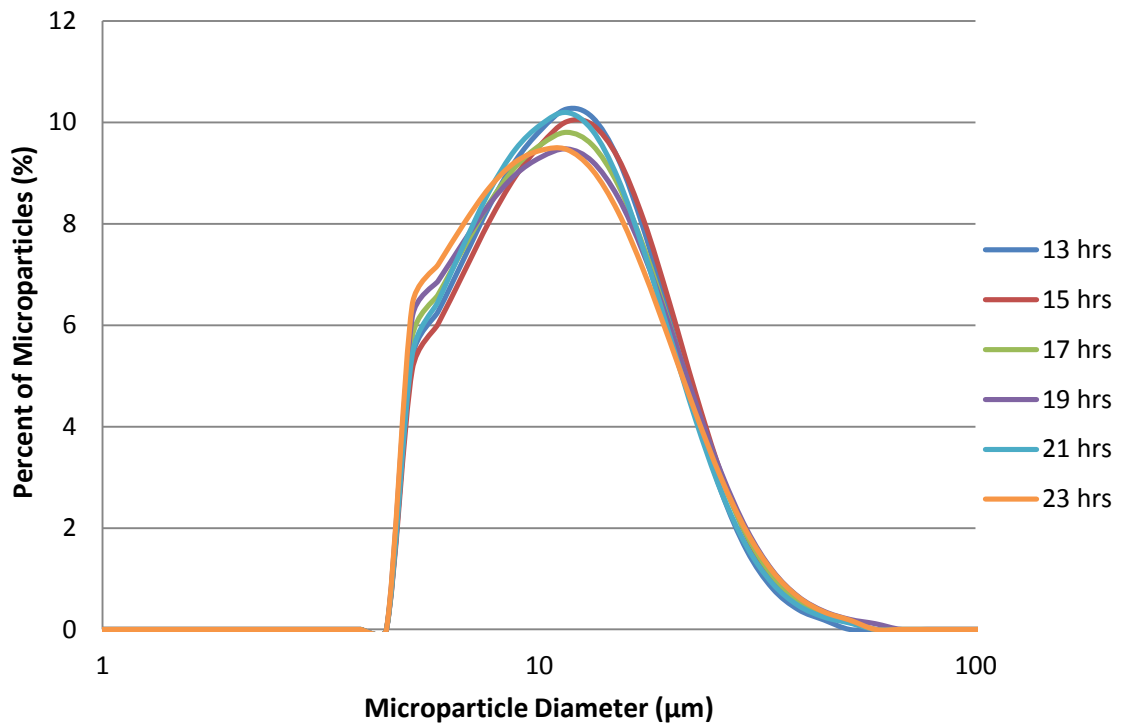
Figure 3.7. Effect of stir time on microparticle size measured by laser diffraction. The average diameters of microparticles fabricated with 3% (w/v) PLGA and 10% (w/v) PVA and stir times 13, 15, 17, 19, 21, and 23 hours were 11.94 ± 5.97 µm, 12.32 ± 6.39 µm, 12.13 ± 6.50 µm, 12.25 ± 6.86 µm, 11.94 ± 6.23 µm, and 12.01 ± 6.66 µm, respectively.
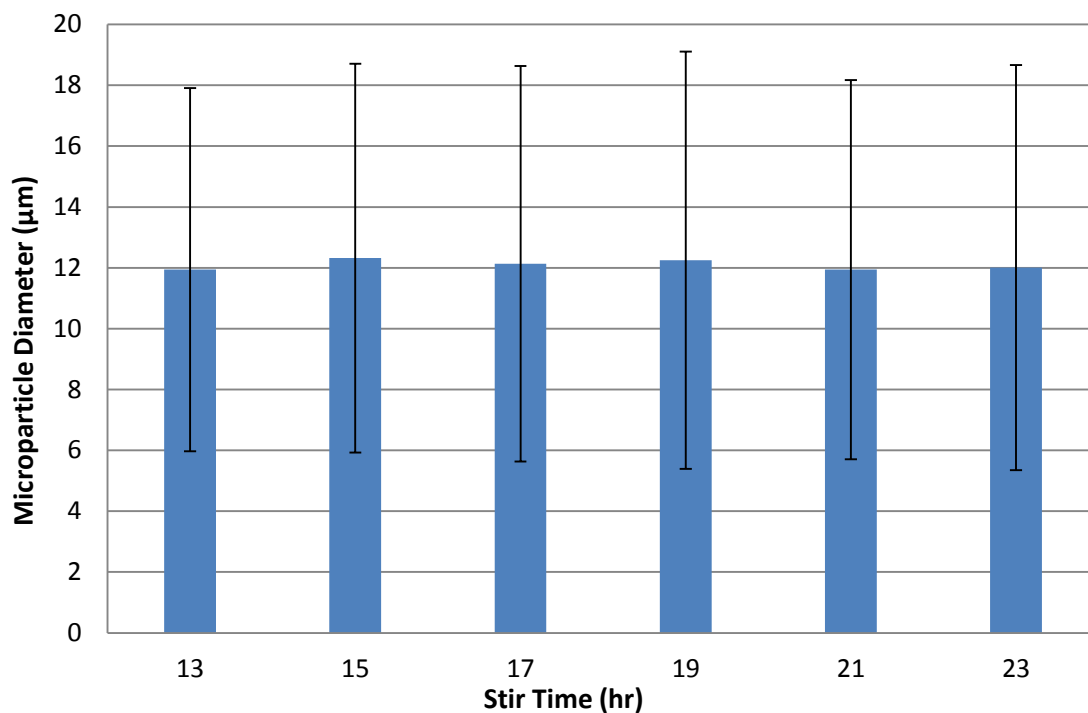
Figure 3.8. Mean size (μm) and standard deviations for OVA-loaded microparticles. Microparticles were synthesized with 3% (w/v) PLGA and 10% (w/v) PVA, corresponding to the size distributions shown in **Figure 3.7**.

All of the tested stir times yielded microparticles near our desired size target of 10 μm in diameter. ANOVA and individual Tukey tests were used to determine the statistical significance between groups. Because there was no definite n provided for the laser diffraction data, we chose an arbitrary, yet purposely high n of 5000. Using an ANOVA test, a significant difference (defined as $p < 0.05$) was found between at least one pair of time points ($p = 0.007$); however, Tukey tests showed no significant difference between any of the pairs of time points.

Laser diffraction was also used to determine the effect of % (w/v) PVA on mean microparticle diameter. Microparticles were synthesized using Method 1 and 10 % (w/v) PLGA. Then, laser diffraction data from three separate experiments, including that shown

in **Figure 3.9**, synthesized using each PVA concentration were compiled (**Figure 3.10**). In this case, there was a significant decrease in microparticle size when stir time was increased ($p = 0.001$) using both an ANOVA and a Tukey Test between each group, which will be discussed further in Chapter 6. Additionally, when % (w/v) PVA concentration was increased, a significant decrease in mean microparticle diameter was observed ($p = 0.001$), again using both an ANOVA and a Tukey Test between each group.
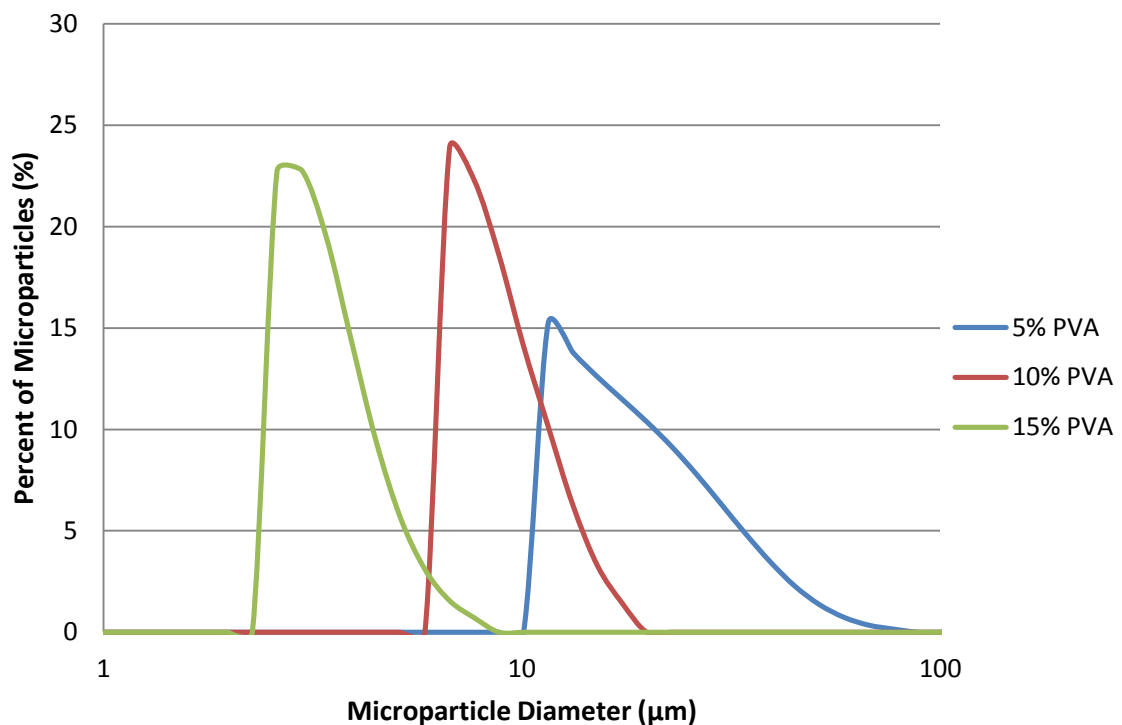
Figure 3.9. Sample distributions of OVA-loaded microparticles fabricated using Method 1. Microparticles were synthesized with 3% (w/v) PLGA and varying (w/v) PVA concentrations and stirred for 12 hours. The means and standard deviations of these distributions are included in **Figure 3.10**.
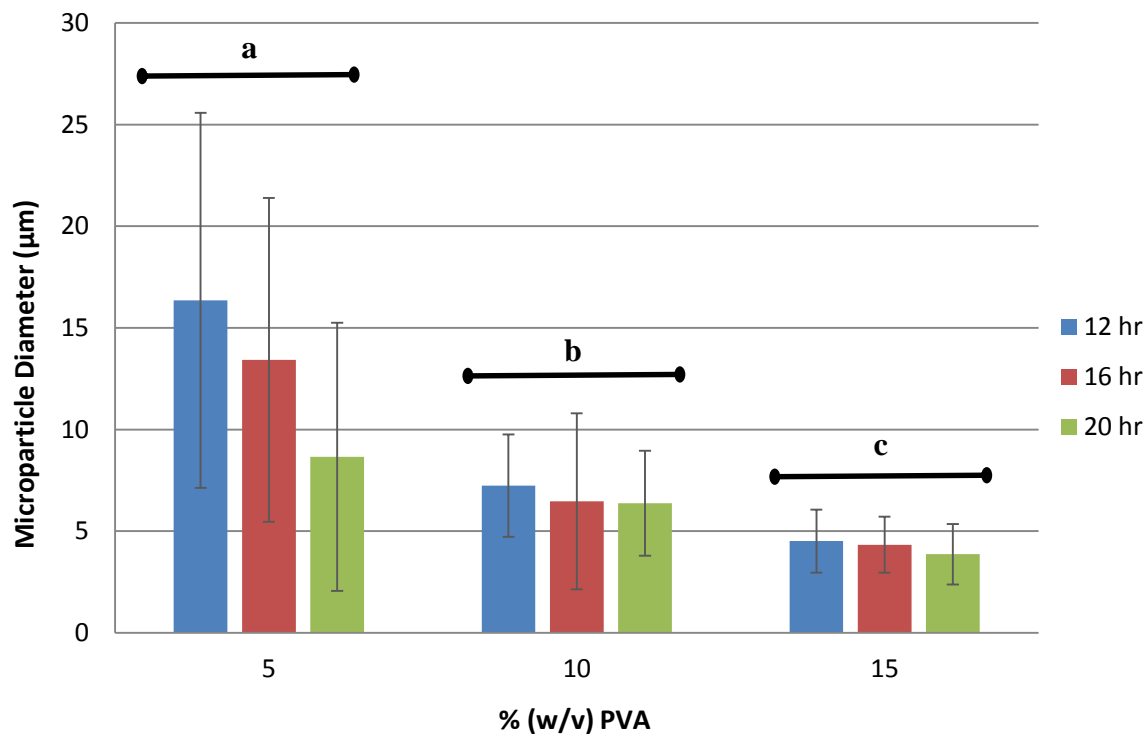
Figure 3.10. Effect of % (w/v) PVA and stirring time on microparticle size. Mean size (µm) and standard deviations of OVA-loaded 3% (w/v) PLGA microparticles at varying (w/v) PVA concentrations and stir times, fabricated using Method 1. Using Tukey Tests, statistically different treatment groups are labeled as 'a', 'b', and 'c' using p < 0.05. There was no statistical difference between microparticles made using different stir times. However, there was a statistically significant difference between microparticles made using different (w/v) PVA.

Figure 3.11 represents the size distribution of 10 mg/mL OVA-loaded microparticles fabricated with Method 2 using 10 % (w/v) PLGA and three different PVA concentrations.
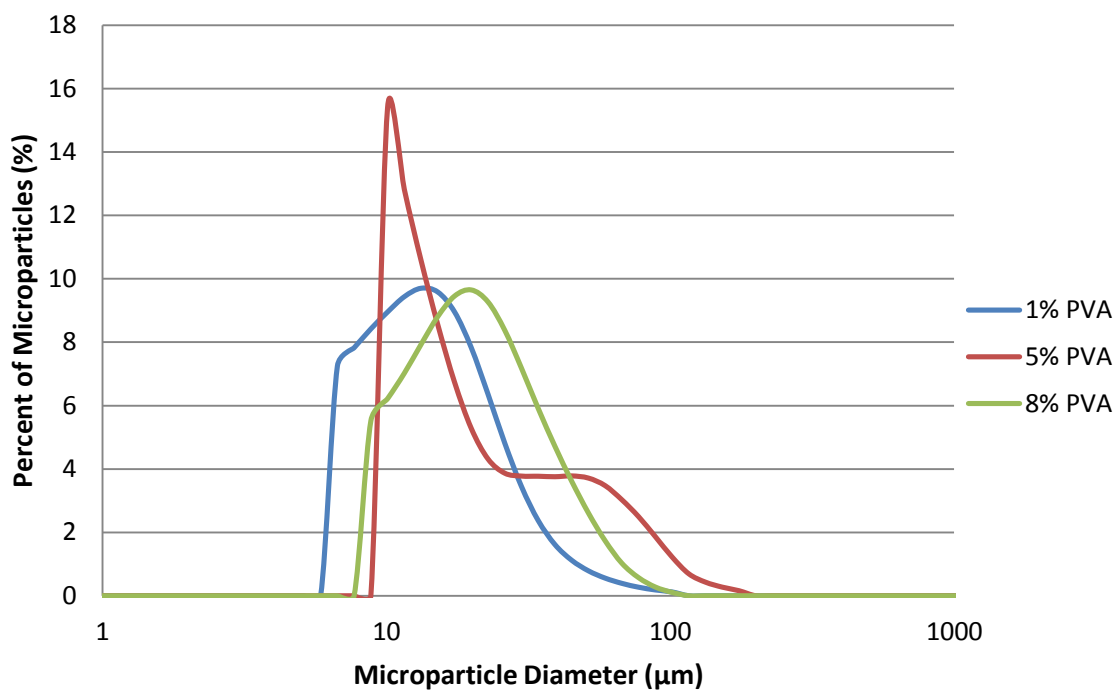
Figure 3.11. Sample distributions of OVA-loaded microparticles fabricated using Method 2. Microparticles were synthesized using 10 mg/mL OVA-loaded microparticles with 10% (w/v) PLGA and varying PVA concentrations (1, 5, and 8%). 5.1

Figure 3.12 shows the mean size ranges of all 10, 50, and 100 mg/mL OVA-loaded microparticles at 1, 5, and 8 % PVA and 10 % (w/v) PLGA. In this instance, there was no statistically significant difference between mean microparticle diameters fabricated using different concentrations of OVA (p = 0.953) or % (w/v) PVA (p = 0.990). Overall, the microparticles were larger in diameter than those fabricated using Method 1.
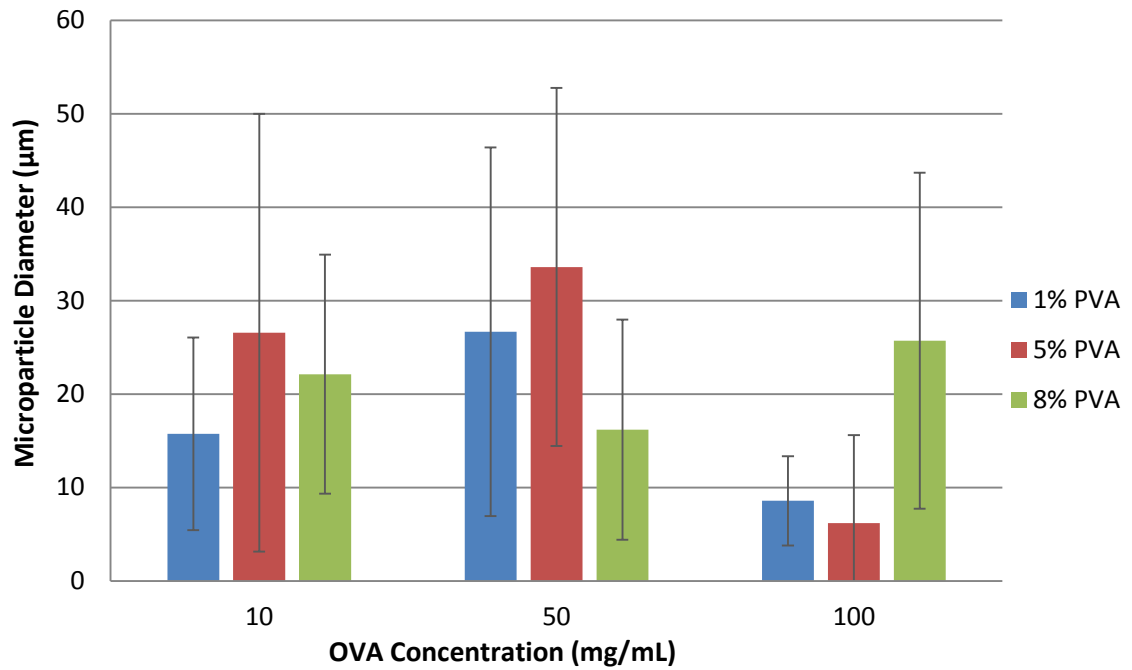
Figure 3.12. Effect of % (w/v) PVA and OVA concentration on microparticle size. Mean size (µm) and standard deviations of 10% (w/v) PLGA microparticles at varying OVA and (w/v) PVA concentrations.

Chapter 4: Microparticle Encapsulation, Release, and Degradation

4.1 Objectives

Controlled release of loaded microparticles is essential for delivery of the vaccine over time. In this study, we analyzed the encapsulation efficiency, release profile, and degradation characteristics of our loaded microparticles. Analysis of encapsulation was completed at the outset to determine the amount of model protein loaded into the microparticles. Encapsulation information was then used to calculate the amount of microparticles necessary to complete a study characterizing the release profiles of microparticles loaded with varying concentrations of protein. Following this, a measurement of PLGA degradation was completed to determine if sufficient degradation was achieved throughout our study time and whether variations in protein concentration affected degradation.

4.2 Methods

4.2.1 Encapsulation

We used two main methods of determining encapsulation efficiency, direct and indirect. Direct methods entailed taking a sample of microparticles and measuring the encapsulated protein content. Indirect methods involved measuring the concentration found in samples of supernatant of microparticles, then subtracting this value from the concentration expected if no encapsulation had occurred to determine percent encapsulation.

4.2.1.1 Direct

The direct method analyzed OVA encapsulation by dissolving the microparticles in an organic solvent, centrifuging the sample, and performing a BCA assay on the supernatant to measure the amount of OVA in the solution.[70] The organic solvents dimethyl sulfoxide (DMSO), methylene chloride (DCM), acetonitrile, and chloroform were tested to find the most accurate way of measuring encapsulation.[70–73] The direct method for measuring encapsulation was as follows:

5 mg OVA-loaded microparticles (10 mg/mL OVA, 3% PLGA, 10% PVA) were added to a glass vial. 5 mg unloaded microparticles (3% PLGA, 10% PVA) were weighed into a separate glass vial as a control.

For chloroform, 1.0 mL chloroform was added to both vials and the polymer was dissolved by vortexing for 5 minutes. After mixing, 1.0 mL deionized water was added to both vials, and the vials were placed on a shaker for 20 minutes. The organic and aqueous phases were then separated by centrifugation at 1000 x *g* for 10 minutes, and the top aqueous layer was collected for BCA analysis.

For DMSO, 0.5 mL DMSO were added to both vials and the polymer was dissolved by vortexing for 15 minutes. Then, 0.5 mL DCM was added to both vials, and after gentle mixing, 0.5 mL PBS was added to both vials and vortexed. The organic and aqueous phases were separated by centrifugation at 2000 RPM for 1 hour, and the top aqueous layer was collected for BCA analysis.

For acetonitrile, 1.0 mL DMSO was added to both vials. The vials were allowed to sit until the polymer was dissolved. Both vials were centrifuged at 3600 RPM for 20 minutes, and the pellet was then resuspended in 1 mL PBS for BCA analysis.

4.2.1.2 Indirect

Three assays were used to measure protein encapsulation indirectly, the Micro Bicinchoninic Acid (BCA) Assay, the Macro Bradford Assay, and the Micro Bradford Assay. These three methods are complimentary. The differing methods were used to optimize the encapsulation procedure.

For the Micro BCA Assay, supernatants were collected during the first centrifugation step of the microparticle fabrication process. To determine the amount of OVA in the supernatant, known concentrations of ovalbumin were prepared to create standards (0, 0.5, 1, 2.5, 5, 10, 20, 40, and 200 µg/mL). In a 96-well plate, 150 µL of each sample and standard were added. 150 µL working reagent was added and thoroughly mixed. The working reagent was made per Thermo Scientific's protocol for the Micro BCA assay. The plate was then incubated at 37 °C for 2 hours. The plate was then read at 562 nm in a plate reader.

For the Macro Bradford Assay, supernatants were again collected during the first centrifugation step of the microparticle fabrication process. Standards were created using known concentrations of OVA (0, 25, 125, 250, 500, 750, 1000, 1500, 2000 µg/mL). 30 µL of each sample and standard were pipetted into a microcentrifuge tube followed by 1.5 mL working reagent from Thermo Scientific. Each tube was let incubate for 10 minutes at room temperature. 200 µL of each sample was pipetted into a 96-well plate. The samples were then read at 595 nm on a plate reader.

Lastly, for the Micro Bradford Assay, supernatants were collected during the first centrifugation step of the microparticle fabrication process as in the previously described assays. Standards were created using known concentrations of ovalbumin (0, 2, 3, 4, 5,

7.5, 10, 20, 40, 50, 60, 75, 100, 125 µg/mL). 500 µL of each supernatant or standard was pipetted into a microcentrifuge tube followed by 500 µL working reagent from Thermo Scientific. Each tube was incubated at room temperature for 10 minutes. 200 µL of each sample was pipetted into a 96-well plate and the absorbance was read at 595 nm using a plate reader. Because the standard curve was quartic, concentration of OVA was plotted as a function of absorbance to calculate percent encapsulation.

4.2.2 Release

To determine the release profile of the microparticles, eight time points over a period of 14 days were used (0 hr, 1 hr, 4 hr, 6 hr, 24 hr, 48 hr, 7 days, and 14 days). Microparticles were fabricated with Method 2 using 0 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL OVA as described in Chapter 3. For each group, 250 mg microparticles were suspended in 5 mL deionized water and incubated at 37 °C in a shaker. At each time point, 200 µL were removed from each test tube and centrifuged at 3600 RPM for 25 minutes. The supernatant was collected, flash frozen, and stored at -20 °C. The remaining microparticle pellet was resuspended in 200 µL deionized water and added back to the test tube. This method measures and accumulation of OVA released over the 14 days. To determine the amount of OVA in the supernatant, the Bradford Assay was used as described previously in section 4.2.1.

4.2.3 Degradation

Gel permeation chromatography (GPC) was utilized to determine the degradation rate of the PLGA microparticles. Samples of microparticles containing varying concentrations of OVA were collected at two time points (day 0 and day 21). At the

conclusion of the 21 day study period, 3 mg lyophilized microparticles were suspended in 1 mL tetrahydofuran (THF). This mixture was filtered to remove insoluble materials and pipetted into glass vials for GPC analysis. A blank THF vial was analyzed initially to provide a background reading. Two standards, of known polystyrene molecular numbers, were also dissolved and analyzed using GPC.

4.3 Results

4.3.1 Encapsulation

4.3.1.1 Direct

For each of the organic solvents tested, 5 mg OVA-loaded microparticles fabricated using Method 1 was tested in triplicate. The concentration of OVA for each solvent was determined from a standard BCA curve obtained at the time of analysis, and averaged. Total OVA content was back-calculated using the volume of the aqueous layer removed for the Micro BCA assay. Loading efficiencies were expressed as a measure of our calculated OVA content over the original amount of OVA added to the fabrication mixture used in Method 1 (**Figure 4.1**).
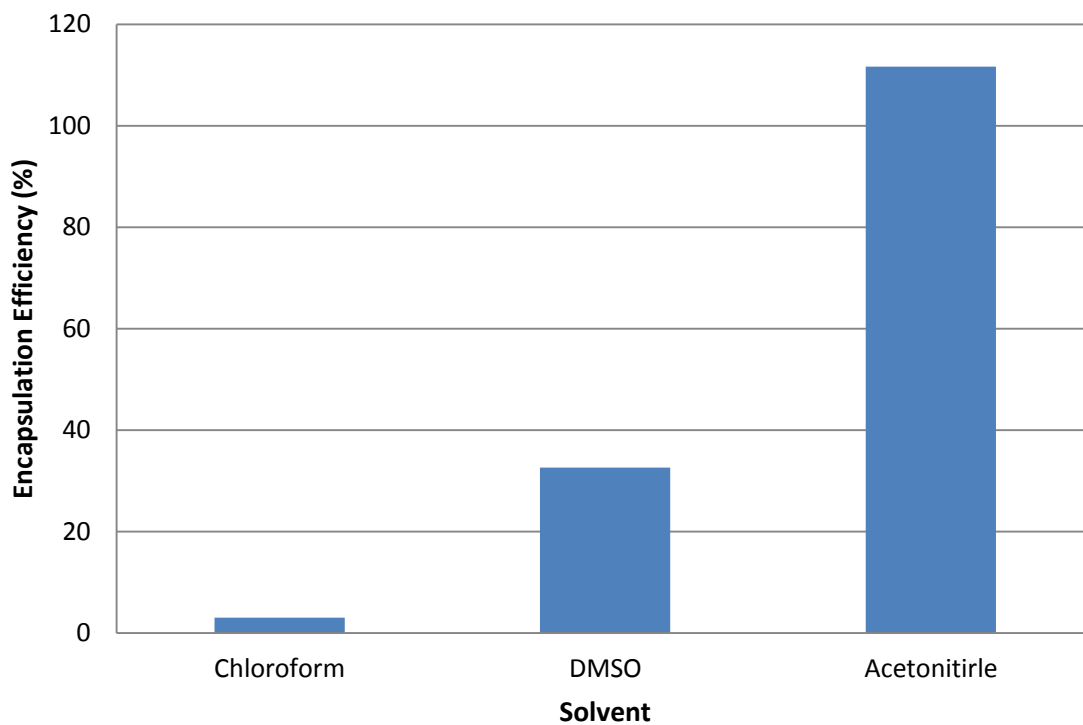
Figure 4.1. Encapsulation efficiency measured by direct encapsulation methods. Microparticles were fabricated using Method 1, with 3% (w/v) PLGA, 10% (w/v) PVA, and 10 mg/mL OVA. Their encapsulated OVA content was determined utilizing the following solvents: chloroform, dimethyl sulfoxide (DMSO), and acetonitrile, respectively.

The data showed that the concentrations obtained ranged from 1.03 μg/mL to 38.11 μg/mL, within the captured range of the Micro BCA assay. The solvent protocol that yielded the highest loading efficiency was acetonitrile at 111.7%, followed by DMSO at 32.60%, and finally chloroform, with only 3.2% loading.

4.3.1.2 Indirect

The Micro BCA assay was the first method used to analyze the supernatants of the microparticles in order to determine variables in microparticle fabrication that affected encapsulation. The stir times and percent (w/v) PVA were compared for the microparticles made using fabrication Method 1 (**Figure 4.2**). The optical density

indicates the amount of OVA left in the supernatant. Additionally, when encapsulation

rates were calculated, they were extremely low (data not shown).
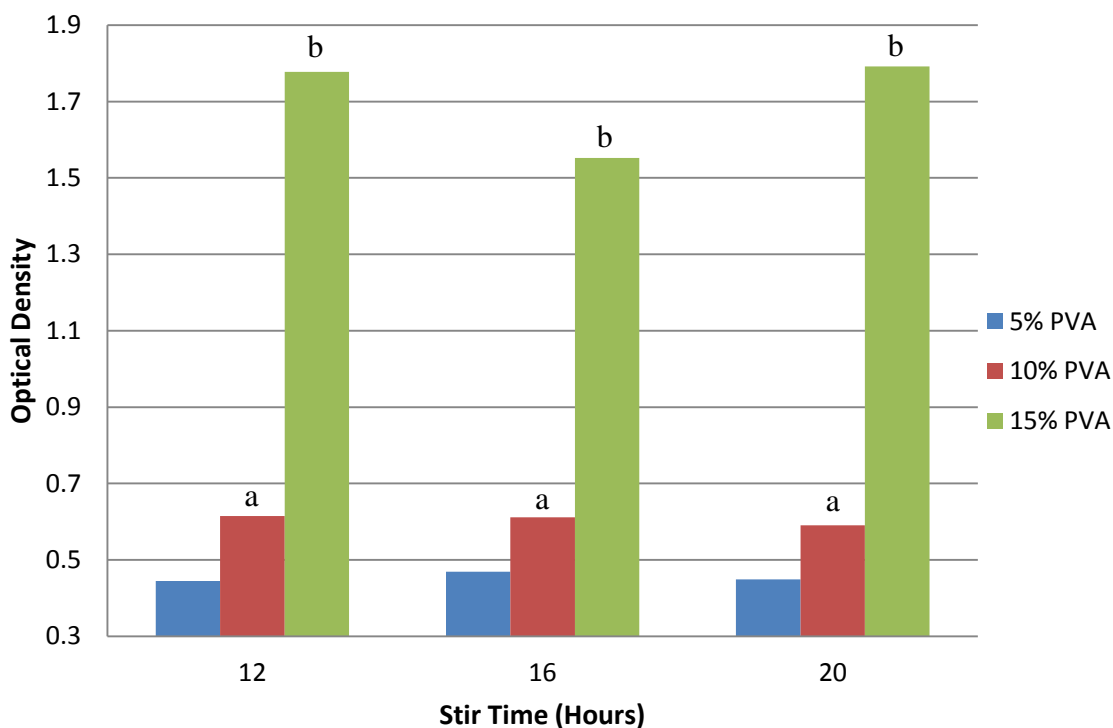
Figure 4.2. Effect of stir time and % (w/v) PVA on OVA encapsulation measured by the BCA Assay. There was significantly greater encapsulation of OVA for microparticles fabricated with 5% and 10% than 15% (w/v) PVA (p = 0.001), but there was no significant difference of encapsulation based on stir time (p = 0.961). Using Tukey Tests, statistically different treatment groups are labeled as 'a' and 'b' using p < 0.05.

The Macro Bradford Assay was completed on microparticles using fabrication

method 2. The concentration of PVA was varied from 1%, 5%, and 8% (w/v). The

concentration of OVA introduced in the supernatant was also varied from 40 µg/mL, 200

µg/mL, and 400 µg/mL. The absorbances from the assay were converted to

concentrations using a standard curve. The results were at the lower end of the working

range of the assay based on µg/mL as seen in **Figure 4.3**. Therefore, the micro assay was used to analyze other batches and encapsulation efficiencies were not calculated. The 8% (w/v) PVA was also partially gelled during the experiment so we believe that the calculated concentration was not accurate.
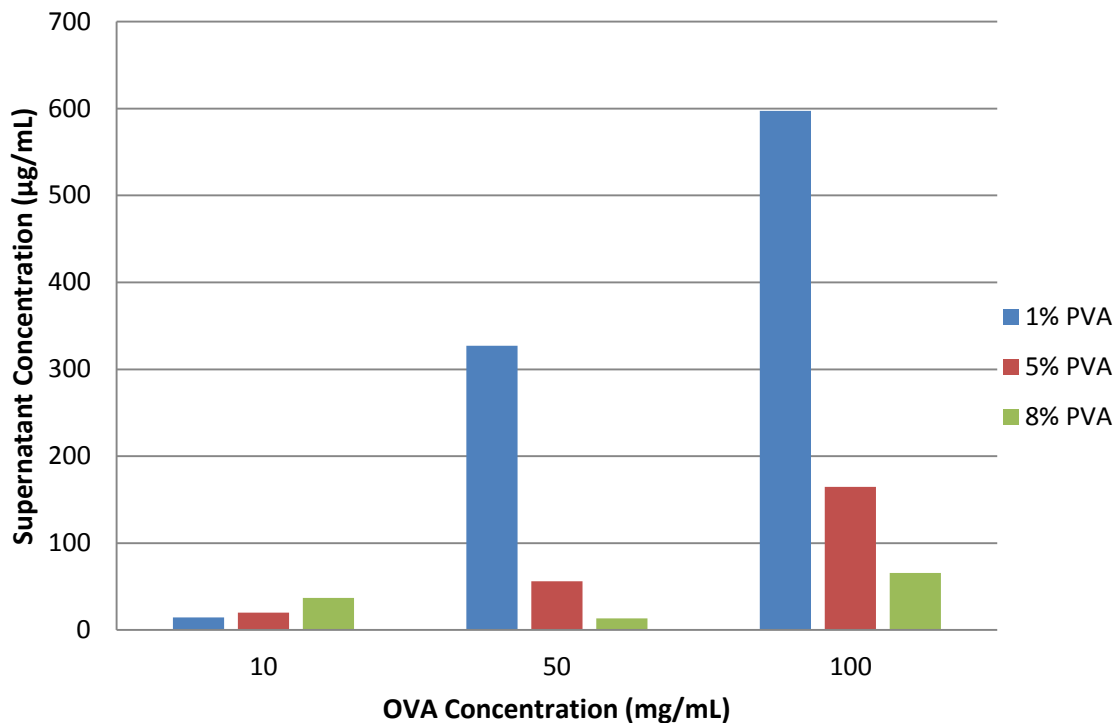
Figure 4.3. **Concentration of OVA in supernatant using the Macro Bradford Assay**. Microparticles were fabricated using Method 2 with 10% (w/v) PLGA and varying concentrations (w/v) % PVA. Lower OVA concentration in the supernatant implies that OVA was encapsulated in the microparticles.

Using the Micro Bradford Assay encapsulation studies were attempted on microparticles made with 1%, 5%, and 8% (w/v) PVA using fabrication Method 2. However, supernatants from microparticles fabricated with 8% (w/v) PVA solidified and could not be measured. We decided to continue with the 5% (w/v) PVA microparticles only.

There was a significant difference in encapsulation efficiency between microparticles made with 10 mg/mL and 100 mg/mL OVA (p = 0.0416) and between microparticles made with 50 mg/mL and 100 mg/mL OVA (p = 0.033). We concluded that microparticles made with 100 mg/mL OVA yielded the highest encapsulation rate of of 87.5 ± 15.2 %, while microparticles fabricated with 10 mg/mL and 50 mg/mL encapsulated only 59.1 ± 56.7 % and 55.4 ± 15.1 %, respectively (**Figure 4.4**).
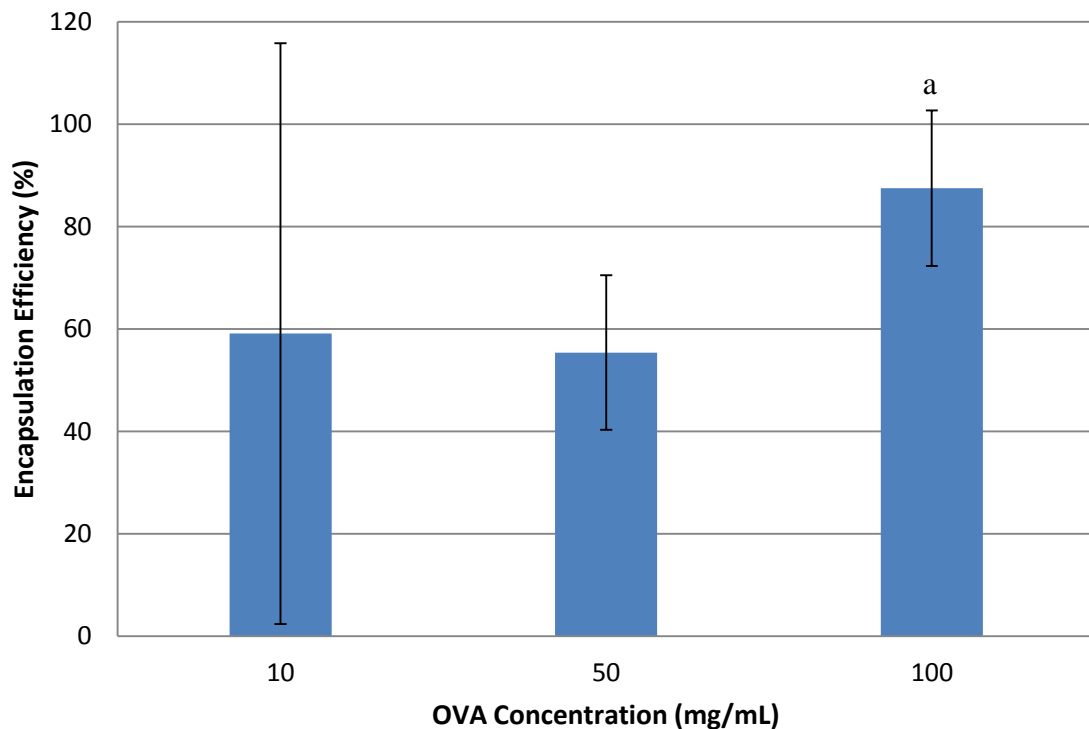
Figure 4.4. Encapsulation efficiencies of microparticles loaded with varying concentrations of OVA. Microparticles were fabricated using Method 2, with 10% (w/v) PLGA, 5% (w/v) PVA, and varying concentrations of OVA (10, 50, and 100 mg/mL). These values were calculated using the Micro Bradford Assay. There is a significant difference between the 10 mg/mL OVA and the 100 mg/mL OVA microparticles (p = 0.0416). There is also a significant difference between the 50 mg/mL and the 100 mg/mL OVA microparticles (p = 0.033). This is indicated using 'a'.

4.3.2 Release

The release study conducted over 14 days shows the amount of OVA released into the supernatant. To analyze the data, a standard curve was constructed using dilutions of OVA. The release profile was unusual since the assay indicated there was OVA in the unloaded microparticles. Therefore, a normalized graph was made to correct for the discrepancy in the zero point (**Figure 4.5**). There was no OVA in the 0 mg/mL OVA-loaded microparticles, so it was used as the baseline; therefore, calculated values for each time point were subtracted from the other corresponding values to get an overall difference. **Figure 4.6** shows the amount of OVA released compared to the total amount of OVA in the microparticles. This was calculated using the amount of OVA encapsulated into the microparticles for each batch (**Figure 4.4**) and the averaged weight of each batch. The average microparticle batch weight was 145.2 ± 53.1 g, 109.6 ± 54.7 g, and 124.1 ± 58.7 g corresponding to 10 mg/mL, 50 mg/mL, and 100 mg/mL. The released culminated at 14 days with the values of 0.0 ± 0.0 µg, 3.84 ± 0.003 µg, 3.51 ± 0.006 µg, and 2.03 ± 0.003 µg corresponding to 0 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL, respectively (**Figure 4.5**).
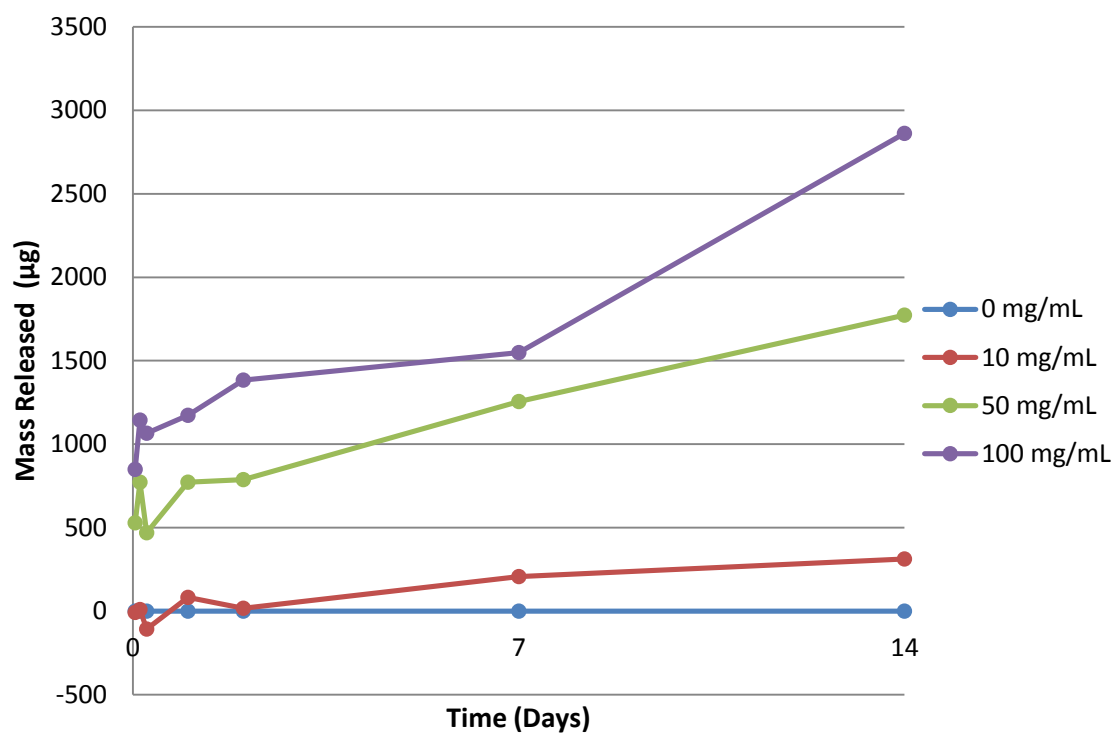
Figure 4.5. Release of OVA from microparticles over 14 days. Microparticles were fabricated using Method 2, with 10% (w/v) PLGA, 5% (w/v) PVA, and varying concentrations of OVA (0, 10, 50, and 100 mg/mL). Release data was normalized to the 0 mg/mL OVA-loaded microparticle control. The error bars are too small to visualize on the graph.
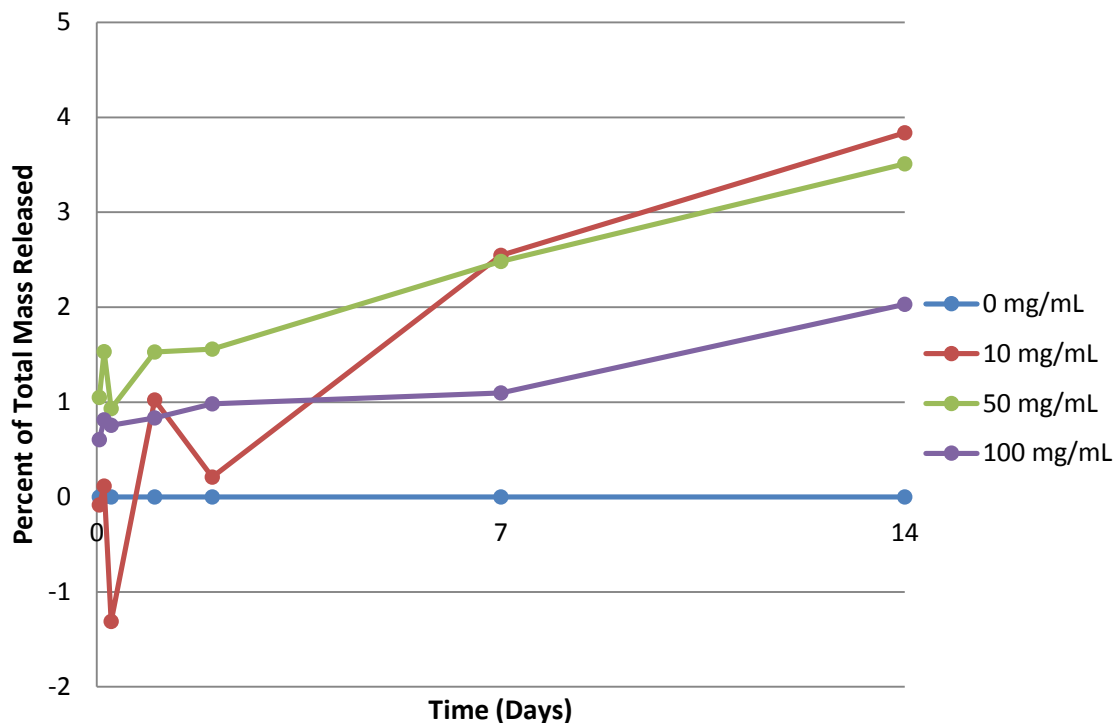
Figure 4.6. Percent release of total OVA from microparticles over 14 days.
Microparticles were fabricated using Method 2, with 10% (w/v) PLGA, 5% (w/v) PVA,
and varying concentrations of OVA (0, 10, 50, and 100 mg/mL). Release data was
normalized to the 0 mg/mL OVA-loaded microparticle control. The error bars are too
small to visualize on the graph.

4.3.3 Degradation

Degradation studies were completed over a 21-day period to observe the
degradation of PLGA over time. To analyze the data, two standards were created with
known polystyrene molecular numbers and run through GPC. Samples collected from
day 0 and day 21 time points for microparticles containing different concentrations of
OVA (0 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL) were then analyzed using these
standards to calculate the molecular number of PLGA in the samples. Similar degradation
rates were observed for the different OVA concentrations (**Figure 4.7**). The
polydispersity indexes for the samples on day 0 are: 2.04, 2.07, 2.11, 2.01, corresponding

to 0 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL of fabrication OVA concentration, respectively. The polydispersities for the samples on day 21 are: 2.17, 1.92, 1.88, and 1.86, corresponding to 0 mg/mL, 10 mg/mL, 50 mg/mL, and 100 mg/mL of fabrication OVA concentration, respectively.
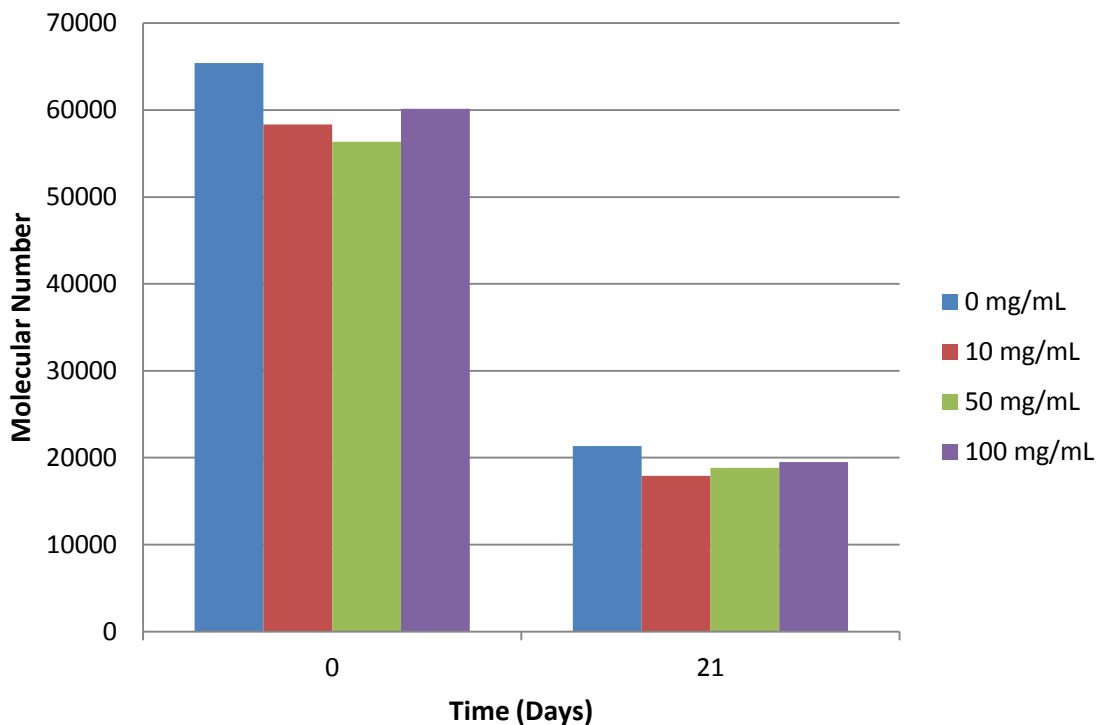
Figure 4.7. Microparticle degradation over 21 days. Microparticles were fabricated using Method 2, with 10% (w/v) PLGA, 5% (w/v) PVA, and varying concentrations of OVA (0, 10, 50, and 100 mg/mL). Similar degradation profiles were observed for each OVA concentration.

Chapter 5: Cell Response to Proteins, Microparticles, and Gardasil®

5.1 Objectives

Murine dendritic cells were used to evaluate the immunogenicity of microparticles. Several studies were conducted to compare unloaded, OVA-loaded and Gardasil®-loaded microparticle treatment groups to control groups, OVA alone and Gardasil® alone. Immune response of JAWSII dendritic cells was measured using a TNF-α assay, a cytokine released in response to antigen uptake and presentation by dendritic cells to T-lymphocytes.

5.2 Methods

5.2.1 Cell Culture

JAWS II cells (ATCC) were grown as semi-adherent cells in Alpha Minimum Essential Media (Life Technologies) with 4 mM L-glutamine (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldich), murine GM-CSF (PeproTech), and fetal bovine serum (FBS) (Life Technologies) as per the manufacturer's specifications at 37 °C and 5% CO_2 in T-25 culture dishes with 5 mL complete media. Once culture dishes were at least 80% confluent, cells were lifted from the culture dish using 0.25% trypsin-EDTA and plated into 96-well plates at $1x10^5$ cells/well.[67]

5.2.2 TNF-α Assay

The following solutions were mixed according to the protocol for mouse TNF-α assay kit (**Appendix E – TNF-α ELISA Protocol**) (Biolegend): Coating Buffer (8.4 g $NaHCO_3$, 3.56 g Na_2CO_3, add DI H_2O to fill one liter), Assay Diluent (10% FBS in PBS, PBS (8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add DI water to 1.0 L, pH

to 7.4), Wash buffer: 0.05% Tween-20 in PBS. After induction with each experimental treatment group for 24 hours, media was collected and the TNF-α kit was used, according to the protocol, to quantify TNF-α production. Absorbance was then read at 450 nm and 570 nm. To calculate TNF-α production in the cells, the absorbance from the standards was used to generate a quadratic standard curve. TNF-α production was then back calculated using the generated curve.

5.2.3 OVA Sensitivity

Several studies were conducted without the use of the microparticles or vaccine to first verify the precision and accuracy of the chosen cell culture method and TNF-α assay. Preliminary studies used OVA as the model protein.

Using the seeding density of $1x10^5$ cells per well, a range of OVA concentrations were tested in duplicate. For the first study, the cells were induced with solutions of 0, 0.1, 0.5, 1, 10, 50 and 100 mg/mL OVA dissolved in media. In the first study, the media was collected after 24 hrs and tested for TNF-α concentrations. The second OVA sensitivity study was conducted to test the lower bounds of OVA sensitivity using concentrations of 0.01, 0.1 and 0.2 mg/mL. Samples from the second study incubated for 36 hrs prior to performing the TNF-α assay on the cell media.

5.2.4 Response to Microparticles

In order to assess the immunogenicity of our vaccine delivery system, JAWSII TNF-α production was measured in response to incubating five treatment groups: OVA protein, unloaded PLGA microparticles, OVA-loaded PLGA microparticles, Gardasil®-loaded PLGA microparticles, and Gardasil® alone. JAWSII dendritic cells were plated in

96 well plates at 1×10^5 cells/well as per the manufacturer's recommendations and allowed to reach confluency. As determined from the OVA sensitivity study, an OVA concentration of 0.05 mg/mL was deemed appropriate as the primary determinant of the final concentration of each treatment group. All treatment groups were placed into solutions of complete media and kept sterile. Once confluent, the media was removed from each well and replaced with 200 μL of each treatment in media. Following a 24-hour incubation period, the media was collected for a TNF-α ELISA assay and stored at -20 °C until ready for use.

5.3 Results

5.3.1 OVA Sensitivity

As shown in **Figure 5.1**, the cells induced with 100 mg/mL OVA produced the highest TNF-α response (669.34 ± 25.20 pg/mL TNF-α). The next highest response was observed in cells induced with 50 mg/mL OVA (396.35 ± 218.65 pg/mL TNF-α) followed by 0.1 mg/mL OVA (375.24 ± 295.14 pg/mL TNF-α). The three lowest responses were observed in cells induced with 0.5 mg/mL OVA (171.08 ± 37.37 pg/mL TNF-α), 1 mg/mL OVA (214.10 ± 21.04 pg/mL TNF-α), and 10 mg/mL OVA (179.83 ± 130.22 pg/mL TNF-α). The untreated media did not produce a measurable response of TNF-α.
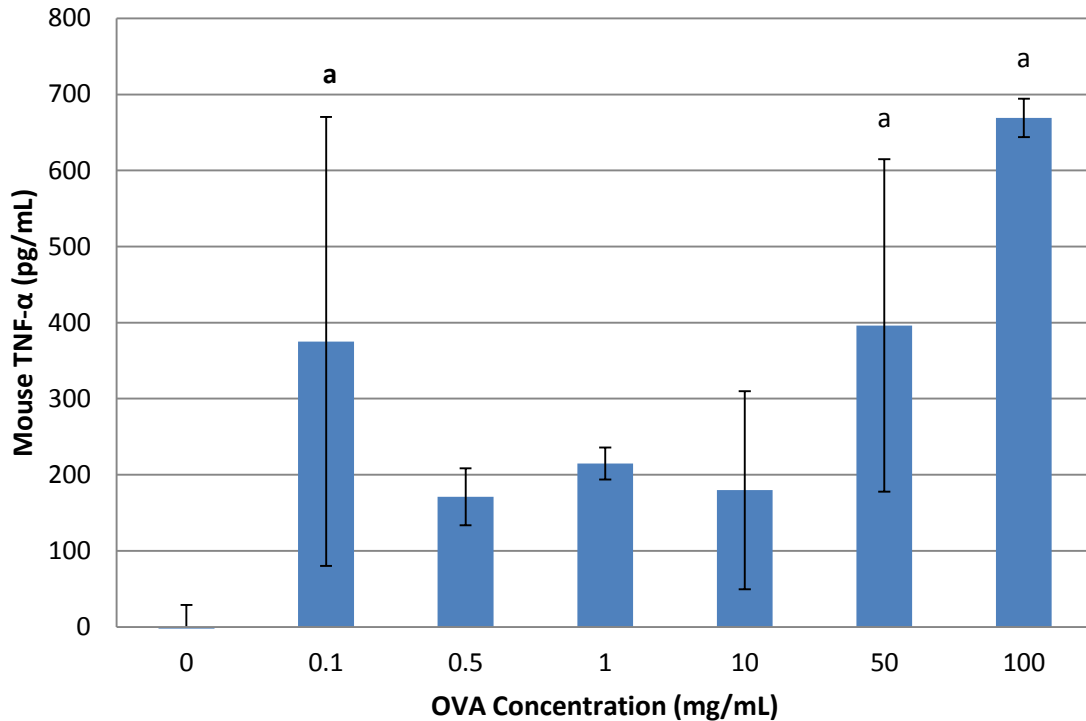
Figure 5.1. TNF-α production of the OVA Sensitivity Study 1. JAWSII TNF-α production in response to induction with 0, 0.1, .5, 1, 10, 50 and 100 mg/mL OVA. Using an ANOVA and Tukey Tests, treatment groups statistically different from the control are labeled as 'a' using $p < 0.05$.

The TNF-α production resulting from the second study is shown in **Figure 5.2**. Concentrations of 0.05, 0.5 and 1 mg/mL OVA produced 323.98 ± 39.79 pg/mL TNF-α, 390.06 ± 55.92 pg/mL TNF-α, and 434.13 ± 163.20 pg/mL TNF-α. The calculated response from untreated media was 23.36 ± 5.26 pg/mL TNF-α. Each concentration of OVA used was determined to be statistically different than the control, including the response from 0.05 mg/mL OVA ($p = 0.015$). There was not a statistically significant difference found between each concentration.
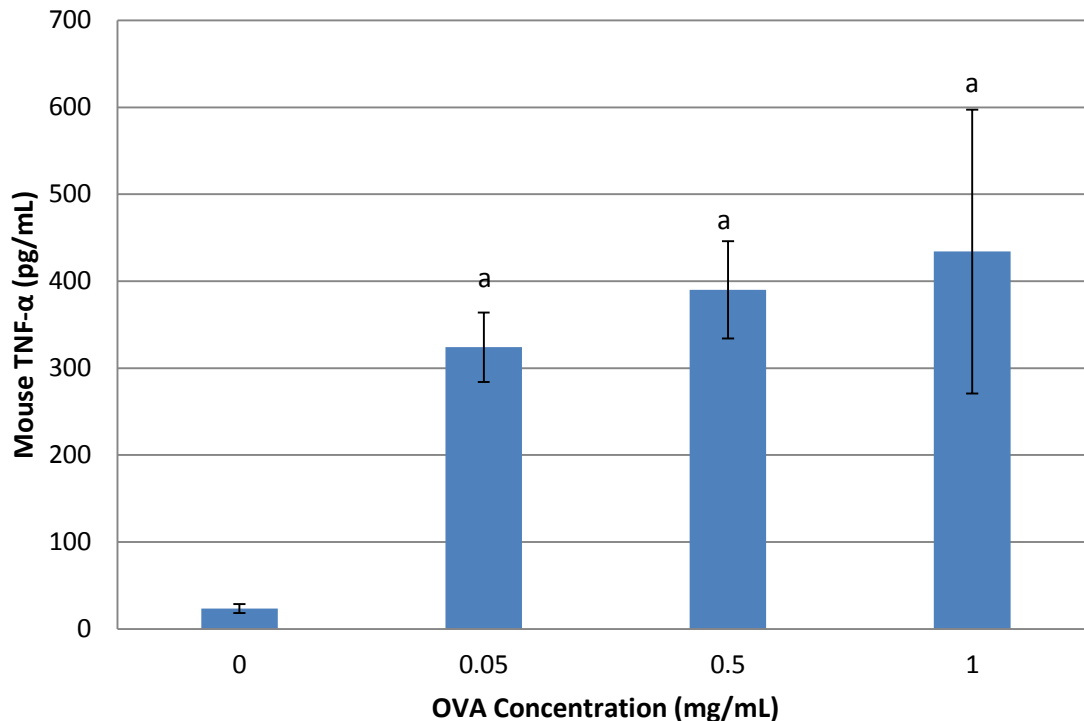
Figure 5.2. TNF-α production of the OVA Sensitivity Study 2. JAWSII TNF-α production in response to induction with 0, 0.05, 0.5, and 1 mg/mL OVA. Using ANOVA and Tukey Test, OVA concentration groups 0.05, 0.5, and 1 mg/ml are statistically different from the control (p = 0.0147, p = 0.00462, p = 0.00201, respectively) and are labeled as 'a' to indicate p < 0.05.

5.3.2 Response to Microparticles

Figure 5.3 shows the TNF-α production in response to media alone, OVA alone, unloaded microparticles, OVA-loaded microparticles, Gardasil®-loaded microparticles, and Gardasil® alone. OVA-loaded microparticles were able to elicit the largest concentration of TNF-α (698.7 ± 0.2 pg/mL TNF-α) followed by OVA protein control (572.4 ± 0.2 pg/mL TNF-α). Unloaded PLGA microparticles, Gardasil®-loaded PLGA microparticles, and Gardasil® control treatment groups elicited similar cellular production of TNF-α (111.4 ± 0.5 pg/mL, 101.5 ± 0.4 pg/mL and 15.37 ± 0.04 pg/mL TNF-α, respectively. The results also show that Gardasil®-loaded microparticles produce

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a cellular response that is statistically similar to that of unloaded microparticles.
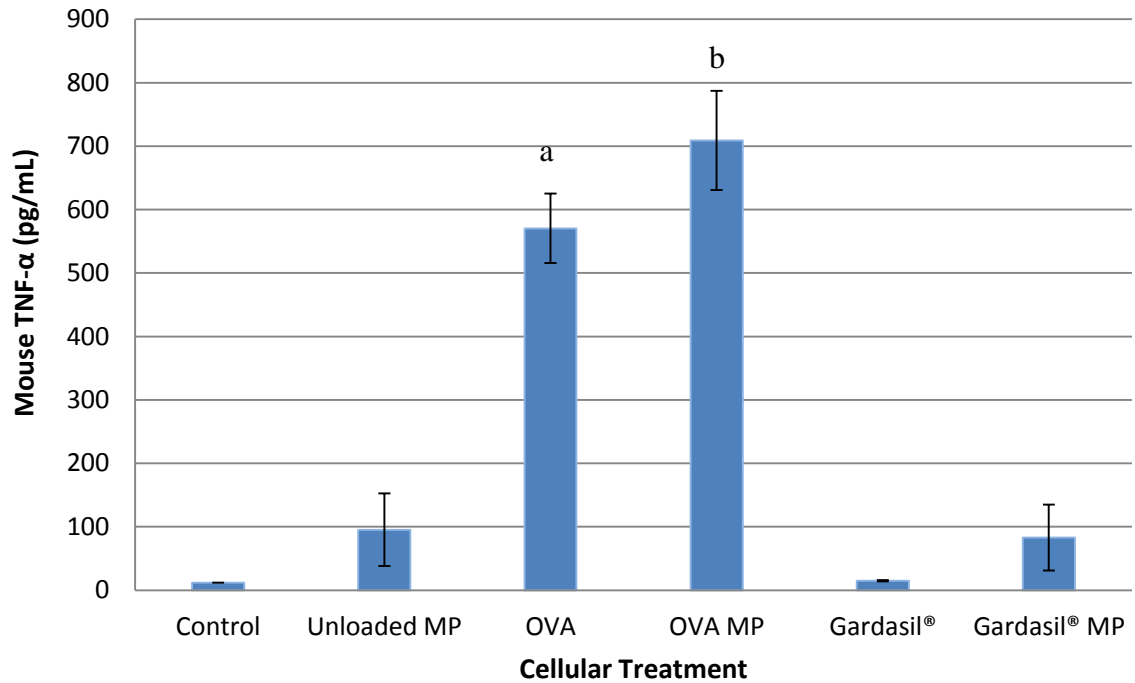
Figure 5.3. TNF-α production in response to various microparticle (MP) treatments. Using ANOVA and Tukey Test, statistically significant treatment ($p < 0.05$) groups are depicted in this graph using 'a' and 'b'.

Chapter 6: Discussion

The overarching question guiding our research was: To what degree can a controlled-release, poly(D,L-lactide-co-glycolic acid) microparticle vaccine delivery system induce an immune response to HPV virus-like particles, the antigenic component of the HPV vaccine? This broad research question was then divided into more specific questions. The first question asked which parameters in microparticle synthesis will yield microparticles in the desired range of 1-10 μm. The second question inquired which microparticle synthesis parameters will yield the desired encapsulation efficiency without sacrificing the desired size. The third question related to determining the release and degradation profiles of the microparticles. The fourth question asked if the protein-loaded PLGA microparticles induce an immune response in murine dendritic cells and how does this response compare to the response induced by protein alone. Fifth, we questioned if the vaccine-loaded PLGA microparticles induce an immune response in murine dendritic cells and how does this response compare to the response induced by the Gardasil® HPV vaccine.

6.1 Microparticle Fabrication and Characterization

Microparticle characterization answered the research question regarding which parameter variations in microparticle synthesis will yield microparticles with a diameter of 1-10 micrometers. The parameters affecting size were determined through light microscopy and laser diffraction. Using light microscopy and a MATLAB program, samples stirred for 19, 21, and 23 hours showed similar size distributions, while microparticles stirred for 17 hours were noticeably smaller (**Figures 3.4** and **3.5**).

However, all four plots showed a similar overall shape, with most microparticle diameters 20 µm or less. The average microparticle size was in the desired size range. However, the distribution was not uniform due to the chosen magnification. For this reason, laser diffraction was used to sample a larger population of microparticles in order to determine microparticle size distribution.

The opaqueness of the microparticles formulated with Gardasil® in **Figure 3.6** suggests that they were loaded. Although at this stage it was uncertain whether the microparticles were loaded with HPV vaccines. Despite this image not being used to calculate a microparticle size distribution, we observed that the microparticles were larger (approximately 80 µm in diameter) than those loaded with OVA.

Using laser diffraction, we were able to collect a more accurate size distribution by sampling a larger number of microparticles in solution (**Figures 3.7** and **3.8**). In **Figure 3.7**, we observed that, regardless of stir time, synthesis of 3% (w/v) PLGA and 10% (w/v) PVA microparticles using fabrication Method 1 yielded microparticle diameters close to our desired size target of 10 µm. Using an ANOVA test, we observed a significant difference (defined as $p < 0.05$) between at least one pair of time points ($p = 0.007$). However, because a Tukey test showed no significant difference between any of the pairs of time points, we concluded that stir time was not a significant factor in determining microparticle size.

We also used laser diffraction to evaluate the effect of percent (w/v) PVA (**Figure 3.9** and **3.10**) on the diameters of microparticles fabricated using Method 1. We observed a statistically significant decrease in microparticle diameter as stir time increased ($p = 0.001$), which contradicted our previous results that stir time did not affect microparticle

size. We also observed a statistically significant decrease in microparticle diameter as percent (w/v) PVA increased (p = 0.001). However, although these p-values were identical, we observed by visual inspection that percent (w/v) PVA had a stronger effect on microparticle size than stir time did. In addition, as **Figure 3.10** demonstrates, it was only with the microparticles synthesized with 5% PVA that showed a definite effect between different stir times and mean diameter, thus heavily affecting the calculated p-value. Another general observation was that the microparticle distributions were narrower as the mean size decreased. Again, all percentages of PVA yielded microparticles near our desired size target of 10 μm in diameter.

Using fabrication Method 2, we observed a greater overall microparticle size, without percent (w/v) PVA or concentration of OVA causing a trend in microparticle size (**Figure 3.11** and **3.12**). Overall, as mentioned, there was no significant trend of the mean diameters of the microparticles synthesized with the various concentrations of OVA (p = 0.953) or percent (w/v) PVA (p = 0.990). However, we were willing to sacrifice control of microparticle size for shorter fabrication times and higher encapsulation rates, as further explained in Chapter 6.

6.1.2 Limitations

One way to improve the synthesis of microparticles would have been to utilize a probe sonicator or homogenizer to create each emulsion.[19] This would have decreased both the average microparticle diameter and the range of microparticle diameters observed using laser diffraction. During this study, we used a bath sonicator, which sends sound waves to the outside of each emulsification. A probe sonicator would have been able to homogenize each emulsification more thoroughly by sending sound waves

directly to it. Furthermore, during the stage of loaded microparticle synthesis, it was difficult to determine if (and how much) the microparticles were loaded with either OVA or Gardasil® because we were still in the process of trying to determine the best method of determining encapsulation rate.

6.2 Microparticle Encapsulation, Release, and Degradation

The encapsulation studies answered the research question: which parameter variations in microparticle synthesis will yield protein-loaded microparticles of appropriate size, while maintaining high encapsulation efficiency? The factors affecting encapsulation efficiency are PVA concentration and OVA concentration in the original formulation. The degradation and release study answers the research question: Will the degradation and release profiles of protein-loaded microparticles be comparable to that of a multi-dose vaccine? All of the formulations of microparticles had a uniform slow release. The fastest release profile that could be extracted is from the 10 mg/mL with 5% PVA microparticles that would deliver 100% of the contents in 1.00 years.

6.2.1 Encapsulation

For the direct encapsulation method, in which we measured the amount of OVA loaded into the microparticles directly by using organic solvents to dissolve the microparticles, we obtained inconsistent results (**Figure 4.1**). For example, our data suggested that over 100% loading efficiency was found when acetonitrile was used as the organic solvent. This result may be due to the presence of polymer in the aqueous layer.

Problems with the direct method using DMSO also arose with repeated studies. Since follow-through studies were unsuccessfully able to resolve this issue, we were led

to eventually reject the direct method as a reliable and accurate measurement of microparticle encapsulation and thus decided to measure encapsulation efficiency indirectly by measuring the amount of OVA left in the supernatant after the microparticles were made and back calculating to the amount of OVA that was encapsulated within the microparticles.[74,75]

For the BCA Assay as seen in **Figure 4.2,** there was no noticeable change in encapsulation over time, but there was a change in encapsulation efficiency with varying concentrations of PVA in the fabrication technique. It was concluded that 15% PVA had a significantly lower concentration than 10% and 5% PVA ($p = 0.001$). Encapsulation efficiency increasing as the PVA in the fabrication process decreased was also observed in a study on A-PLGA microspheres.[76] Therefore, concentrations of PVA higher than 15% were not further used in order to maximize the encapsulation efficiency of the microparticles. The BCA assay was able to show relative differences among the different formulations of microparticles; however, when encapsulation rates were calculated, they were extremely low (data not shown). Therefore, we began using Method 2 to fabricate microparticles in order to increase encapsulation rates.

Furthermore, the Bradford assay is a commonly accepted method like the BCA assay. The range of the Macro Bradford assay is from 125 μg/mL to 2000 μg/mL of protein. The majority of data gathered from this study was too low to accurately use the standard curve to analyze. The data mostly resulted in noise using this assay. This issue caused us to switch to the Micro Bradford assay, which has an optimal reading range of 1 μg/mL to 25 μg/mL, but it can be extended up to 125 μg/mL with the appropriate standards. The data that was in the reading range of the Macro Bradford assay, the 1%

PVA, showed very high amount of OVA in the supernatant (**Figure 4.3**), which indicates a low amount of OVA in the microparticles. Therefore, 1% PVA is not optimal for the microparticle formulation process.

For the Micro Bradford Assay, the initial OVA concentration formulation was compared (**Figure 4.4**). This study was conducted to maximize encapsulation using the 3 different OVA concentrations in the fabrication step (10 mg/mL, 50 mg/mL, and 100 mg/mL). The 100 mg/mL yielded the highest encapsulation efficiency as compared to the 10 mg/mL and 50 mg/mL (p = 0.0416 and p = 0.033, respectively). This showed that using a higher concentration of OVA led to higher efficiencies in encapsulation. Therefore, a higher concentration of VLPs than what is present in a dose of the Gardasil® vaccine may need to be used to obtain sufficient encapsulation within the microparticles.

6.2.2 Release

The release profile of the OVA from the microparticles (**Figure 4.5**) did not result in a typical release profile from a biodegradable polymer with a burst release followed by a slow increasing release of the contents.[77,78] The curve slowly rose over the first week, without a noticeable burst release. The lack of burst release is ideal for a vaccine. A desired profile for release would be if the VLPs slowly release rather than expel all contents at once. Therefore, the microparticle formulation tested is ideal for creating a slow release profile over 14 days. In addition, the maximum amount of OVA released into the solution was 3.84 % ± 0.006 % of the total OVA in the microparticles as seen in **Figure 4.6** for 10 mg/mL OVA concentration in the formulation. This release profile seems to be steadily releasing OVA into the solution at a very slow rate. If there is constant, steady release 100% of the contents would be release in 1.00 years. This

characteristic would be ideal for a vaccine to allow a slow controlled release of the VLPs. A study conducted using a similar fabrication protocol with PLGA showed a trend between increased PVA concentrations in the fabrication process lead to slower release profiles.[79] This study used a maximum PVA concentration of 0.1% to achieve a slower release profile; in our study we used 5% PVA, which would create a drastically slower release profile following the same trend. Therefore, it can be predicted that our microparticles will release slower than their microparticles, which at about 9 days achieved 10% of protein released.[79] Our data was consistent with their trend.

6.2.3 Degradation

For the microparticle degradation studies, we expected to observe a significant reduction in PLGA molecular number over the 21 day period, coinciding with the OVA release observed in the release studies. We did not expect degradation rates to vary based on the concentration of the loaded protein. The reduction of PLGA molecular number shown in **Figure 4.7** confirms these expectations. Degradation rates were similar between differing OVA concentration, with PLGA molecular number being reduced by greater than a factor of 3 over the 21 day study period.

6.2.4 Limitations

The standard for administering the three doses of HPV vaccines is over 6 months. The study conducted for release was 14 days and degradation was only 21 days with OVA. Therefore, the VLP loaded microparticles need to be tested *in vivo* to determine the exact release profile. The microparticle formulation may need to be altered slightly to get

a shorter release period. Therefore, a definitive conclusion cannot be made regarding how the release profile would be in humans.

6.3 Cellular Response to Proteins, Microparticles, and Gardasil®

The cell response studies answered research question 4: Will protein-loaded PLGA microparticles induce an immune response in murine dendritic cells and how does this response compare to the response induced by protein alone. The protein-loaded PLGA microparticles did produce an immune response. The cell studies also answered research question 5: Will vaccine-loaded PLGA microparticles induce an immune response in murine dendritic cells and how does this response compare to the response induced by the Gardasil® HPV vaccine. The vaccine-loaded microparticles did produce an immune response and the microparticles increased the immune response than just the vaccine alone.

6.3.1 OVA Sensitivity

For the OVA sensitivity studies, we expected that if the OVA concentration was within a range above minimum detection levels and below saturation, the cells would produce an increasing response with increasing OVA. However, the TNF-α production shown in **Figure 5.1** did not produce such a response. The cells induced with 0.5 mg/mL OVA showed a lower TNF-α response than the cells induced with 0.1 mg/mL OVA, and the 1 mg/mL samples produced more than the 10 mg/mL samples. These results could be explained by high variance within samples. For this reason, a second sensitivity study was conducted. Since it appeared that even 0.1 mg/mL was able to produce a TNF-α

response, which was within the range of the assay, the second study used even lower concentrations.

The results of the second OVA sensitivity study were more conclusive than the first sensitivity study. TNF-α production calculated using the shown in **Figure 5.2** for the second OVA sensitivity study had much lower standard deviations than was seen in the first sensitivity study. TNF-α production appeared to be saturated for all concentrations tested. Most importantly, the data shows that even a 0.05 mg/mL OVA solution is able to induce a TNF-α response of 323.98 ± 39.79 pg/mL. Because this concentration was demonstrated to be sufficient, the following studies conducted using microparticles aimed to use an encapsulated OVA concentration of 0.05 mg/mL.

6.3.2 Response to Microparticles

One study was conducted using all treatment groups: OVA, unloaded microparticles, OVA-loaded microparticles, Gardasil® alone, and Gardasil®-loaded microparticles. Statistically significant TNF-α production ($p < 0.05$) in response to these groups was observed, but more studies will need to be conducted in order to verify these results.

Furthermore, we opted to use the HPV vaccine, Gardasil®, as the source of VLPs as compared to the pure VLPs. Since the vaccine also contains adjuvant components, we expected that the vaccine treatment groups would stimulate a greater immune response in comparison to the OVA treatment groups. In sharp contrast, the OVA control and unloaded microparticle treatment groups produced a greater TNF-α response than the Gardasil® control and Gardasil®-loaded microparticles ($p < 0.05$). The OVA control resulted in 572.37 ± 90.46 pg/mL TNF-α produced and the unloaded microparticles resulted in 111.39 ± 53.91 pg/mL TNF-α produced. In comparison, the Gardasil® control

and Gardasil®-loaded resulted in 15.37 ± 0.59 pg/mL and 101.48 ± 39.25 pg/mL TNF-α produced, respectively.

These results can best be explained by the fact that HPV VLPs have a much higher molecular weight than the OVA protein, so fewer VLPs were needed to make up the same concentration. Moreover, only 40 µg VLPs are in 500 µL of the vaccine solution. Since only 800 µL of the vaccine was used in the fabrication of Gardasil®-loaded microparticles, less than 80 µg VLPs were incorporated into the entire batch of microparticles. Unlike the OVA-loaded microparticles, we did not conduct encapsulation analysis on the Gardasil®-loaded microparticles. Therefore, the amount of VLPs in the microparticles and released from the microparticles could not be estimated. Likely, the fewer number of VLPs reduced the interactions between the cells and VLPs in comparison to the interactions between the cells and OVA, resulting in a milder immune response.

6.3.3 Limitations

JAWSII dendritic cells were used to effectively compare the immune response to OVA, microparticles, and Gardasil®. JAWSII and measurement of TNF-α production were indicative of the innate immune response rather than the adaptive immune response. Vaccines stimulate the adaptive immune response, but since the innate immune response is essential for a functional adaptive immune response, the results from this work can be used to predict future work involving the adaptive immune response.

Though these studies address the immunogenicity of the microparticle formulation, these studies do not address the time-course over which the microparticles were intended to release the antigen. This is because the incubation time of the treatment

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groups with the cells was standardized to 24 hours, a much shorter time than the dosage regimen of the actual HPV vaccine. More work will need to be done to investigate the microparticles' immunogenicity in combination with controlled release over an extended time course.

6.4 Future Directions

Our team has multiple recommendations for extending this project. First, a co-culture of B-cells and T-cells could be utilized to measure antibody production against VLPs alone and VLP-loaded microparticles. B-cells and T-cells are involved in active immunity, which is what vaccines stimulate. Another advantage of this method is the ability to measure specific antibody production against VLPs instead of cytokines indicative of a general inflammatory response. After this second *in vitro* cell study, the ultimate goal would be to test our vaccine delivery system *in vivo* in a mouse model, testing for antibody production against the VLPs in the mouse.

Additionally, in order to fully mimic the immunostimulatory profile of existing HPV vaccines, a three-component, single-dose vaccine containing VLPs with an adjuvant and two different VLP-loaded microparticle populations could be developed. The VLPs alone would stimulate the immune system immediately, while the microparticle formulations could be manipulated to release at three months and at six months. Using this method would prevent immunotolerance, the phenomenon that occurs when the immune system is subjected constantly to the same antigen and eventually doesn't respond to it as well as expected.

Chapter 7: Conclusion

Our studies show that protein encapsulated in microparticles can be delivered over a long period of time and elicit an immune response, indicating that VLP-loaded microparticles could be used to eliminate the need for a multi-dose HPV vaccine. We were successful in addressing our research questions concerning size, encapsulation, controlled release, and cellular response. The microparticles fabricated with 10% and 15% PVA using Method 1 produced microparticles with a mean diameter ranging from 3-12 μm, near the target of <10 μm as required for dendritic cell uptake. Although increasing PVA decreased the size of our microparticles, increased concentrations of PVA negatively affected encapsulation leading to the development of Method 2. With 5% PVA and a 100 mg/mL OVA solution, we were able to achieve high encapsulation of 87.5 ± 15.2%. A release study using OVA-loaded microparticles fabricated with Method 2 was able to gradually release protein over the course of our 14-day study. Since less than 4% of the measured encapsulation protein was released at this time, this indicates that the microparticles are likely to keep releasing well beyond that time and possibly reach the desired six months of release or longer. The cellular uptake results indicated that cells induced with OVA- or Gardasil-loaded microparticles were able to produce a higher immune response than cells induced with OVA or Gardasil alone, suggesting that the microparticles could also be acting as an adjuvant. Overall, these results help substantiate the hypothesis that controlled-release microparticles could be used to formulate a single-dose HPV vaccine.

In this study, the effects of stir times and concentrations of PVA and OVA were examined in PLGA microparticles synthesized using two different methods. It was shown

that stir time did not have a significant effect on microparticle size, while the percentage of PVA did. In addition, the concentration of OVA did not have a statistically significant effect on the microparticle size. In terms of stir times, within the 13 to 23 hour stir time, all microparticles were around 12 μm in mean microparticle diameter. Contrarily, as the concentration of PVA in the aqueous phase increased, it was shown that there was a significant decrease in the microparticle size. As well, a number of previous reports demonstrated that by increasing the PVA concentration in the external aqueous phase, the size of the nanoparticles decreases.[80–82]   Lastly, no trend was observed between concentration of OVA and microparticle size. Future work should consider much shorter stir times during the synthesis of the microparticles and its effect on microparticle size.

Furthermore, the release profile of the microparticles was ideal for a slow controlled-release vaccine, only releasing a maximum of 3.8% in the first 2 weeks. The majority of microparticles synthesized by others have a burst release.[79]   This release profile may even be too slow for the vaccine, which ideally would have 100% release by week 24 (6 months). This release profile was for OVA-loaded microparticles in distilled water. Therefore, the release profile may be altered when tested *in vivo* with the VLPs. The degradation rate may also be altered when tested *in vivo*. The molecular weight and molecular number decreased by 2/3 over 21 days. This degradation rate was rapid suggesting that the microparticle formulation may need to be altered.

Additionally, the release profile is heavily dependent on the encapsulated model protein. For our studies, we used OVA rather than using the VLPs. There is a significant difference in size between OVA and VLPs, which may affect both encapsulation and

release. Further studies need to be done to determine the release profile of the VLP loaded microparticles.

Murine dendritic cells were used to evaluate the immunogenicity of the microparticles. TNF-α production in response to microparticle, vaccine, and control treatment groups was measured as a means of quantifying the innate immune response. Cells produced TNF-α in response to unloaded microparticles, attesting to the efficacy of microparticles as a vaccine adjuvant. Future work should consider utilizing different cell lines, such as macrophages and T-cells to investigate the adaptive immune response, which is the branch of the immune response that reacts to vaccines. Additionally, the time-course of cell studies should be designed to match the release and degradation profiles of the microparticles, which should represent the time-course of the typical dosage regimen of the vaccine.

Overall, our study added to the growing body of knowledge about the viability of microparticles for vaccine delivery systems used for HPV by using OVA as a model protein. By manipulating parameters of the double emulsion fabrication method, we were able to achieve a mean microparticle size diameter of 12 μm, just above our 10 μm target. By further optimizing PVA concentration or possibly using a homogenizer, further research could develop a synthesis method with more microparticles in the size range needed for dendritic cell uptake. We were also able to achieve a high encapsulation, with 88% efficiency for microparticles fabricated with a 100 mg/mL OVA solution. These microparticles were able to demonstrate a prolonged release over the course of 14 days, without appearing to have a significant burst release. Finally, OVA-loaded microparticles were able to produce a stronger immune response than OVA alone as measured by TNF-

α production. Microparticles loaded with Gardasil, however, did not produce a significantly different amount of TNF-α than unloaded microparticles. The methods investigated for fabrication of microparticles loaded with OVA and characterizing their size, encapsulation, release and immunogenicity will reduce the amount of trial and error required to achieve an optimal microparticle formation of HPV VLP loaded microparticles for controlled-release vaccine delivery.

Appendices

Appendix A – Microparticle Fabrication: Method 2 Protocol

<u>Preparing the % (w/v) PLGA Solution</u>
Materials: 2 autoclaved test tubes (per batch), glass pipette, aluminum foil, parafilm, test tube rack, PLGA, DCM
    (1) Weigh out 100 mg PLGA and place in glass test tube.
    (2) Repeat step 1 for 2 test tubes total.
    (3) Add 1 mL DCM in chemical hood (use glass pipette or glass syringe). Do not vortex or mix. Leave overnight in the hood to allow the PLGA to dissolve overnight.
    (3) Cover the glass test tubes with small squares of aluminum foil.
    (4) Cover the aluminum foil with parafilm and create a tight seal. Avoid having the parafilm exposed directly to the DCM solution.
    (5) Before using place the test tubes under the sterile hood UV light for 15 minutes to disinfect.

<u>Preparing the % (w/v) PVA Solution</u>
Materials: PVA, 500 mL bottle, water, stir bar, heat/stir plate
    (1) Heat 500 mL Millipore water in bottle (set at ~130°C on the heated stir plate)
    (2) Weigh out 25 g PVA.
    (3) Wait until the water has reached ~70°C (maintain the temperature between 70-80°C throughout the process).
    (4) While stirring the water with a magnetic stirbar, slowly sprinkle in the PVA.
    (5) Set the temperature at ~180°C and allow the solution to stir while covered with aluminum foil or a lightly placed bottle cap on bottle.
    (6) Once the solution is clear and the PVA has dissolved, take the bottle off of the stir plate and let it cool to room temperature.
    (7) If a significant amount of water has evaporated, refill the bottle to the 500 mL line with more water.
    (8) Store the PVA solution in a glass bottle until needed.
    (9) Autoclave PVA solution in a glass bottle using liquid 9 setting.

<u>Preparing the OVA Solution</u>
Materials: OVA, distilled water, two 15mL centrifuge tubes, 3mL syringe, 18 gauge needle, green microfilter
    (1) Weigh out 300 mg OVA into 15 mL plastic centrifuge tubes.
    (2) Add 1.5 mL distilled water and vortex the solution to dissolve and mix the OVA.
    (3) Sterilize the solution.
        (a) Use 18 gauge needle and 3 mL syringe to extract the OVA solution into syringe the syringe.
        (b) Replace the needle with the microfilter while the solution is still in the syringe.
        (c) Expel the solution through the microfilter into a new and sterile 15 mL centrifuge tube.

Mixing the OVA Solution into the PLGA/DCM Solution
Materials: sonicator, ice, OVA solution, PLGA/DCM solution, micropipette
    (1) Fill the bath sonicator ¾ full with water and ice.
    (2) While in hood, add 400uL OVA solution into each glass test tube containing the PLGA/DCM solution.
    (3) Cover the glass test tube and vortex the resulting solution 2-3 times for 5 seconds each until the solution turns opaque.
    (4) Sonicate the test tubes in the ice water sonicator for 2 minutes.

Mixing the OVA/PLGA/DCM Mixture into the PVA Solution
Materials: sonicator, ice, 500mL beaker, stir bar, micropipette, stir plate
    (1) Add 200 mL PVA solution to a new and autoclaved beaker with a stir bar in the hood.
    (2) Add 3 mL PVA solution to the OVA/PLGA/DCM glass test tube and vortex immediately until it turns opaque and contains particles within it.
    (3) Repeat step 2 for the other test tube.
    (4) Sonicate the glass test tubes in the ice bath sonicator for 2 minutes.
    (5) Vortex each glass test tube for 10 seconds.
    (6) Sonicate the glass test tubes in the ice bath sonicator for another 2 minutes
    (7) Vortex each glass test tube again for 10 seconds.
    (8) Using sterile pipettes in the hood, add 3 mL PVA solution to each test tube.
    (9) Pour contents of the two tubes into the 200mL 5 % (w/v) PVA beaker while it's stirring at 360 RPM.
    (10) If necessary, use the sterile pipette to wash the excess solution from the test tube into the beaker.
    (11) Allow the solution to stir for 4 hours uncovered.

Washing and Collecting the Microparticles
Materials: 50mL Falcon tubes, micropipette, microcentrifuge tubes, electric pipette, plastic pipettes, sterilized distilled water, sterile exacto knife, sterile tube for collected particles
    (1) Turn off the stir plate and remove the beaker.
    (2) Pour the contents into 50 mL Falcon tubes up to the 45mL line.
    (3) Centrifuge the Falcon tubes at 1000g for 15 min with ramp down of 9 and ramp up of 9.
    (4) Remove 1 mL supernatant from each Falcon tube and save it in labeled microcentrifuge tubes for later encapsulation analysis.
        (a) Store the supernatants in the freezer.
    (5) Remove the remaining supernatant and discard it as waste.
    (6) Fill each Falcon tube with 45 mL sterile, distilled water.
    (7) Invert the tubes until the microparticle pellet has dispersed into the water.
    (8) Centrifuge the Falcon tubes at 1000 *xg* for 15 minutes.
    (9) Remove and discard the supernatant.
    (10) Repeat steps 6-9 twice more, for a total of 3 washes.
    (11) After the last wash, leave a small volume of water, ~1mL, to help transfer particles into one sterile and labeled Falcon tube.

(12) Cut the tip of a 1000 µL pipette tip with an exacto knife.
(13) Resuspend the microparticles into the water remaining in the Falcon tube and transfer the microparticles into the new and labeled Falcon tube.
(14) Flash freeze the Falcon tube containing the microparticles with nitrogen and lyophilize the microparticles for later use.

Appendix B – MATLAB Sizing Program

% Matlab Size Code

```
clear all
clf
clc
close all
format compact

% This is a sample of the matlab code used to calculate size of the microparticles based
% on microscope images

%% Loading Image File
root=['C:\Documents and Settings\rez\My Documents\Other\']; % Folder Name with a
backslash % at the end
for i=1:1 % put 1: however many images you took
  name=['PLGA_in_PBS_40x_3-8-2013_',num2str(i),'.TIF']; % File name
  eval(['file' num2str(i) '=' 'name;']); % setting a file number equal to each image
  eval(['I' num2str(i) '=' 'imread([root,file' num2str(i) ']);']); % reading the image to I1, I2,
I3, etc
end

%% Cell Count
A=ones(size(1));
image=[I1]; % Storing the Images, for multiple images, use a for loop
myfilter = fspecial('gaussian',[3 3], 0.5); % Sets a Filter, to change the options do help
fspecial
myfilteredimage = imfilter(image, myfilter, 'replicate'); % applying the filter to the
images
level=graythresh(myfilteredimage); % thresholding the image
BW1=im2bw(I1,level+.06); % applies the threshold plus an arbitrary .06 to clean up the
image
[centers, radii] = imfindcircles(BW1 ,[1 100]);
numberofcells=size(centers); % Counts number of cells by size of matrix
A(1,1)=numberofcells(1); % Stores the number of cells in a large matrix...The 1 indicates
the first row

%% Plotting the data
figure(1)
subplot(1,3,1)
imshow(I1)
title('original image')
subplot(1,3,2)
imshow(BW1)
title('thresholded image in black and white')
```

```
subplot(1,3,3)
imshow(BW1)
hold on
viscircles(centers, radii,'EdgeColor','b'); % plots blue outlines around cells
title('image with particles counted')
hold off

figure(2)
plot(radii,'b+')
NumberofParticle=A
U=radii';
AverageSizeofParticles=mean(U)
hold on
y=numberofcells+1;
t=linspace(1,numberofcells(1),numberofcells(1));
plot(t,AverageSizeofParticles,'r-')
title('Particle Distribution')
hold off

conversionfactor=50/212;% conversion factor from microscope pixels --> um

figure(5)
radii_um=radii.*conversion factor;
plot(radii_um,'b+')
U_um=radii_um';
AverageSizeofParticles_um=mean(U_um)
hold on
plot(t,AverageSizeofParticles_um,'r-')
title('Particle Distribution')
ylabel('Number of Particles')
xlabel('Size of Particles (um)')
hold off

figure(6)
title('Histogram of Particle Size')
ylabel('Number of Particles')
xlabel('Size of Particles (um)')
hist(radii,50)
```

Appendix C – BCA Assay Protocol

INSTRUCTIONS

Micro BCA™ Protein Assay Reagent Kit

PIERCE
a brand of ꞬꞬꞬ
3747 N. Meridian Road
P.O. Box 117
Rockford, IL 61105

23235   0412w

Number	Description
23235	**Micro BCA™ Protein Assay Reagent Kit**, sufficient reagents for 480 tube assays or 3,200 microplate assays
23231	**Micro BCA™ Reagent A (MA)**, 240 ml, containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH
23232	**Micro BCA™ Reagent B (MB)**, 240 ml, containing bicinchoninic acid (4.0%) in water
23234	**Micro BCA™ Reagent C (MC)**, 12 ml, containing 4.0% cupric sulfate, pentahydrate in water
23209	**Albumin Standard Ampules, 2 mg/ml**, 10 x 1 ml ampules containing bovine serum albumin (BSA) at a concentration of 2.0 mg/ml in a solution of 0.9% saline and 0.05% sodium azide

Storage: Upon arrival store at room temperature. Product shipped at ambient temperature.

This product is guaranteed for one year from the date of purchase when handled and stored properly.

Note: If either Reagent MA or Reagent MB precipitates upon shipping in cold weather or during long-term storage, dissolve precipitates by gently warming and stirring solutions. Discard any reagent that shows discoloration or evidence of microbial contamination.

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Introduction

The Pierce Micro BCA™ Protein Assay Reagent Kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. An adaptation of Pierce's BCA Protein Assay Reagent Kit (Product No. 23227), the Micro BCA™ Kit has been optimized for use with dilute protein samples (0.5–20 µg/ml). The unique method (U.S. Patent No. 4,839,295) utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment.[1] A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA.[2] Studies with di-, tri- and tetrapeptides suggest that the extent of color formation is caused by more than the mere sum of individual color-producing functional groups.[2]

Telephone: 800-8-PIERCE (800-874-3723) or 815-968-0747 • Fax: 815-968-7316 or 800-842-5007
www.piercenet.com • Customer Service: cs@piercenet.com • Technical Assistance: ta@piercenet.com

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The Micro BCA™ Protein Assay Reagent Kit uses concentrated reagents and a protocol that utilizes an extended incubation time at an elevated temperature (60°C). The result is an extremely sensitive colorimetric protein assay in a test tube or microplate assay format.

Preparation of Standards and Working Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a fresh set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as your sample. Each 1 ml ampule of 2.0 mg/ml Albumin Standard is sufficient to prepare a set of diluted standards such that three replicates of each dilution may be included in the Test Tube Procedure.

Table 1: Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	4.5 ml	0.5 ml of Stock	200 µg/ml
B	8.0 ml	2.0 ml of vial A dilution	40 µg/ml
C	4.0 ml	4.0 ml of vial B dilution	20 µg/ml
D	4.0 ml	4.0 ml of vial C dilution	10 µg/ml
E	4.0 ml	4.0 ml of vial D dilution	5 µg/ml
F	4.0 ml	4.0 ml of vial E dilution	2.5 µg/ml
G	4.8 ml	3.2 ml of vial F dilution	1 µg/ml
H	4.0 ml	4.0 ml of vial G dilution	0.5 µg/ml
I	8.0 ml	0	0 µg/ml = Blank

B. Preparation of the Micro BCA™ Working Reagent (WR)

1. Use the following formula to determine the total volume of WR required:

   (# standards + # unknowns) (# replicates) (volume of WR per sample) = total volume WR required

   Example: for the standard Test Tube Procedure with 3 unknowns and 2 replicates of each sample:

   (9 standards + 3 unknowns) (2 replicates) (1 ml) = 24 ml WR required (round up to 25 ml)

   Note: 1 ml of the WR is required for each sample in the Test Tube Procedure, while only 150 µl of WR reagent is required for each sample in the Microplate Procedure.

2. Prepare fresh WR by mixing 25 parts of Micro BCA™ Reagent MA and 24 parts Reagent MB with 1 part of Reagent MC (25:24:1, Reagent MA:MB:MC). For the above example, combine 12.5 ml of Reagent MA and 12.0 ml Reagent MB with 0.5 ml of Reagent MC.

   Note: When Reagent MC is initially added to Reagents MA and MB, a turbidity is observed that quickly disappears upon mixing to yield a clear-green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

C. Procedure Summary (Test Tube Procedure)
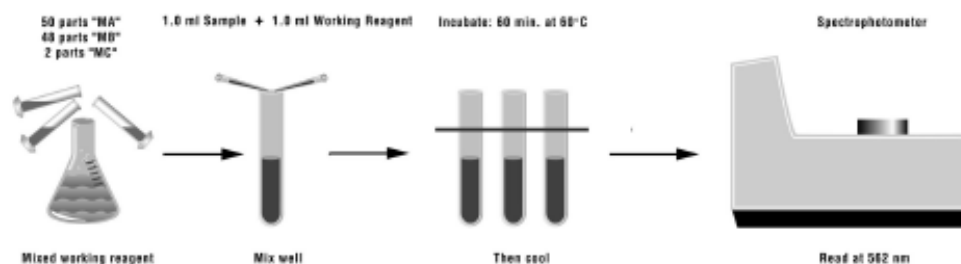
50 parts "MA"
48 parts "MB"
2 parts "MC"

1.0 ml Sample + 1.0 ml Working Reagent

Incubate: 60 min. at 60°C

Spectrophotometer

Mixed working reagent   Mix well   Then cool   Read at 562 nm

2

Test Tube Procedure (linear working range of 0.5-20 µg/ml)

1. Pipette 1.0 ml of each standard and unknown sample replicate into an appropriately labeled test tube.

2. Add 1.0 ml of the WR to each tube and mix well.

3. Cover tubes and incubate at 60°C in a water bath for 1 hour.

4. Cool all tubes to RT.

5. With the spectrophotometer set to 562 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples within 10 minutes.

    Note: Because the Micro BCA™ Assay does not reach a true end point, color development will continue even after cooling to RT. However, because the rate of color development is low at RT, no significant error will be introduced if the 562 nm absorbance readings of all tubes are made within 10 minutes of each other.

6. Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.

7. Prepare a standard curve by plotting the average Blank-corrected 562 nm reading for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedure (linear working range of 2-40 µg/ml)

1. Pipette 150 µl of each standard or unknown sample replicate into a microplate well.

2. Add 150 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.

3. Cover plate and incubate at 37°C for 2 hours.

4. Cool plate to RT.

5. Measure the absorbance at or near 562 nm on a plate reader.

    Notes:
    a. Wavelengths from 540-590 nm have been used successfully with this method.
    b. Because plate readers use a shorter light path length than cuvette spectrophotometers, 562 nm readings are lower with the Microplate Procedure than with the Test Tube Procedure. Consequently, the lower limit of detection is greater (2.0 µg/ml) in the Microplate Procedure.

6. Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.

7. Prepare a standard curve by plotting the average Blank-corrected 562 nm reading for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

    Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
No color in any tubes	Sample contains a copper-chelating agent	Dialyze, desalt, or dilute sample Increase the copper concentration in the working reagent (more Reagent MC) Remove interfering substances from sample using Pierce Product No. 23215
Blank absorbance is OK, but standards and samples show less color than expected	Strong acid or alkaline buffer, alters working reagent pH	Dialyze, desalt or dilute sample
	Color measured at the wrong wavelength	Measure the color at 562 nm

Continued next page

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Troubleshooting, Continued		
Problem	**Possible Cause**	**Solution**
Color of samples appear darker than expected	Protein concentration is too high Sample contains lipids or lipoproteins	Dilute sample Add 2% SDS to the sample to eliminate interference from lipids[3] Remove interfering substances from sample using Pierce Product No. 23215
All tubes (including blank) are dark purple	Sample contains a reducing agent Sample contains a thiol Sample contains biogenic amines (catecholamines)	Dialyze or dilute sample Remove interfering substances from sample using Pierce Product No. 23215
Need to read color at a different wavelength	Colorimeter does not have 562 nm filter	Color may be read at any wavelength between 540 nm and 590 nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering Substances

Certain substances are known to interfere with the Micro BCA™ Assay including those with reducing potential, chelating agents, and strong acids or bases. Because they are known to interfere with protein estimation at even minute concentrations, avoid the following substances as components of the sample buffer:

Ascorbic Acid	Hydrogen Peroxide	Iron	Reducing Sugars
Catecholamines	Hydrazides	Lipids	Tryptophan
Cysteine	Impure Glycerol	Phenol Red	Tyrosine
EGTA	Impure Sucrose	Reducing Agents	Uric Acid

Other substances interfere to a lesser extent with protein estimation using the Micro BCA™ Protein Assay Reagent Kit. These have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Test Tube Procedure are listed in Table 2.

In Table 2, substances were compatible at the indicated concentration in the Test Tube Procedure if the error in protein concentration estimation (of BSA at 1,000 μg/ml) caused by the presence of the substance in the sample was less than or equal to 10%. The substances were tested using freshly prepared WR for each run. The Blank-corrected 562 nm absorbance readings (for the 1,000 μg/ml BSA standard + substance) were compared to the net 562 nm readings of the same standard prepared in 0.9% saline.

B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Micro BCA™ Protein Assay may be eliminated or overcome by several methods.

1. Remove the interfering substance by dialysis or gel filtration.

2. Dilute the sample until the substance no longer interferes. This works if the starting protein concentration of the sample is high.

3. Precipitate proteins with acetone or trichloroacetic acid (TCA). The liquid containing the substance that interfered is discarded and the protein pellet is easily solubilized directly in the alkaline Micro BCA™ WR.[4] A protocol for performing this on samples to be assayed with BCA Protein Assay Reagent is available at the Pierce web site. Alternatively, use Pierce Product No. 23215 (see Related Pierce Products below).

4. Increase the amount of copper in the WR (prepare WR using a greater proportion of Reagent MC; e.g., MA:MB:MC equal to 25:24:2 or 25:24:3), which may eliminate interference by copper chelating agents.

   Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

Table 2: Compatible Substance Concentrations in the Micro BCA™ Protein Assay (see text for details)

Substance	Compatible Concentration	Substance	Compatible Concentration
Salts/Buffers		**Detergents**	
ACES, pH 7.8	10 mM	Brij®-35	5.0%
Ammonium sulfate	——	Brij®-56, Brij®-58	1.0%
Bicine, pH 8.4	2 mM	CHAPS (CHAPSO)	1.0% (5.0%)
Bis-Tris, pH 6.5	0.2 mM	Deoxycholic acid	5.0%
Borate (50 mM), pH 8.5 (#28384)	1:4 dilution*	Lubrol® PX	1.0%
B-PER® Reagent (#78248)	1:10 dilution*	Nonidet P-40 (NP-40)	5.0%
Calcium chloride in TBS, pH 7.2	10 mM	Octyl β-glucoside	0.1%
Na-Carbonate/Na-Bicarbonate (0.2 M), pH 9.4 (#28382)	undiluted	Octyl β-thioglucopyranoside	5.0%
		SDS	5.0%
Cesium bicarbonate	100 mM	Span® 20	1.0%
CHES, pH 9.0	100 mM	Triton® X-100	5.0%
Na-Citrate (0.6 M), Na-Carbonate (0.1 M), pH 9.0 (#28388)	1:500 dilution*	Triton® X-114	0.05%
		Triton® X-305, X-405	1.0%
Na-Citrate (0.6 M), MOPS (0.1 M), pH 7.5 (#28386)	1:500 dilution*	Tween®-20, Tween®-80	5.0%
		Tween®-60	0.5%
Cobalt chloride in TBS, pH 7.2	——	Zwittergent® 3-14	——
EPPS, pH 8.0	100 mM	**Chelating agents**	
Ferric chloride in TBS, pH 7.2	0.5 mM	EDTA	0.5 mM
Glycine	n/a	EGTA	——
Guanidine-HCl	4 M	Sodium citrate, pH 4.8 (or pH 6.4)	5 mM (16.7 mM)
HEPES, pH 7.5	100 mM	**Reducing & Thiol-Containing Agents**	
Imidazole, pH 7.0	12.5 mM	N-acetylglucosamine in PBS, pH 7.2	——
MES, pH 6.1	100 mM	Ascorbic acid	——
MES (0.1 M), NaCl (0.9%), pH 4.7 (#28390)	1:4 dilution*	Cysteine	——
MOPS, pH 7.2	100 mM	Dithioerythritol (DTE)	——
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted	Dithiothreitol (DTT)	——
Nickel chloride in TBS, pH 7.2	0.2 mM	Glucose	1 mM
PBS; Phosphate (0.1 M), NaCl (0.15 M), pH 7.2 (#28372)	undiluted	2-Mercaptoethanol	1 mM
		Thimerosal	——
PIPES, pH 6.8	100 mM	**Misc. Reagents & Solvents**	
RIPA lysis buffer; 50 mM Tris, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	1:10 dilution*	Acetone	1.0%
		Acetonitrile	1.0%
Sodium acetate, pH 4.8	200 mM	Aprotinin	1 mg/L
Sodium azide	0.2%	DMF	1.0%
Sodium bicarbonate	100 mM	DMSO	1.0%
Sodium chloride	1 M	Ethanol	1.0%
Sodium citrate, pH 4.8 (or pH 6.4)	5 mM (16.7 mM)	Glycerol (Fresh)	1.0%
Sodium phosphate	100 mM	Hydrazide (Na_2BH_4 or $NaCNBH_3$)	——
Tricine, pH 8.0	2.5 mM	Hydrochloric Acid	10 mM
Triethanolamine, pH 7.8	0.5 mM	Leupeptin	10 mg/L
Tris	50 mM	Methanol	1.0%
TBS; Tris (25 mM), NaCl (0.15 M), pH 7.6 (#28376)	1:10 dilution*	Phenol Red	——
		PMSF	1 mM
Tris (25 mM), Glycine (192 mM), pH 8.0 (#28380)	1:10 dilution*	Sodium Hydroxide	50 mM
		Sucrose	4%
Tris (25 mM), Glycine (192 mM), SDS (0.1%), pH 8.3 (#28378)	undiluted	TLCK	0.1 mg/L
		TPCK	0.1 mg/L
Zinc chloride in TBS, pH 7.2	0.5 mM	Urea	3 M
		o-Vanadate (sodium salt) in PBS, pH 7.2	1 mM

* Diluted with ultrapure H_2O. A blank indicates that substance is incompatible with the assay.

Related Pierce Products

Number	Description
23208	**Pre-Diluted Protein Assay Standards: Bovine Serum Albumin Fraction V (BSA) Set**, 7 x 3.5 ml of dilutions in the range of 125-2,000 µg/ml
23212	**Bovine Gamma Globulin Standard Ampule**s, 2 mg/ml, 10 x 1 ml
23213	**Pre-Diluted Protein Assay Standards: Bovine Gamma Globulin Fraction II (BGG) Set**, 7 x 3.5 ml of dilutions in the range of 125-2,000 µg/ml
23227	**BCA Protein Assay Reagent Kit**, working range of 20-2,000 µg/ml
23236	**Coomassie® Plus Protein Assay Reagent Kit**, working range of 1-1,500 µg/ml
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**, sufficient reagents to pre-treat 500 samples to remove interfering substances prior to total protein quantitation

Additional Information

A. Please visit the Pierce web site for additional information on this product including:

- Frequently Asked Questions
- Tech Tip protocol: TCA or Acetone Elimination of Interfering Substances
- Application notes and more complete reference list

B. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins (Table 3). These differences relate to amino acid sequence, pI, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods utilize BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Pierce Albumin Standard (BSA) (Product No. 23209) provides a consistent standard for protein estimations. Nevertheless, individual proteins, including BSA and IgG, differ slightly in their color responses in the Micro BCA™ Assay (Figure 1). Therefore, if great accuracy is required, the standard curve should be prepared from a pure sample of the target protein to be measured.

Figure 1: Typical color response curves for BSA and BGG using the Test Tube Procedure.

Micro BCA™ Reagent Protein Assay
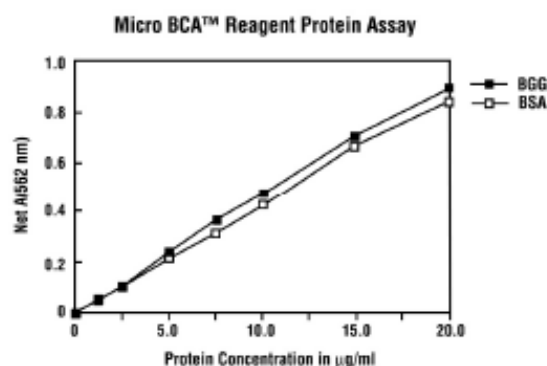
Table 3: Protein-to-Protein Variation

562 nm absorbance ratios for proteins relative to BSA using the Test Tube Procedure.
Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.80
α-Chymotrypsinogen, bovine	0.99
Cytochrome C, horse heart	1.11
Gamma globulin, bovine	0.95
IgG, bovine	1.12
IgG, human	1.03
IgG, mouse	1.23
IgG, rabbit	1.12
IgG, sheep	1.14
Insulin, bovine pancreas	1.22
Myoglobin, horse heart	0.92
Ovalbumin	1.08
Transferrin, human	0.98
Average ratio	**1.05**
Standard Deviation	**0.12**
Coefficient of Variation	**11.4%**

C. Alternative Total Protein Assay Reagents

If interference by a reducing substance or metal-chelating substance contained in the sample cannot be overcome, try the Pierce Coomassie® Plus Protein Assay Reagent Kit (Product No. 23236), which is less sensitive to such substances.

D. Cleaning and Re-using Glassware

Care must be exercised when re-using glassware. The Micro BCA™ WR is sensitive to metal ions, especially copper ions. All glassware must be cleaned and then given a thorough final rinse with ultrapure water.

References

1. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150, 76-85.
2. Wiechelman, K., Braun, R. and Fitzpatrick, J. (1988). Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.* 175, 231-237.
3. Kessler, R. and Fanestil, D. (1986). Interference by lipids in the determination of protein using bicinchoninic acid. *Anal. Biochem.* 159, 138-142.
4. Brown, R., Jarvis, K. and Hyland, K. (1989). Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal. Biochem.* 180, 136-139.

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Appendix D – Bradford Assay Protocol

INSTRUCTIONS

Thermo
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Coomassie Plus™ (Bradford) Assay Kit

23236

Number	Description
23236	**Coomassie Plus (Bradford) Assay Kit**, sufficient reagents for 630 test tube or 3160 microplate assays

Kit Contents:

Coomassie Plus (Bradford) Assay Reagent, 950mL, containing coomassie G-250 dye, methanol, phosphoric acid and solubilizing agents in water; store at 4°C

> **Caution**: Phosphoric acid is a corrosive liquid.

Albumin Standard Ampules, 2mg/mL, 10 × 1mL ampules, containing bovine serum albumin (BSA) at 2.0mg/mL in a solution of 0.9% saline and 0.05% sodium azide; store unopened ampules at room temperature (Available separately as Product No. 23209)

Storage: Upon receipt store each component as indicated. Product shipped at ambient temperature.

Note: Discard any reagent that shows discoloration or evidence of microbial contamination.

Table of Contents

Introduction

The Thermo Scientific™ Coomassie Plus™ Kit is a quick and ready-to-use coomassie-binding, colorimetric method for total protein quantitation. This modification of the well-known Bradford method greatly reduces the tendency of coomassie reagents to give nonlinear response curves by a formulation that substantially improves linearity for a defined range of protein concentration. In addition, the Coomassie Plus Reagent results in significantly less protein-to-protein variation than is observed with other Bradford-type coomassie formulations.

When coomassie dye binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465nm to 595nm with a concomitant color change from brown to blue. Performing the assay in either test tube or microplate format is simple: Combine a small amount of protein sample with the assay reagent, mix well, incubate briefly and measure the absorbance at 595nm. Protein concentrations are estimated by reference to absorbances obtained for a series of standard protein dilutions, which are assayed alongside the unknown samples.

Preparation of Standards and Assay Reagent

A. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably in the same diluent as the sample(s). Each 1mL ampule of Albumin Standard is sufficient to prepare a set of diluted standards for either working range suggested in Table 1. There will be sufficient volume for three replications of each diluted standard.

Table 1. Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
Dilution Scheme for Standard Test Tube and Microplate Protocols (Working Range = 100–1500µg/mL)			
A	0	300µL of Stock	2000µg/mL
B	125µL	375µL of Stock	1500µg/mL
C	325µL	325µL of Stock	1000µg/mL
D	175µL	175µL of vial B dilution	750µg/mL
E	325µL	325µL of vial C dilution	500µg/mL
F	325µL	325µL of vial E dilution	250µg/mL
G	325µL	325µL of vial F dilution	125µg/mL
H	400µL	100µL of vial G dilution	25µg/mL
I	400µL	0	0µg/mL = Blank

| Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
Dilution Scheme for Micro Test Tube or Microplate Protocols (Working Range = 1–25µg/mL)			
A	3555µL	45µL of Stock	25µg/mL
B	6435µL	65µL of Stock	20µg/mL
C	3970µL	30µL of Stock	15µg/mL
D	3000µL	3000µL of vial B dilution	10µg/mL
E	2500µL	2500µL of vial D dilution	5µg/mL
F	1700µL	1700µL of vial E dilution	2.5µg/mL
G	4000µL	0	0µg/mL = Blank

B. Mixing and Equilibrating the Coomassie Plus Reagent

Mix the Coomassie Plus Reagent solution immediately before use by gently inverting the bottle several times (Do not shake the bottle to mix the solution). Remove the amount of reagent needed and equilibrate it to room temperature (RT) before use.

Note: Coomassie Plus Reagent contains additives that retard formation of dye-dye and dye-protein aggregates that tend to form in all coomassie-based protein assay reagents. If left undisturbed, the aggregates will become large enough over time to be visible. For example, when left overnight in a clear glass tube, the reagent forms dye-dye aggregates that are visible as a dark precipitate in the bottom of the tube with nearly colorless liquid above. Dye-dye aggregates can form over several hours in stored reagent while dye-protein-dye aggregates form within seconds. Fortunately, gentle mixing completely disperses the aggregates. Therefore, it is good practice to mix the Coomassie Plus Reagent before dispensing and to mix each tube or plate immediately before measuring absorbances.

Procedure Summary

(Standard Test Tube Protocol):

0.05 ml Sample
+
1.5 ml Coomassie Plus Reagent

Mix well

Spectrophotometer

Read at 595 nm

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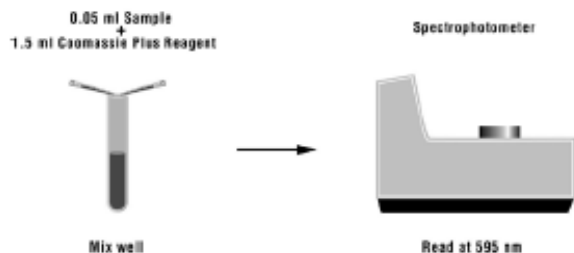
Test Tube Procedures

A. Standard Test Tube Protocol (Working Range = 100-1500µg/mL)

Note: The linear working range with BSA = 125-1000µg/mL; the linear working range with IgG = 125-1500µg/mL.

1. Pipette 0.05mL of each standard or unknown sample into appropriately labeled test tubes.

2. Add 1.5mL of the Coomassie Plus Reagent to each tube and mix well.

3. Optional: For the most consistent results, incubate samples for 10 minutes at room temperature (RT).

4. With the spectrophotometer set to 595nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.

5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

B. Micro Test Tube Protocol (Working Range = 1-25µg/mL)

1. Pipette 1.0mL of each standard or unknown sample into appropriately labeled test tubes.

2. Add 1.0mL of the Coomassie Plus Reagent to each tube and mix well.

3. Optional: For the most consistent results, incubate samples for 10 minutes at room temperature (RT).

4. With the spectrophotometer set to 595nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.

5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Microplate Procedures

A. Standard Microplate Protocol (Working Range = 100-1500µg/mL)

1. Pipette 10µL of each standard or unknown sample into the appropriate microplate wells (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).

2. Add 300µL of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 seconds.

3. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).

4. Measure the absorbance at or near 595nm with a plate reader.

5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

   Note: When compared to the Standard Test Tube Protocol, 595nm measurements obtained with the Microplate Protocols are lower because the light path is shorter. Consequently, this may increase the minimum detection level of the assay. If higher 595nm measurements are required, use 15µL of standard or sample and 300µL of Coomassie Plus Reagent per well.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

B. Micro Microplate Protocol (Working Range = 1-25µg/mL)

1. Pipette 150µL of each standard or unknown sample into the appropriate microplate wells.

2. Add 150µL of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 seconds.

3. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).

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4. Measure the absorbance at or near 595nm on a plate reader.

5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration (µg/ml). Using the standard curve, determine the protein concentration estimate for each unknown sample.

   Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

Troubleshooting

Problem	Possible Cause	Solution
Absorbance of Blank is OK, but remaining standards and samples yield lower values than expected	Improper reagent storage	Store reagent refrigerated
	Reagent still cold	Allow reagent to warm to RT
	Absorbance measured at incorrect wavelength	Measure absorbance near 595nm
Absorbances of Blank and standards are OK, but samples yield lower values than expected	Sample protein (peptide) has a low molecular weight (e.g., less than 3000)	Try the BCA Protein Assay
A precipitate forms in all tubes	Sample contains a surfactant (detergent)	Dialyze or dilute sample or remove interfering substances from sample using Product No. 23215
	Samples not mixed well or left to stand for extended time, allowing aggregates to form with the dye	Mix samples immediately prior to measuring absorbances
All tubes (including Blanks) are dark blue	Strong alkaline buffer raises pH of formulation, or sample volume too large, thereby raising reagent pH	Dialyze or dilute sample. Remove interfering substances from sample using Product No. 23215
Need to read absorbances at a different wavelength	Spectrophotometer or plate reader does not have 595nm filter	Color may be read at any wavelength between 575nm and 615nm, although the slope of standard curve and overall assay sensitivity will be reduced

A. Interfering substances

Certain substances are known to interfere with coomassie-based protein assays including most ionic and nonionic detergents, which reduce color development and can cause precipitation of the assay reagent. Other substances interfere to a lesser extent. These have only minor (tolerable) effects below a certain concentration in the original sample. Maximum compatible concentrations for many substances in the Standard Test Tube Protocol are listed in Table 2 (see last page). Substances were compatible in the Standard Test Tube Protocol if the error in protein concentration estimation (of BSA at 1000µg/mL) caused by the presence of the substance in the sample was less than or equal to 10%. The Blank-corrected 595nm absorbance measurements (for the 1000µg/mL BSA standard + substance) were compared to the net 595nm absorbances of the 1000µg/mL BSA standard prepared in 0.9% saline.

B. Strategies for eliminating or minimizing the effects of interfering substances

The effects of interfering substances in the Coomassie Plus Assay may be overcome by one of several methods.

- Remove the interfering substance by dialysis or desalting.
- Dilute the sample until the substance no longer interferes.
- Precipitate proteins with acetone or trichloroacetic acid (TCA). Upon precipitation the liquid containing the substance that interfered is discarded and the protein pellet is solubilized in a small amount of ultrapure water or directly in the Coomassie Plus Reagent. Alternatively, use Product No. 23215 (see Related Thermo Scientific Products).
  Note: For greatest accuracy, the protein standards must be treated identically to the sample(s).

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Related Thermo Scientific Products

15041	**Pierce 96-Well Plates – Corner Notch**, 100/pkg
15075	**Reagent Reservoirs**, 200/pkg.
15036	**Sealing Tape for 96-Well Plates**, 100/pkg
23208	**Pre-Diluted Bovine Serum Albumin (BSA) Set**, 7 × 3.5mL
23209	**Bovine Serum Albumin Standard Ampules**, 2mg/mL, 10 ×1mL
23212	**Bovine Gamma Globulin Standard Ampules**, 2mg/mL, 10 × 1mL
23213	**Pre-Diluted Bovine Gamma Globulin Fraction II (BGG) Set**, 7 × 3.5mL
23227	**Pierce BCA Protein Assay Kit**, working range of 20-2000µg/mL
23235	**Micro BCA™ Protein Assay Kit**, working range 0.5-20µg/mL
23215	**Compat-Able™ Protein Assay Preparation Reagent Set**

Additional Information

A. Please visit the web site for additional information on this product including:

- Tech Tip #9: Quantitate immobilized protein

- Application notes and more complete reference list

B. Response characteristics for different proteins

Each of the commonly used total protein assay methods exhibits some degree of varying response toward different proteins. These differences relate to amino acid sequence, isoelectric point, structure and the presence of certain side chains or prosthetic groups that can dramatically alter the protein's color response. Most protein assay methods utilize BSA or immunoglobulin (IgG) as the standard against which the concentration of protein in the sample is determined. Thermo Scientific Bovine Serum Albumin Standard (BSA) (Product No. 23209) provides a consistent standard for protein estimations. Nevertheless, individual proteins, including BSA and IgG, differ slightly in their color responses in the Coomassie Plus Assay (Figure 1). For greatest accuracy, the standard curve should be prepared from a pure sample of the target protein to be measured.

Table 3 shows typical protein-to-protein variation in color response. All proteins were tested at 1000µg/mL using the Standard Test Tube Protocol. The average net color response for BSA was normalized to 1.00 and the average net color response of the other proteins is expressed as a ratio to the response of BSA. The protein-to-protein variation observed with the Coomassie Plus Reagent is significantly less than that seen with other Bradford-type coomassie formulations.

**C. Measuring Absorbances at Wavelengths other than 595nm**

If a photometer or plate reader is not available with a 595nm filter, the blue color may be measured at any wavelength between 570nm and 610nm. The maximum sensitivity of the assay occurs when the absorbance of the dye-protein complex is measured at 595nm. Measuring the absorbance at any wavelength other than 595nm will result in a lower slope for the standard curve and may increase the minimum detection level for the protocol.

D. Effect of Temperature on 595nm Absorbance

Absorbance measurements at 595nm obtained with the Coomassie Plus Reagent are dependent on the temperature of the reagent to some extent. As the reagent temperature increases to RT, the 595nm measurements will increase. Therefore, it is important that the Coomassie Plus Reagent remain at a constant temperature (i.e., RT) during the assay.

E. Cleaning and Re-using Glassware

Care must be exercised when cleaning glassware that will be used again for protein assays. Thorough cleaning often requires the use of a detergent such as Thermo Scientific PCC-54 Detergent (Product No. 72288), which must be completely removed in the final rinse. Coomassie dye will stain glass or quartz cuvettes. Disposable polystyrene cuvettes are a convenient alternative.

Coomassie Plus Reagent Protein Assay
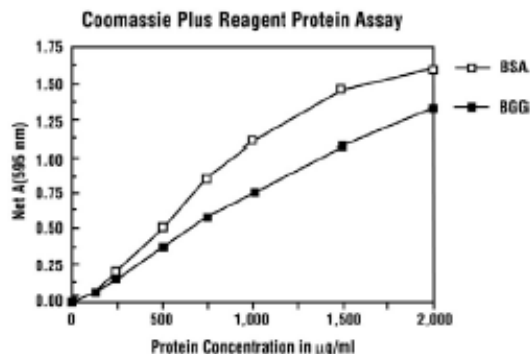
Figure 1. Typical color response curves for BSA and BGG using the Standard Test Tube Protocol.

Table 3. Protein-to-Protein Variation. Absorbance ratios (595nm) for proteins relative to BSA using the Standard Test Tube Protocol in the Coomassie (Bradford) Assay.

Ratio = (Avg "test" net Abs.) / (avg. BSA net Abs.)

Protein Tested	Ratio
Albumin, bovine serum	1.00
Aldolase, rabbit muscle	0.74
α-Chymotrypsinogen, bovine	0.52
Cytochrome C, horse heart	1.03
Gamma globulin, bovine	0.58
IgG, bovine	0.63
IgG, human	0.66
IgG, mouse	0.62
IgG, rabbit	0.43
IgG, sheep	0.57
Insulin, bovine pancreas	0.67
Myoglobin, horse heart	1.15
Ovalbumin	0.68
Transferrin, human	0.90
Average ratio	**0.73**
Standard Deviation	**0.21**
Coefficient of Variation	**28.8%**

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Table 2. Compatible substance concentrations in the Coomassie Plus Protein Assay (see text for details).

Substance	Compatible Concentration	Substance	Compatible Concentration
Salts/Buffers		**Detergents**	
ACES, pH 7.8	100mM	Brij™-35	0.062%
Ammonium sulfate	1.0M	Brij-56 (Brij-58)	0.031%(0.016%)
Asparagine	10mM	CHAPS, CHAPSO	5.0%
Bicine, pH 8.4	100mM	Deoxycholic acid	0.4%
Bis-Tris, pH 6.5	100mM	Lubrol™ PX	0.031%
Borate (50mM), pH 8.5 (#28384)	undiluted	Octyl β-glucoside	0.5%
B-PER™ Reagent (#78248)	1/2 dilution*	Nonidet P-40 (NP-40)	0.5%
Calcium chloride in TBS, pH 7.2	10mM	Octyl β-thioglucopyranoside	3.0%
Na-Carbonate/Na-Bicarbonate (0.2M), pH 9.4 (#28382)	undiluted	SDS	0.016%
Cesium bicarbonate	100mM	Span™ 20	0.5%
CHES, pH 9.0	100mM	Triton™ X-100, X-114	0.062%
Na-Citrate (0.6M), Na-Carbonate (0.1M), pH 9.0 (#28388)	undiluted	Triton X-305, X-405	0.125%(0.025%)
Cobalt chloride in TBS, pH 7.2	10mM	Tween™-20	0.031%
EPPS, pH 8.0	100mM	Tween-60	0.025%
Ferric chloride in TBS, pH 7.2	10mM	Tween-80	0.016%
Glycine	100mM	Zwittergent™ 3-14	0.025%
Guanidine-HCl	3.5M	**Chelating agents**	
HEPES, pH 7.5	100mM	EDTA	100mM
Imidazole, pH 7.0	200mM	EGTA	2mM
MES, pH 6.1	100mM	Sodium citrate	200mM
MES (0.1M), NaCl (0.9%), pH 4.7 (#28390)	undiluted	**Reducing & Thiol-Containing Agents**	
MOPS, pH 7.2	100mM	N-acetylglucosamine in PBS, pH 7.2	100mM
Modified Dulbecco's PBS, pH 7.4 (#28374)	undiluted	Ascorbic acid	50mM
Nickel chloride in TBS, pH 7.2	10mM	Cysteine	10mM
PBS; Phosphate (0.1M), NaCl (0.15M), pH 7.2 (#28372)	undiluted	Dithioerythritol (DTE)	1mM
PIPES, pH 6.8	100mM	Dithiothreitol (DTT)	5mM
RIPA lysis buffer; 50mM Tris, 150mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS, pH 8.0	1/40 dilution*	Glucose	1.0M
Sodium acetate, pH 4.8	180mM	Melibiose	100mM
Sodium azide	0.5%	2-Mercaptoethanol	1.0M
Sodium bicarbonate	100mM	Potassium thiocyanate	3.0M
Sodium chloride	5.0M	Thimerosal	0.01%
Sodium citrate, pH 4.8 or pH 6.4	200mM	**Misc. Reagents & Solvents**	
Sodium phosphate	100mM	Acetone	10%
Tricine, pH 8.0	100mM	Acetonitrile	10%
Triethanolamine, pH 7.8	100 mM	Aprotinin	10 mg/L
Tris	2.0M	DMF, DMSO	10%
TBS; Tris (25mM), NaCl (0.15M), pH 7.6 (#28376)	undiluted	Ethanol	10%
Tris (25mM), Glycine (192mM), pH 8.0 (#28380)	undiluted	Glycerol (Fresh)	10%
Tris (25mM), Glycine (192mM), SDS (0.1%), pH 8.3 (#28378)	1/4 dilution*	Hydrochloric Acid	100mM
Zinc chloride in TBS, pH 7.2	10mM	Leupeptin	10mg/L
		Methanol	10%
		Phenol Red	0.5mg/mL
		PMSF	1mM
		Sodium Hydroxide	100mM
		Sucrose	10%
		TLCK	0.1mg/L
		TPCK	0.1mg/L
		Urea	3.0M
		o-Vanadate (sodium salt), in PBS, pH 7.2	1mM

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Appendix E – TNF-α ELISA Protocol

Mouse TNF-α

ELISA MAX™ Standard Sets

Cat. No. 430901 (5 plates)
         430902 (10 plates)
         430903 (20 plates)

BioLegend's ELISA MAX™ Standard Sets contain the capture and detection antibodies, recombinant protein standard, and Avidin-HRP required for the accurate quantification of natural and recombinant mouse TNF-α. These sets are cost-effective and designed for experienced ELISA users. Optimization of reagent concentrations and assay conditions may be required.

It is highly recommended that the instruction sheet be read in its entirety before using this product. Use the recommended assay protocol, microwell plates, buffers, diluent, and substrate solution to obtain desired assay results. Do not use this set beyond the expiration date.

Materials Provided

1. Mouse TNF-α ELISA Capture Antibody (200X)
2. Mouse TNF-α ELISA Detection Antibody (200X)
3. Mouse TNF-α Standard
4. Avidin-HRP (1000X)
5. Instruction Sheet
6. Lot-Specific Instruction/ Analysis Certificate

Introduction

Mouse TNF-α (Tumor Necrosis Factor-alpha) is a potent multifunctional cytokine which can exert regulatory, inflammatory and cytotoxic effects on a wide range of normal lymphoid and non-lymphoid cells and tumor cells. TNF-α is secreted by macrophages, monocytes, neutrophils, T-cells (primarily CD4+ T cells), NK-cells, and many transformed cell lines.

Principle of the Test

BioLegend's ELISA MAX™ Standard Set is a sandwich Enzyme-Linked Immunosorbent Assay (ELISA).

Troubleshooting

High Background:

- Background wells were contaminated.
- Matrix used had endogenous analyte.
- Plate was insufficiently washed.
- TMB Substrate Solution was contaminated.

No signal:

- Incorrect or no antibodies were added.
- Avidin-HRP was not added.
- Substrate solution was not added.
- Wash buffer contained sodium azide.

Low or poor signal for the standard curve:

- Standard was incompletely reconstituted or was stored improperly.
- Reagents were added to wells with incorrect concentrations.
- Plate was incubated with inappropriate temperature, timing or agitation.

Signal too high, standard curves saturated:

- Standard was reconstituted with less volume than required.
- One or more reagent incubation steps were too long.
- Plate was incubated with inappropriate temperature, timing, or agitation.

Sample readings out of range:

- Samples contain no or below detectable levels of analyte.
- Samples contain analyte concentrations greater than highest standard point.

High variations in samples and/or standards:

- Pipetting errors may have occurred.
- Plate washing was inadequate or nonuniform.
- Samples were not homogenous.
- Samples or standard wells were contaminated.

References Using This Set

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For other technical resources, please visit:
www.biolegend.com/support or
email: techserv@biolegend.com

Calculation of Results

Plot the standard curve on log-log axis graph paper with cytokine concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown cytokine concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding cytokine concentration. If the samples were diluted, multiply by the appropriate dilution factor. The data is best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If a test sample's absorbance value falls outside the standard curve ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.
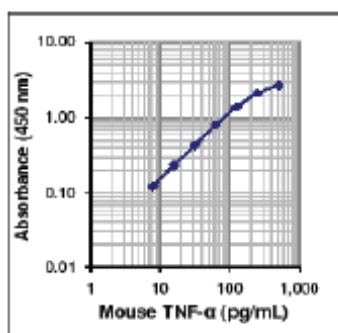
Performance Characteristics

Specificity: No cross reactivity was observed when this kit was used to analyze multiple human, mouse and rat recombinant proteins.

Materials to be Provided by the End-User

- Microwell plates: 96-well Nunc MaxiSorp™ is recommended.
- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Deionized (DI) water
- Coating Buffer: 8.4 g NaHCO$_3$, 3.56 g Na$_2$CO$_3$, add DI H$_2$O to 1.0 L, pH to 9.5 (BioLegend Cat. No. 421701 is recommended.)
- Assay Diluent: 10% Fetal Bovine Serum or 1% BSA in Phosphate-Buffered Saline (PBS) (BioLegend Cat. No. 421203 is recommended.)
- PBS: 8.0 g NaCl, 1.16 g Na$_2$HPO$_4$, 0.2 g KH$_2$PO$_4$, 0.2 g KCl, add DI water to 1.0 L, pH to 7.4
- Wash Buffer: Phosphate-Buffered Saline (PBS) + 0.05% Tween-20 (BioLegend Cat. No. 421601 is recommended.)
- Wash bottle or automated microplate washer
- TMB Substrate Solution – BioLegend Cat. No. 421101 is recommended.
- Stop Solution (2 N H$_2$SO$_4$)
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Absorbent paper

Storage Information

- Store kit components at 4°C.
- After reconstitution of the lyophilized standard with Assay Diluent, aliquot into polypropylene vials and store at -70°C. Do not repeatedly freeze/thaw the recombinant protein standard as loss of activity may occur.
- Prior to use, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/support/#msds).
2. TMB substrate solution is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling

Cell Culture Supernatant: If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at < -20°C. Avoid repeated freeze/thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at 1,000 X *g*. Remove serum layer and assay immediately or store serum samples at < -20°C. Avoid repeated freeze/thaw cycles. Serum specimens should be clear and non-hemolyzed.

Plasma: Collect blood sample in a citrate, heparin or EDTA containing tube. Centrifuge for 10 minutes at 1,000 X *g* within 30 minutes of collection. Assay immediately or store plasma samples at < -20°C. Avoid repeated freeze/thaw cycles. Plasma specimens should be clear and non-hemolyzed.

Reagent Preparation

Do not mix reagents from different sets or lots. Avidin-HRP, Mouse TNF-α Standard, and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

1. Dilute the pre-titrated Capture Antibody 1:200 in Coating Buffer. For one plate, dilute 60 μL Capture Antibody in 11.94 mL Coating Buffer.

2. Reconstitute the lyophilized standard with 0.2 mL of Assay Diluent, re-cap vial, and mix well. Allow the reconstituted standard to sit for 15 minutes at room temperature, then invert/vortex to mix.

3. Prior to use, prepare 1,000 μL of the top standard at a concentration of 500 pg/mL from stock solution in Assay Diluent (refer to Lot-Specific Instruction/Analysis Certificate).

4. Dilute the pre-titrated Biotinylated Detection Antibody 1:200 in Assay Diluent. For one plate, dilute 60 μL Detection Antibody in 11.94 mL Assay Diluent.

5. Dilute Avidin-HRP 1:1000 in Assay Diluent. For one plate, dilute 12 μL Avidin-HRP in 11.99 mL Assay Diluent.

6. Prepare all other reagents required for the assay including TMB Substrate Solution. Refer to reagent description in the section "Materials to be Provided by the End-User".

Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

1. One day prior to running the ELISA, dilute Capture Antibody in Coating Buffer. Add 100 μL of this Capture Antibody solution to all wells of a 96-well plate provided in the set. Seal plate and incubate overnight at 4°C.

2. Bring all reagents to room temperature (RT) prior to use. It is strongly recommended that all standards and samples be run in duplicate or tripli-cate. A standard curve is required for each assay.

3. Wash plate 4 times with at least 300 μL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. All subsequent washes should be performed similarly.

4. To block non-specific binding and reduce background, add 200 μL Assay Diluent per well.

5. Seal plate and incubate at RT for 1 hour with shaking at 200 rpm on a plate shaker.

6. While plate is being blocked, prepare standard dilutions and appropriate sample dilutions (if necessary).

7. Prepare 1,000 μL of top standard at 500 pg/mL from stock solution in 1X Assay Diluent (refer to Lot-Specifications/Analysis Certificate). Perform six two-fold serial dilutions of the 500 pg/mL top standard with Assay Diluent in separate tubes. After diluting, the mouse TNF-α standard concentrations are 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, and 7.8 pg/mL, respectively. Assay Diluent serves as the zero standard (0 pg/mL).

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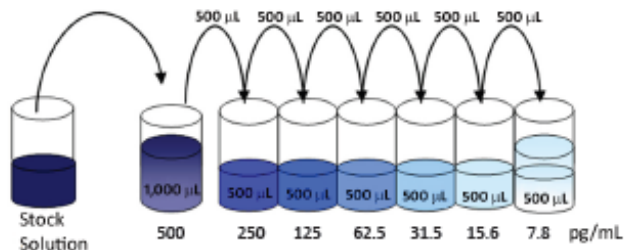
8. Wash plate 4 times with Wash Buffer.

9. Add 100 µL/well of standard dilutions and samples to the appropriate wells. If needed, samples can be further diluted with Assay Diluent before adding 100 µL/well diluted samples.

10. Seal plate and incubate at RT for 2 hours with shaking.

11. Wash plate 4 times with Wash Buffer.

12. Add 100 µL of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking.

13. Wash plate 4 times with Wash Buffer.

14. Add 100 µL of diluted Avidin-HRP solution to each well, seal plate and incubate at RT for 30 minutes with shaking.

15. Wash plate 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.

16. Add 100 µL of TMB Substrate Solution and incubate in the dark for 15-30 minutes or until the desired color develops*. Positive wells should turn blue in color. It is not necessary to seal the plate during this step.

17. Stop reaction by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.

18. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

*Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate readers.

Assay Procedure Summary

Day 1

Add 100 µL diluted Capture Antibody solution to each well, incubate overnight at 4° C

Day 2

1. Wash plate 4 times

2. Add 200 µL Assay Diluent to block, incubate at room temperature for 1 hour with shaking

3. Wash plate 4 times

4. Add diluted standards and samples to the appropriate wells, incubate at room temperature for 2 hours with shaking

5. Wash plate 4 times

6. Add 100 µL diluted Detection Antibody solution to each well, incubate at room temperature for 1 hour with shaking

7. Wash plate 4 times

8. Add 100 µL diluted Avidin-HRP solution to each well, incubate at room temperature for 30 minutes with shaking

9. Wash plate 5 times, soaking for 30 seconds to 1 minute per wash

10. Add 100 µL of TMB Substrate Solution to each well, incubate in the dark for 15-30 minutes or until the desired color develops

11. Add 100 µL Stop Solution to each well

12. Read absorbance at 450 nm and 570 nm

For more information about BioLegend ELISA MAX™ Sets and LEGEND MAX™ ELISA Kits with precoated plates, visit **www.biolegend.com.**

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Glossary

Adaptive immunity – specific immunity that results in immunological memory in the form of antibodies, B cells, and T cells

Adjuvant – a substance that is added to a vaccine to assist in antigen uptake and immune response generation

Antibody – a large Y-shaped protein that identifies and binds to foreign objects in the body

Antigen – a substance that is not pathogenic itself, but originates from a pathogen; it is what the immune system recognizes as foreign, or "nonself," and therefore potentially infections

Antigen-presenting cells (APCs) – cells of the immune system, including dendritic cells, macrophages, and B-cells, that phagocytose foreign antigens and display them on their surfaces as complexes with surface molecules

B cell – a type of lymphocyte in the immune system that can when activated can produce antigen-specific antibodies

Biodegradable – the ability to disintegrate or fall apart over time within a biological context, such as the body

Cervarix™ - a vaccine against two types of HPV that is given in three doses over 6 months

Cervical cancer – cancer of the cervix in the female

Copolymer – a type of polymer composed of two different monomers that are linked covalently linked together

Cytokine –proteins produced in the body that function in cell signaling

Dendritic cell – a cell found in the immune system that is an APC with the most effective range of antigen presentation

Emulsion – a comprehensive term of several similar techniques used to synthesize micro and nanoparticles. This method involves submerging the materials in solvents and using the varying chemical properties of the materials and interactions to create the particles

Gardasil® - a vaccine against four types of HPV that is given in three doses over 6 months

Human papillomavirus (HPV) – a virus that is highly implemented in causing cervical and other cancers

Hydrophilic – having the tendency to associate with water

Hydrophobic – having the tendency to not associate with water

Immunogenicity – the ability of a substance to elicit an immune response

Immunoglobulin – see "Antibody"

Innate immunity – nonspecific immunity that is required for functional and effective adaptive immune response

Interferon-gamma (IFN-γ) – a cytokine produced in during an immune response that is needed in both innate and adaptive immunity

JAWSII – an immortalized cell line of immature dendritic cells from mice

Kinetics – the measurement and study of rates of chemical and biochemical reactions

Live attenuated antigen – a virus or bacterium that retains its ability to reproduce in the host but is unable to cause disease in most patients. This is due to the fact that the virus or bacterium has been subjected to "serial passage," or grown for generations in cells in

which they have difficulty reproducing, forcing it to adapt to the only available host cells and lose its strength against its natural host

Microparticle – an object that is defined by size classification (on the microscale), and is sometimes used for transport or vaccine delivery

Nanoparticle - sub microparticles that are less than 1000 nm in diameter

Ovalbumin (OVA) – a protein found in the egg white of chicken eggs that is commonly used as a model protein in vaccine studies

Pathogen – a disease-causing microorganism or particle such as a bacterium, virus, or prion

Pharmacokinetics – the movement of drugs within the body

Poly(D,L-lactic-co-glycolic acid) (PLGA) – a synthetic, biodegradable polymer that has been extensively researched for use in microspheres and other biomedical applications

Polymer – a large molecule typically composed of repeating structural units bonded by covalent bonds

Polysaccharide – long carbohydrate molecules composed of repeating monomers, or subunits, which appear on the outer coating of many bacterial pathogens

Protein – a macromolecule made of amino acids and joined together by peptide bonds

T cell – a type of lymphocyte in the immune system that can help B cells, kill infected cells, and instill immunological memory

Tumor necrosis factor-alpha (TNF-α) – a cytokine primarily produced by macrophages in the immune response that can result in systemic inflammation and fever

Toll-like receptor (TLR) - a type of protein that spans the plasma membrane of an APC that recognize and bind to antigens

Vaccine – a pharmacological method to immunize an individual against a specific disease

Virus-like particles (VLPs) – self-assembled complexes of viral proteins that are not virulent and can be used as the antigenic component of vaccines, such as in the HPV vaccines, Cervarix™ and Gardasil®

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