GELATIN NANOPARTICLES FOR USE AS A VACCINE ADJUVANT IN INTRANASAL IMMUNIZATIONS

By

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To my family for their love and support

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GELATIN NANOPARTICLES FOR USE AS VACCINE ADJUVANT IN INTRANASAL IMMUNIZATIONS

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Vaccine adjuvants are used to increase the immune response in the delivery of subunit antigens. Currently the only FDA approved adjuvants are aluminum based and must be delivered parenterally. Nasal mucoadhesive vaccine administration can decrease cost, increase efficiency and increase patient compliance. The purpose of this study was to develop a mucoadhesive gelatin nanoparticle >500 nm in diameter that can be used to encapsulate a model protein antigen. The particles were prepared by nanoprecipitation of a gelatin solution with acetone. Thiol groups were incubated with gelatin to increase mucoadhesivness at 20, 40, and 80 mg per 1 gram of gelatin. The thiolation chemistry was characterized using UV-Vis and x-ray photoelectron spectroscopy (XPS). The total amount of sulfur present in the gelatin was determined to be 7.48, 30.53, and 46.75 mmol/gram respectively. However XPS analysis revealed that there was no substantial difference between *surface* sulfur content of the unmodified gelatin nanoparticles and the gelatin nanoparticles modified with 80 mg of iminothiolane. Particle size, charge and morphology were determined using laser light diffraction, atomic force microscopy microscopy and electron microscopy. The average diameter of the unmodified gelatin was 171 nm. The average diameter of the thiolated gelatin nanoparticles was 275 nm. The polydispersity index was approximately 0.61 ± 0 .04 for all

nanoparticles. The zeta (ζ) potential of the unmodified gelatin nanoparticles was -21.5 ± 2.0 mV and the ξ-potential of the modified gelatin nanoparticles was -25.2 ± 1.5, -27.3 ± 0.8, and -28.6 ± 3.0 mV for the 20, 40, and 80 thiolated gelatin nanoparticles. Particle encapsulation efficiency (EE) and release kinetics were conducted using fluorescein isothiocyanate – bovine serum albumin (FITC-BSA) as a model antigen. The EE of the nanoparticles increased from 35.0% (unmodified gelatin) to 82.5% (highest modified gelatin). Particles encapsulated with FITC-BSA released < 20% of their payload over an 8 hour period at 37°C in phosphate buffered saline before a plateau was reached. To observe *in vitro* activity, the nanoparticles were incubated with mucus producing human nasal epithelial cells, RPMI 2650, at 37°C for 24 hours. Confocal microscopy revealed that there was no uptake of any of the gelatin nanoparticles by epithelial cells. The unmodified and thiolated gelatin nanoparticles were incubated with human nasal mucus. Mucoadhesiveness was evaluated by measuring the fluorescence of the nanoparticles remaining in the suspension after centrifugation of the mucus solution. There was an 8% decrease in percent nanoparticles remaining but there was no significant decrease in particle remaining due to thiolation.

CHAPTER 1
INTRODUCTION

Introduction

The origin of modern day vaccination began in the 18th century when in 1796 a country doctor named Edward Jenner first noticed that milkmaids infected with cowpox did not become infected with smallpox. Smallpox was a disease that had killed 10% of Europe's population, and the survivors were left with disfiguring scars and blindness.[1, 2] His injection of a young boy with the fluid from a cowpox lesion was the first documented attempt to provide protection from disease through inoculation. More than 200 years later there are 27 preventable diseases that have available vaccines and vaccines were a $10.6 billion dollar industry.[3, 4]

Historically there have been two main branches of vaccine production. One is the development of live, attenuated vaccines. These are vaccines where non-virulent strains of the target microorganism are used or have been made non-pathogenic by modification of their genome. Vaccines for smallpox, measles, mumps, rubella, and cholera fall into this class. The second most recognized branch is the inactivated, whole organism group. These are vaccines where the microorganism is killed by heat or chemical means and its entire structure is used in the production of the vaccine. Influenza, pertussis, anthrax, and hepatitis A are diseases represented by this class.[5] Both of these categories are strongly immunogenic and are capable of generating sufficient antibody production to provide protection against disease.

In the early 20th century, a third type of vaccine called the subunit vaccine arose. These vaccines are made from a fragment of the microorganism, such as a protein, polysaccharide, DNA strand or toxin. They can be used separately or in conjugation, such as with the type B influenza vaccine. Diphtheria and tetanus toxoids were the first subunit vaccines to be developed. These types of vaccines are ideal because some of the risks that accompany the live

and inactivated vaccines are eliminated but they lack their potency because of reduced immunogenic effect.[6] They must be aided by a biological or chemical agent commonly referred to as an adjuvant.

Adjuvants can enhance the effectiveness of a vaccine in a number of ways. The most significant is the increased immune response provided by the adjuvant. The second role is sustained release at a specific site over an extended period of time. And the final role is through selected targeting to specific cell types that are crucial in invoking immune memory.[6] Currently in the United States the only licensed adjuvants are aluminum based products such as aluminum hydroxide, aluminum phosphate and potassium aluminum sulfate. The efficacy of aluminum based adjuvants varies greatly on the type of antigen delivered.[6, 7] The drawbacks of using aluminum adjuvants are that they can induce severe tissue reactions and hypersensitivity, they cannot induce cell-mediated immunity, they cannot be processed for long-term storage by means of lyophilization or freezing, and perhaps most significantly immunization through oral or intranasal routes are not possible with aluminum adjuvants.[6]

Immunization through mucosal routes has significant advantages over intramuscular and subcutaneous routes because pathogen encounter is highest at the mucosa, the majority of antibodies are produced in mucosa tissue, local and systemic immunity can be generated from mucosal sites, mucosal sites have an abundance lymphocytes and antigen presenting cells, and vaccine delivery can be made without the use of needles.[8] Out of the common mucosal entry routes: oral, genital, and nasal, the nasal tract has the most impervious pathway. Intranasal immunization is a desirable option because it is highly permeable, protects the vaccine from exposure to enzymatic degradation and acidic environments, guards against loss of product due to dilution, and generates a robust immune response. Additionally the increased antigen to

activity ratio combined with the lack of barriers for use work to lower cost for widespread availability.[8, 9]

Adjuvants for intranasal immunization must go beyond the minimum criteria established for general use adjuvants and meet additional objectives established for introduction through a respiratory route. Nanoparticles that have been considered for vaccine delivery include lipid-based carriers such as liposomes, lipoproteins, and nano-sized complexes comprised of saponin and cholesterol. The investigation into the use of polymers for vaccine delivery has yielded several systems, some of which have approached phase I clinical trials.[6, 9] Of these polymer systems, the polyesters poly (lactic acid) and poly (lactic-co-glycolic acid) are the most heavily studied. These biodegradable polymers were the pioneers in polymeric vaccine carrier research because of their biocompatibility, immunostimulatory properties, and previous FDA approval for biomedical use. However these particles are not suitable for protein based vaccines, they are not optimized for intranasal immunization and have not shown conclusively that they are more efficient than the aluminum adjuvants.[6]

Gelatin nanoparticles have been used to encapsulate antibiotics, growth factors, plasmids, oligonucleotides, and iron oxides.[10-13] Research has been proposed for gelatin nanoparticles to treat HIV, lung cancer, and breast cancer. The use of gelatin nanoparticles for mucosal vaccination has been somewhat restricted since gelatin is not inherently mucoadhesive. There have been several attempts made to increase gelatin's mucoadhesivness through surface modification. The cationization of gelatin has been achieved by coupling gelatin with amine containing compounds such as ethylenediamine, spermidine, and more recently cholaminechloride hydrochloride, a quaternary amine.[14, 15] These studies showed that the addition of positively charged amine groups will increase the electrostatic interactions between

the polymer and the negative surfaces it encounters in a biological environment, thereby improving surface adhesiveness, cell transfection, and DNA binding among other things. A different approach centered on adding sulfhydryl (thiol) functional groups to a polymer to increase mucoadhesiveness has also been shown to be effective. Thiols work by forming covalent bonds with cysteine present in the mucosal layer. Thiolation of a polymer also has the additional functions of improving enhancing cell permeation and uptake.[16] To date thiolated polymers have be created using gelatin, chitosan, polyacrylic acid, and polycarbophil.[16-19] However many of the thiolated polymers were intended for use in nanoapplications, and none so far have been prepared for larger polymeric particles that could used to develop a vaccine adjuvant.

Specific Aims

The purpose of this research is to fabricate and characterize a crosslinked, biocompatible, biodegradable, particle that shows an increased adhesiveness for mucosal tissue and is in a size range capable of phagocytosis by a macrophage *in vivo*. To complete this objective the gelatin nanoparticles will be crosslinked with glutaraldehyde to increase stability and extend the length of biodegradability. To improve the adhesiveness of the particles, gelatin nanoparticles will be surface modified with 2-Iminothiolane and characterized for size, surface charge, and degree of thiolation. This research will be unique in the fact that gelatin particles modified with thiol functional groups have not been evaluated for use as a vaccine adjuvant, and there has not been a study demonstrating the increased mucoadhesiveness by an ex vivo examination of surface force adhesion using atomic force microscopy. There will a number of approaches used to attempt to fabricate these particles. Gelatin particles will be fabricated and characterized on the basis of size, surface charge morphology, and distribution. Thiolated gelatin particles will be fabricated and characterized for size, morphology, degree of thiolation, and release kinetics of a model

therapeutic agent. The amount of adhesion for both particles will be evaluated by a mucus binding assay and the cell particle affinity will be evaluated using confocal microscopy.

Specific Aim 1: Synthesis and Characterization of Thiolated Crosslinked Gelatin Nanoparticles

The formation of gelatin particles without modification will be evaluated under a number of experimental conditions that effect size, dispersion, charge, and stability. Thiolated gelatin will be prepared by reacting gelatin with 2-iminothiolane hydrochloride at varying amounts and nanoparticles will be formed using the investigated variables. Nanoparticles will be prepared at approximately a 0.5 micron size range from the thiolated gelatin. Particles will be loaded with fluorescently labeled ovalbumin as the model antigen for release studies. The size of these particles will be determined using the Nanotrec. Size and morphology will be determined using microscopy. Topography of the particle will be determined using atomic force microscopy. The nanoparticle will be analyzed for chemical analysis using x-ray photoelectron spectroscopy. The amount of protein released from the particle will be determined by fluorescence and the degree of thiolation will be determined by a colorimetric analysis using a spectrophotometer.

Specific Aim 2: Comparative Examination of Mucoadhesive Properties of Gelatin Particles

Gelatin particles containing different degrees of thiolation will be used for this study. Nasal epithelial cells will be grown in culture and incubated with thiolated and unmodified gelatin nanoparticles to determine the affinity of the particle for the cell. The mucus produced from these epithelial cells will be extracted from the surface and used to determine the percentage of gelatin particles that bind to the mucus after incubation relative to the degree of modification.

CHAPTER 2
BACKGROUND

Vaccines

Description

Vaccines are preventative therapeutic agents designed to stimulate the immune system to respond as if there were an actual infection present. They accomplish this by introducing the actual infectious organism, in a non-dangerous state, or a fragment of the organism into the host to stimulate the adaptive immune response.[6] Over 6 million deaths a year are prevented worldwide due to vaccination programs, only potable water has made a greater impact on human health. The Center for Disease Control (CDC) lists that there are currently 28 vaccine-preventable diseases. CDC initiatives in United States have designated 17 of these for prevention or elimination. Currently, in the U.S. there are 53 vaccines licensed for use, but the market for new vaccines, adjuvants, and improvements in manufacturing and storage is growing despite fewer companies entering the vaccine market.[20] The vaccines of the future are expected to reduce cost, improve safety, improve stability and storage, and to increase potency and decrease side effects.

Types

The three major categories of vaccines, which are live-attenuated, inactivated, and subunit.[5] Live-attenuated vaccines are composed of pathogens that are highly immunogenic but generally harmless. Live-attenuated viruses are generated by several means and can be sub-categorized as: wild-type production, natural attenuation, chemical attenuation, or cross-species inoculation. The earliest approach at creating a live vaccine was via cross-species inoculation when Jenner attempted to vaccinate humans against smallpox by using a cowpox virus.[21] This method is based on the theory that a microorganism that is pathogenic in one mammal can

induce an immune response in a mammal of a differing species while not actually causing a disease state. Naturally attenuated viruses are a second method of producing live vaccines that are non-harmful. These vaccines inherently have strains of both a virulent and innocuous form. They have been developed for diseases arising from Type 2 polio and rotavirus.[5] Other sub-types of live-attenuated including the wild-type rely on natural or artificial manipulation of the virus genome to be made safe. Vaccines for measles, mumps, and rubella, tuberculosis and cholera are live-attenuated. These vaccines have the ability produce a strong immune response, provide lasting immunity, and do not require an adjuvant to be effective. However it is difficult to produce bacterial vaccines by this method.[5]

Inactivated vaccines, the second category of vaccines, generate adequate immune protection but their immune response is not as strong as the live-attenuated group. They have the ability to induce Class II MHC, but not Class I MHC. Subsequently stronger and more frequent booster shots are needed. Representative diseases treated in this group are Influenza, Polio, Rabies, Hepatitis A and Pertussis, a bacterial disease.[5]

The third class of vaccines, the subunit, is characterized by the use of a portion of the infecting organism such as a protein or carbohydrate or DNA toxoids. They can be divided into two subgroups, toxoids and extracts which are further subdivided based on a polysaccharide or protein origin.[4] Toxoids are bacterial toxins inactivated by chemicals such as formaldehyde. The first subunit toxoids to be developed were diphtheria (DT) and tetanus toxoids (TT). They were later followed by vaccines for hepatitis B, meningococcal meningitis, pertussis, and influenza. The other subgroup is the extracts. Extracted antigens include polysaccharides, conjugated polysaccharides, proteins, and peptides.[4] The subunit vaccines are less expensive to produce and are safer for use especially in individuals with immunocompromised immune

system.[6] But these vaccines are not as immunogenic as the whole organism groups and require co-delivery by an immunostimulating agent such as adjuvant and booster shots and are poor inducers of a protective T cell response. A growing market exists for the use of adjuvants in subunit vaccination. Worldwide there are currently 77 new subunit vaccines that are at various stages of development that will require some form of adjuvant component in the future. In addition future vaccines for cancer therapy will derive from peptide chains.

Routes of Vaccine Administration

Parenteral

Parenteral is a delivery route where vaccines are delivered by injection through the skin. In vaccination this typically refers to intramuscular, intradermal, and subcutaneous. The majority of vaccines are by intradermal and subcutaneous administration. The specific route depends on the immunogenicity of the vaccine at the injection site, the source of the vaccine (viral versus bacterial), and whether it contains an adjuvant. Intramuscular injections are typically done in the quadriceps muscle of the upper thigh, for infants, or in the deltoid of the upper arm for children and adults. Deeper intramuscular injections are done for vaccines that are made with an adjuvant or can cause a severe reaction at the site of injection. Vaccines for diphtheria-tetanus-pertussis (DTP), hepatitis A and B, influenza type B, tetanus, and plague are delivered by this method. Subcutaneous delivery is typically done for vaccines that generate a high immune response. They are given in the same manner as intramuscular injections, except that their penetration depth is no more than 5/8 – ¼ inch deep. Many live virus vaccines for diseases such as yellow fever, varicella (chicken pox), and measles-mumps-rubella (German measles) are administered through subcutaneous injections.[22]

The liabilities in parenteral administration are primarily cost and patient compliance. Injectable vaccines require needles, syringes, sterilization means, trained personnel, and

facilities, all which will add to the final cost of dispensing the vaccine. Additionally most parenteral vaccines must be administered in a staggered schedule in order to maximize the immune response and minimize adverse reactions. These additional cost are contributing factors that greatly impact the vaccination programs in low and middle income countries, where it is estimated $76 billion US dollars will be spent between 2006 -2015 to meet global WHO initiatives.[23]

Intranasal

Intranasal vaccine delivery is a seldom used method of administration but it may be one of the most effective methods of transmission due to the robust and widespread immune response, high production of IgA and IgG antibodies, and proximity to the invading pathogens.[8] Mucosal tissue, covered by a layer of epithelial cells, line the three primary entry tracts into the body: respiratory, gastrointestinal, and urogenital. When foreign pathogens invade the body, they are concentrated first at mucosal sites. The epithelial cells recognize these pathogens and emit cytokines which alert the dendritic cells and macrophages. An innate and adaptive immune response is then quickly and robustly generated because of the high amounts of T, B, and plasma cells also found in mucosal tissue.[24]

Of the mucosal routes, oral and intranasal are the most practical in terms of ease of use. But intranasal is more preferred because the antigen is exposed to less degradation, smaller amounts of antigen are required, and other mucosal sites in the body can be activated by the intranasal tract. In the nasal cavity the target tissue site to induce mucosal immunity is called Waldeyer's ring. This ring is comprised of the tubal tonsils, palatine tonsils, lingual tonsils, and adenoids, essentially the lymphoid tissue of the pharynx see figure. This oropharyngeal tissue has a high number of follicles for B- and T- cells, antigen presenting cells, and draining lymph nodes which promote the immune response.[8] There are several factors to consider when

formulating particles for nasal delivery; the most significant are size, charge, and hydrophobicity/hydrophilicity of the surface and bulk. Particle sizes ranging from 50 nm to 10 μm have been investigated for several polymeric and non-polymeric carriers. However the only consistency found in the results is that particles 10 μm in size were not effective of transport of the antigens. In one study particles comprised of poly-(vinyl alcohol)-graft-poly-(lactide-co-glycolide) showed higher antibody titers of at ~100 nm and ~500 nm. But in a different study using PLGA particles, showed particles > 1000 nm had a better response. The suggested theory is that the particles are taken up by different pathways dependent upon their size; the smaller particles are taken up by receptor-mediated endocytosis and the larger by phagocytosis. No conclusive statements can be made with the current data, because there have not been studies done factoring in polymer type, specificity of antibodies generated, and arrangement and availability of the antigen in and on the particle.[8] The net charge on a polymer also has a considerable effect of the immunogenicity of the particle and the interactions between the antigen and polymer. Positively charged particles have an advantage considering the mucus surface and cell surface are negatively charged. Additionally positively charged molecules have better entrapment efficiency for a number of negatively charged antigens, mainly DNA and RNA vaccines. However there is a limit to how cationic the particles can be; some highly cationic liposomes exhibited charge-dependent cytoxicity in a cell culture test. Hydrophobic polymers such as poly-(ε-caprolactone) nanoparticles have shown increased in vitro uptake in comparison of less hydrophobic formulations but nanoparticles surface modified with PEG, a hydrophilic group, showed better penetration of the nasal mucosa. Despite the many positives of using mucosal delivery, injectable vaccines have been the preferred method because precise amounts of the antigen can be delivered and quantitatively measured in the blood stream. Determining

the exact dosage and level of response is much more difficult because of the difficulty in capturing mucosal antibodies.[8, 24]

Role of Immunology in Vaccination

Protection from disease or injury from pathogens and foreign objects is a result of a two-tiered counter-attack orchestrated by the immune system. The two components of the immune response are the innate and acquired response.[25]

Innate Immunity

The innate immune response is an early non-specific defense from a microbial attack. It is a three pronged defense system that consist of: a physical barrier, comprised of epithelial tissue; cellular protectors, that seek out and destroy harmful organisms; and plasma proteins, that aid in the identification and destruction of pathogens.[26] Once a microorganism permeates the epithelial layer, it encounters resistance by cellular mechanisms. One of the first reactions is the inflammatory response. Inflammation a rapid response to tissue damage is typically associated with swelling, redness, heat, pain, and loss of function. During this stage the microbe is met by phagocytic cells, macrophages and neutrophils and a type of lymphocytic cell called a Natural killer cell (NK).

Macrophages, which are differentiated monocytes, are the first phagocytic cells to recognize and respond to the foreign object via pattern-recognition receptors, glucan, lipopolysaccharide, integrins, cytokines, and mannose, a receptor unique to macrophages. Upon encountering the pathogens, macrophages are stimulated to release a number of cytokines. The cytokines associated with macrophages are tumor necrosis factor (TNF), interleukins, chemokines, and type 1 interferons (IFN). Several of these cytokines acts to initiate inflammation, this process will be discussed later in the chapter. Macrophages also engulf antigens and present the byproducts of these antigens on the surface for T cells to respond to.

Neutrophils are a type of polymorphonuclear leukocyte that can also recognize structures on or in certain microbes, bind to it, and destroy it upon internalization. Once the receptors bond to the markers, the macrophage has several mechanisms that it uses to destroy the pathogen. During the process of phagocytosis deadly toxins such as nitric oxide, and hydrogen peroxide are generated to destroy the contents inside. In addition to the phagocytic activity the macrophages will also release chemical signals called cytokines and chemokines that promote inflammation and attract other cells to the area.[21]

Natural killer cells (NK) are also lymphatic cells that act as extracellular killing machines. These cells are able to identify infected cells because they lack a surface component called major histocompatibility complex [MHC] class I, that is present on normal healthy cells. When the NK receptors, killer-cell inhibitory receptors, don't interact with the MHC, the NK releases cytotoxic molecules that will destroy the cell.

Complement proteins also respond very quickly to cover the microbe and target it for destruction. The complement system is the protein component of the innate defense system. The proteins are primarily proteolytic enzymes. These enzymes are activated in sequence, referred to as the complement cascade. It can be activated by the two pathways associated with the innate immune system. One, the alternative pathway, is prompted by the absorption of proteins onto the microbe surface. The second pathway is through lectin binding to mannose on microbial surfaces, which in turn stimulates proteins to switch on the classical pathway.

After approximately 24 hours the innate stage of the immune response is followed by the acquired immune response. This phase is initiated by the presence of professional antigen presenting cells that arrive during the early stages of the innate response to phagocize the invading pathogen and stimulate the effector cells of the adaptive immune response

Antigen Presenting Cells

The bridge between innate and acquired immunity is linked by the antigen presenting cells (APCs). Antigen presenting cells are activated dendritic cells (DCs), activated macrophages, and activated B cells.[27] The role of these cells is to activate T cells via class I and class II Major Histocompatibility complex and a co-stimulatory protein located on the cell surface.

As stated in the previous section, macrophages can operate in a phagocytic capacity. However they also have roles in cytokine production and antigen presentation.[28] After they engulf antigens and present the processed fragments on their surface, they can bind to receptors of the helper T cells (T_H). The binding process activates the T cell, which plays a significant role in acquired immunity.

The dendritic cell (DC) is the most potent antigen presenting cell. Therefore many vaccine and adjuvant strategies have directly attempted to target DCs. The cell functions by encapsulating antigens via macropinocytosis or receptor-mediated endocytosis, processing them and presenting a portion of the antigen on its surface to the T cell through both class I and class II Major Histocompatibility complexes (MHC). The internalization of the microbe stimulates the dendritic cell to transform from a naïve cell into a mature effector cell which possesses surface embedded co-stimulatory molecules. Their most significant role in adaptive immunity is their ability to strongly activate both memory T cells and naïve T cells. T cells function to either directly kill infected cells or to activate stimulate B cells to produce antibodies. The mature cell also works in the innate immune system to activate Natural killer cells (NK) and to secrete IL-12 cytokines. In attempts to directly target DCs biomaterial antigen-delivery vehicles have been that induce endocytosis through DC receptors such as mannose, or surface modified with DC-specific antibodies to bind DCs selectively. Additionally studies have shown that in an in vitro test polystyrene spheres showed optimal DC uptake of a particle diameter of 0.5 μm or less. It

was also noted that surface charge effects on particle uptake become more pronounced in particles > 0.5 μm.[21, 29, 30]

Acquired

If a pathogen has been able to evade the defenses of the innate immunity, the second line of defense is the adaptive or acquired immune system. Acquired immunity is moderated by two main cell types, T cells and B cells. Both B and T cells stem from a lymphoid precursor that originates from the bone marrow and are classified as lymphocytes. However B cells differentiate in the bone marrow and T cells differentiate in the thymus.[21] These cells circulate from the blood stream to the lymphatic system, mainly the spleen, lymph nodes and mucosal tissues where they have the highest likelihood of encountering pathogens. The interaction between B cells and pathogens gives rise to humoral immunity and the interaction between T cells and pathogens induces cell-mediated immunity.[31]

Humoral

Humoral immunity is a long-lasting protection that is mediated by B cells that generate antibodies. Its function is to combat microorganisms outside of the cell, therefore its protection is generally limited to the extracellular spaces and fluids of the body.[21] Once an antigen is recognized and bound to the B lymphocyte, a chain of events is begun that culminates in the production of unique antibodies for each antigen that generally continues throughout a lifetime. The first step in this process is B cell – antigen pairing through receptors. In the lymphatic system there exist many clones of B cells with different specificities for different antigens. Undifferentiated B cells, naïve cells, have immunoglobulins (Ig) located on their membrane surface and act as receptors that recognize and bind to the antigens. Immunoglobulins are plasma proteins, composed of two light polypeptide chains and two heavy polypeptide chains with a total molecular weight (MW) of about 150 kDa. There are 5 classes of immunoglobulins,

IgM, IgD, IgG, IgA, and IgE. Only the first two immunoglobulins are bound to the membrane and act as B-cell antigen receptors (BCR) Figure 2-1. These receptors can recognize many different chemical structures and shapes of a variety of macromolecules. This is possible because the antigen recognition region of the receptor is variable

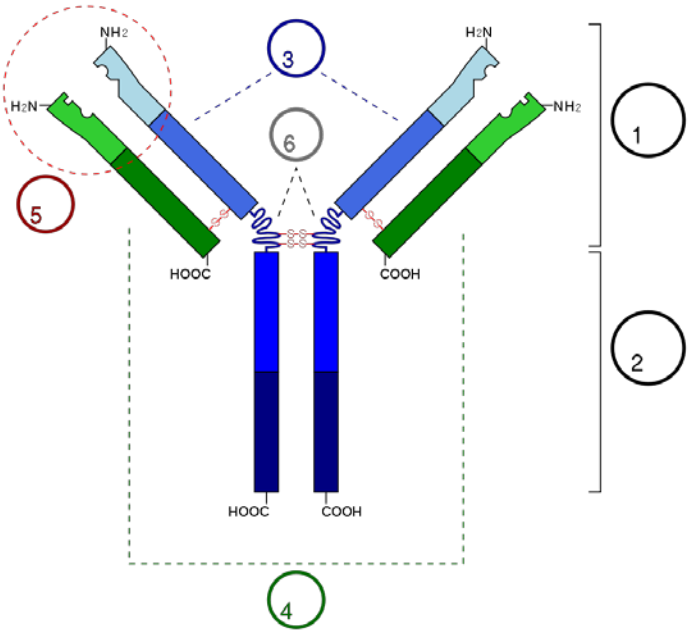


Figure 2-1. Basic antibody structure. Reprinted from Wikipedia.org[32]

When a threshold amount of antigen components such as polysaccharides, lipids, small molecules, and proteins bind to these receptors a sequence of chemical signals will be triggered, that will in turn help to activate the cell. However microbe recognition and binding isn't enough to activate the cell if the antigen is a protein. Additional signaling mechanisms are needed to transform naïve B cells to mature cells. The second step in antibody generation is co-signaling by CD4+ helper T. Helper T cells encounter APCs that have MHC peptide antigens exposed on the surface and binds to them. The coupling process stimulates the naïve helper T cells differentiate into effector CD4+ T cells that can now activate B cells through their class II MHC Figure 2-2.

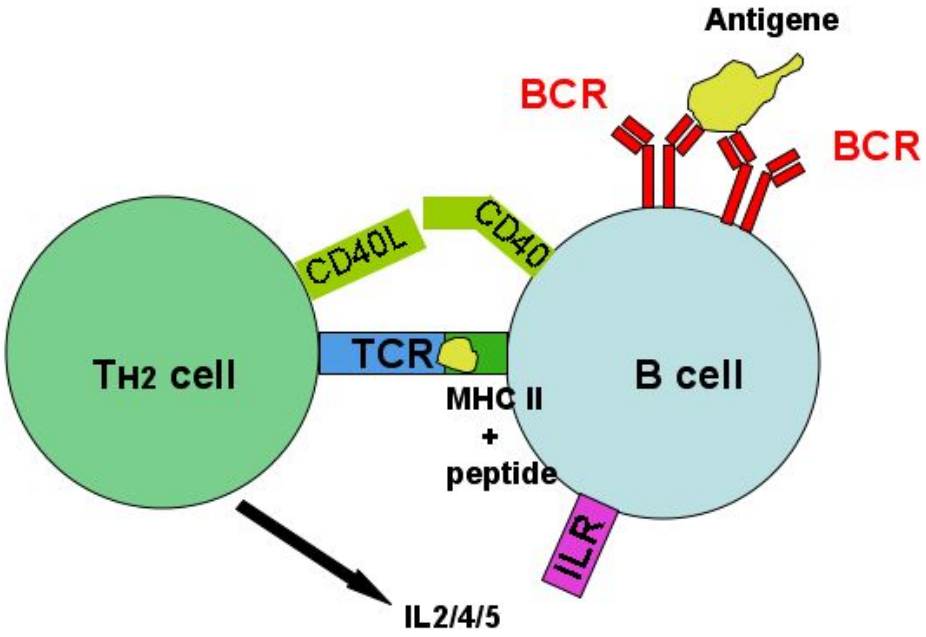


Figure 2-2. B cell binding to antigen and subsequent activation by T helper cell. Reprinted from Wikipedia.org.[33]

Once the B cell has fully activated the final step in antibody production is ready to begin. The activated B cell is prompted to secrete cytokines, IL-4 and IL-5, which cause the cell to undergo proliferation and differentiation into effector cells or memory cells. The effector or plasma cell can then secrete four classes of antibodies, IgM, IgA, IgG, IgE. The function of these antibodies depends on the structure of its heavy chain.

Complement plays a role in adaptive immunity as well as innate immunity. Complement proteins are also co-stimulators of B cell activation. By-products formed after complement activation, such as the C3d form a coating on the microbes that is recognized by a B cell receptor. After the IgM and IgG antibodies enter the blood stream and conjugate on the antigen, the humoral component of the complement protein cascade, called the classical pathway is initiated. The complements goal is to help the antibody in destroying the microbe. A plasma protein, C1, is activated after binding to the Fc portion of the antibody to form a C4b2a complex.

The complex then enzymatically breaks down the C3 protein to the C3b fragment which will stimulate the cascade is almost the same manner as the alternative pathway. The C3b fragment promotes opsonization and phagocytosis, cytolysis, and stimulation of leukocytes to destroy the microbe.[26]

The final role in adaptive immune response is to provide long lasting immune protection. After B lymphocyte activation, the cell could differentiate into antibody producing cells or cells memory cells that circulate within the blood system for years. These cells can be activated very quickly upon a second encounter with an antigen and produce higher levels of antibody titers.[34]

Cell-Mediated

Cell-mediated immunity is governed by the actions of two types of T cells, cytotoxic T lymphocytes (T_C) and helper T lymphocytes (T_H). This type of immunity provides a defense against cells that have been infected by microbes that have managed to evade the innate and humoral protection. The primary duty of the helper cell is to help in the stimulation and activity of other cells, such as the B cell. Cytotoxic T cells function mainly to kill infected cells. Both cell types have T cell receptors, CD4+ for T_H and CD8+ for T_C that recognize antigens presented by the class MHC of antigen presenting cells. T_H cells recognize the class II MHC and for T_C cells recognize the class I MHC. However they take very different paths upon activation. Helper cells are activated by the binding to the APC and by co-stimulator proteins that are found on the APC. Sometimes the co-stimulation is not sufficient or expressed and adjuvants are needed to help to force the APC to express the co-stimulator and to secrete the cytokines that can activate the T cell. After activation the CD4+ helper differentiate into two types of helper cells, T_H1 and T_H2, both cell types secrete a large amount of different cytokines, namely IL-2, IL-4, IL-5, IFN-γ, and TGF-β. These cytokines function primarily to stimulate T cell growth and

activation of other cell types. They also help in the activation of other cells. As discussed earlier CD4+ cells, T_H2, activate B lymphocytes to generate antibodies, however the other CD4+ cell type, T_H1 activates macrophages to kill the microbe contained inside.[26, 34]

When the naïve CD8+ receptor of cytotoxic T cells bind to the MHC of the antigen presenting cell, the T_C is partially activated, co-stimulators are needed for it to become fully mature. On dendritic cells the co-stimulators B7 are highly expressed and once the naïve CD8+ cell ligands to the MHC complex and the stimulator, the cytokine IL-2 is produced. This cytokine will now drive the killer cells differentiate and proliferate into mature cells. These mature effector cells can now target infected cells that display antigen peptide fragments on its surface and cause their death by triggering apoptosis.[21]

The overall role of the adjuvant in both arms of the immune response is helping the vaccine reach more antigen presenting cells and facilitating its uptake.

Vaccine Adjuvants

Current

Adjuvants are vectors designed to aid in increasing the intensity of the antibody response to an antigen.[7] Its main objectives are to augment the immune response, control the release of the antigen and stimulate macrophage activity, and guide the cargo to specific cell types.[6, 7] They are primarily combined with subunit vaccines because of the inability of subunits to provoke a potent immune response unassisted. These formulations are used for both human and livestock inoculations. Adjuvants prepared for use in livestock are primarily oil-water emulsions or entirely oil-based compounds. The most commonly used livestock adjuvant is the Freund's adjuvant. However these are not suitable for use in humans because of the formation of granulomas.[6] Worldwide the only approved adjuvants for human use are aluminum based salts. They have been use for over 70 years and include compounds such as aluminum

hydroxide, aluminum hydroxyphosphate, potassium aluminum sulfate (alum).[6, 35] Aluminum adjuvants have a long history of use, and have been proven to be safe for human use. These adjuvants are prepared by either precipitation or absorption of the antigen onto the aluminum gel. The particles formed are generally < 10 microns in diameter.[35] The exact mechanism of action is not completely understood. The two most accepted theories for increased immune response are by immunostimulation and antigen depot formation.[35] Via the immunstimulation route, alum adjuvants are thought to increase the immune response in 3 ways. The first method is by activation of antigen presenting cells, the second is stimulating the type 2 immune response and activation of the complement cascade. The depot effect of the alum adjuvants describes how the alum is able to slow the release of the antigen from injection site, so that an effective level of antigen is released over a period of several weeks. The biggest drawbacks for using alum adjuvants are that they can be only be delivered by injection, cannot invoke a cell-mediated response, cannot be used with certain vaccines, and cause hypersensitivity.

Alternative Materials

There are several different classes of materials that are recognized as vaccine adjuvants. The materials exist in the forms of emulsions, particulates, liposomes, lipids, polysaccharides, cytokines, and host-derived complexes among others.[36, 37] They are further characterized by the five main modes of action that adjuvants can function in: immunomodulation, presentation, induction of cytotoxic T-lymphocyte response, targeting, and depot generation.[36] Each material type has one or mode that makes it a potential candidate for adjuvancy use. Currently the majority of adjuvants that have made it to U.S. human clinical trial status fall under the classifications of host-derived, toxins, liposomes, and cytokines. The only polymers that appear to be represented at the human clinical trial level are PLG microparticles for use in treating HIV and chitosan for treating Norwalk virus.[6, 38] However three polymer classes, polyesters,

polysaccharides, and polyanhydrides, are being tested in animal models in a variety of forms. The subsequent sections will summarize briefly the non-polymeric adjuvants that have shown substantial promise followed by a more in-depth explanation of the polymers.

Host-derived

An obvious choice to invoke a greater immune response in vaccine delivery is using adjuvants made of microorganisms components. These host-derived adjuvants include virosomes, bacterial lipopolysaccharides, and TLR-2 ligands.[6] The strongest mode of action for this class is through immunomodulation. Virosomes are particles that are comprised from the extraction of a viral envelope through detergent washing and purification.[39] They do not contain DNA, so they cannot infect. But because their proteins appear analogous to infectious antigens, they bind readily to B cell receptors and which will provoke a strong signal to activate differentiation and proliferation. They have been derived from fowlpox, canary virus, and vaccine virus to treat cancers of the breast and prostate and solid tumors.[6]

Toxins

Bacterial toxins derived from diphtheria, cholera, and Escherichia coli have been developed to act as adjuvants to treat HIV, tuberculosis, and hepatitis B.[6] Their mode of action has not been completely investigated, but they act with a high degree of potency at the mucosal surface.

Liposomes

Are small vessels formed by primarily by the association of phospholipids in to micelles that can range from 50 nm- several microns in diameter. Other molecules such as cholesterol, cholate, and surfactants are commonly added to it to moderate the rigidity of the sphere.[37] Liposomes tend not to elicit a potent immune response. Instead their primary mode of action is thought to be in targeting of the antigen to the APC. These particles have gone to clinical trial

incorporating antigens for disease such as malaria, hepatitis A, influenza, meningitides, and shigella.[37]

Cytokines

This class of adjuvants directly targets the immune system. The cytokines commonly used are IL-1 (pro-inflammatory), IL-2 (lymphoproliferative), IL-12, and GM-CSF(granulocyte-macrophage-colony stimulating factor). They are expensive, unstable, and do not survive long in vivo, which limits use in treating infectious disease. However their primary purpose is in immunotherapy for cancer. Currently they are being tested to treat HIV, melanoma, lymphoma, and cancers of the lung, ovary, and prostate.[6, 40]

Polymeric

Poly(lactide-co-glycolide)

Poly(lactide-co-glycolide), PLGA, are polyesters, comprised of alternating monomers of poly(lactic acid) and poly(glycolic acid), Figure 2-3. For over 20 years polyesters have been extensively studied for use as an adjuvant.[40] They are ideal because they are biocompatible, bio-resorbable and its byproducts can be metabolized in the citric acid cycle.[41] The polymer had FDA approval and has been extensively used in the medical device industry in to fabricate sutures, coatings, grafts, inserts, scaffolds, and particulates, both in the microparticle and nanoparticle range.[42, 43] Particles in the micron size range have traditionally been fabricated using oil-in-water emulsion solvent evaporation techniques, double emulsion and coacervation[44]. But later procedures have transitioned into using a spray-drying protocol which improves the loading capacity of water-soluble drugs and particle uniformity and dispersion [45-48] Particles less than 1 micron are typically prepared by, emulsion diffusion, solvent diffusion, emulsion evaporation, and nanoprecipitation mehods.[43] The encapsulated

agent generally is released through bulk erosion of the particle as the polymer's ester bonds are degraded by hydrolysis.[49]

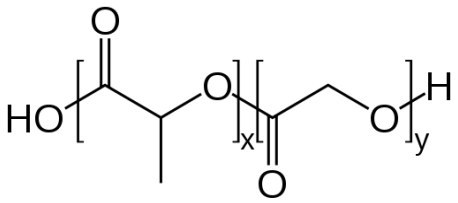


Figure 2-3. Structure of PLGA. Reprinted from Wikipedia.org[50]

Qualities that have made PLGA even more suitable as a vaccine adjuvant are its ability to continuously release antigens over an extended period of time, thereby eliminating the need for a vaccination schedule of repeated injections.[51] This attribute is particularly important in Third World nations where access to supplies and medical staff are limited. PLGA has proven that it is capable of uptake by antigen presenting cells and can specifically target dendritic cells.[41] Additionally PLGA microspheres have been shown to invoke a cytotoxic T-cell response, which is an important step in cell-mediated immunity. The microspheres to date have designed to be administered orally, subcutaneously and intranasally.[3, 52, 53] They have also been used to encapsulate antigens for infectious diseases such as influenza, tuberculosis, malaria, and tetanus.[53-56] However, there are some disadvantages to using PLGA. One is that PLGA is not inherently bioadhesive, it must be modified or combined with other polymers to improve its adhesiveness. This is of importance when attempting to deliver vaccines to mucosal surfaces of the body. The second disadvantage is that PLGA is not soluble in aqueous solutions; therefore encapsulation of water soluble drugs is limited. The third disadvantage is cost and availability. And lastly because PLGA has been so heavily researched, since 2001 there have been 197 patent

applications submitted involving the use of PLGA as a vaccine adjuvant which could limit the potential avenues to obtaining intellectual property rights on upcoming research.

Chitosan

Chitosan is a naturally occurring biodegradable polysaccharide. It is derived from the partial deacetylation of chitin, a substance found in the shells of crustaceans. The polymer is found abundantly throughout the word and is relatively inexpensive. Its chemical structure consists of repeat units of β-(1,4)-linked-D-glucosamine see figure 2.3 substituted with units of N-acetyl-D-glucosamine at varying degrees see figure 2.4. The amino groups on the glucosamine monomer give chitosan a positive charge which contributes to its mucoadhesive characteristics. Chitosan has been found to be non-toxic, and degrades enzymatically first by lysozymes into oligosaccharides, and then is further hydrolyzed by the enzyme N-acetyl-β-D-glucosaminidase.[57, 58] Chitosan is a FDA approved chemical and has found widespread use in the food, cosmetics, pharmaceutical and medical device industry.

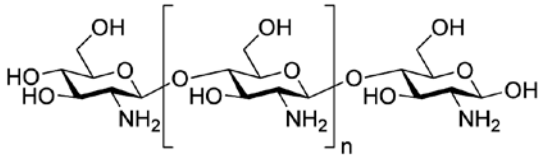


Figure 2-4. Structure of chitosan. Reprinted from Wikipedia.org[59]

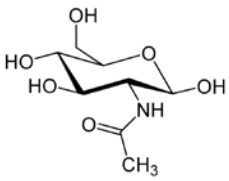


Figure 2-5. Glucosamine unit with acetyl group. Reprinted from Wikipedia.org.[60]

There are several significant biological and chemical properties that make it an ideal candidate for adjuvant use. The mucoadhesive nature of chitosan makes it ideal for binding to

cell and tissue surfaces, and is efficient at paracellular transport. A 1984 chitosan study established that chitosan could stimulate the immune system by strongly activating macrophage and NK cells. A subsequent animal study showed it increased antigen-specific antibody titers when combined with Freund's adjuvant.[61, 62] Chitosan has been formulated for adjuvant use as a solution, hydrogel, powder, microsphere, and nanoparticle, and due to its water solubility, can be prepared without the use of organic solvents that could alter the antigen functionality.[63-66] Formulations have been used to incorporate antigens for influenza, pertussis, diphtheria, and tetanus.[64, 65, 67] They have been tested in animal models and at least one human model. Chitosan appears to be the only other polymer besides PLGA that has made it to human clinical trials. A nasally administered adjuvant for the treatment of Norovirus is currently being tested by LigoCyte Pharmaceuticals Inc.[38] Some of the disadvantages shown when using chitosan as an adjuvant, are that when protein loaded particles are administered through non-mucosal means the immunostimulatory reaction is low.[66] Some studies have indicated that any enhanced immune response by chitosan is due more to the adherence and penetrative abilities of the polymer and not as much to the actual material mediated immune response. Additionally whereas chitosan is soluble in aqueous solutions, it is only under acidic conditions, which made degrade the antigen.

Gelatin

Gelatin is a linear protein that is chemically derived from type I collagen. It is made up of repeating units of approximately 18 amino acids with glycine, proline, and hydroxyproline being the most frequently found Figure 2-6.[68] It is generally soluble in water and insoluble in organic solutions and at low temperatures. Gelatin is amphoteric and has isoelectric point of 7.0 – 9.0 for Type A and 4.7 – 5.2 for Type B, which allows for a range of positively or negatively

charged water soluble molecules can be used with equal effectiveness. Gelatin carriers are degraded through enzymatic hydrolysis by collagenase.[69, 70]

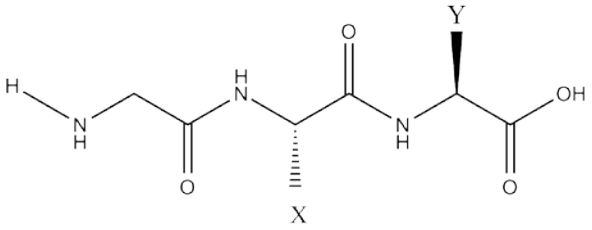


Figure 2-6. Structure of gelatin. X and Y are various amino acid side chains

Gelatin microspheres and nanoparticles have been used to encapsulate a number of proteins, peptides, growth factors, plasmid DNA, chemotherapeutic agents and other pharmaceuticals.[71-74] The first documented instance of gelatin microspheres considered for use as an adjuvant was a 1995 paper that examined whether protein loaded gelatin microspheres could induce an increased antibody response.[75] Their research showed that gelatin increased the level and duration of antibody production of time compared to Freund's adjuvant, and they concluded this was due mainly to the particles release of the antigen over an extended period of time and the ability of macrophages to phagocytize the 2.5 micron sized particles. Following this study only a few studies were published on the use of gelatin microspheres as a vaccine adjuvant.[76] More recently there has been investigation into the use of gelatin nanoparticles as potential adjuvants.[77, 78] Gelatin nanoparticles are typically prepared by a desolvation method, a procedure first refined to its present form by Coester et al.[79] In this technique a nano-sized particle containing the therapeutic agent is precipitated out of an aqueous solution by the addition of an organic solvent. The gelatin nanoparticles that were produced in the later research focused primarily on targeting dendritic cells, where a nano-sized particle would be more ideal. There are several advantages to using gelatin. The first advantage being that it is inexpensive and widely available from a number of different sources. This particularly

important because the total cost benefit ratio of the vaccine must be surpass what is currently available. The second advantage is that gelatin nanoparticles have proven that they can be freeze-dried and rehydrated without appreciable changes in size, agglomeration, or biological activity of its payload over a period of 4 weeks.[13] The disadvantages of gelatin are its lack of mucoadhesiveness in comparison to polymers such as chitosan. However because of the availability of a number of different functional groups on the gelatin surface and this can be improved with surface modification.

CHAPTER 3
PREPARATION AND CHARACTERIZATION OF THIOLATED GELATIN NANOPARTICLES

Introduction

Mucoadhesion refers to the binding of two surfaces to each other, at a mucosal interface in a biological environment. The mucus present at the mucosal surface is primarily composed of a viscoelastic solution of water, glycoproteins, and lipids. In the body this substance is present on the surfaces of the oral, ocular, nasal, gastrointestinal, and vaginal pathways among others. The utilization of these mucosal delivery routes for pharmaceutical applications is ideal due to the desire for localized sustained drug delivery. The adhesion of the drug to mucosal surfaces increases retention time which allows for longer release times, fewer doses, minimization of systemic effects, reduced loss of product and lower cost.

Mucoadhesive Polymers

Common mucoadhesive polymers include chitosan, polyacrylic acid, polycarbophil, lectin, sodium alginate, carbomer, and hyaluronic acid; they can be positively or negatively charged or neutral. These polymers are generally unmodified are not site-specific and are sometimes referred to as first generation.[80, 81] Newer bioadhesives are typically polymers modified with a variety of components that promote cell binding through sugar molecules or increase the positive charge density to be more attractive to negatively charged cell surfaces.[82] More recently, the use of thiolated polymers called thiomers has shown a marked improvement in adhesiveness compared to their unmodified state in polymers such as chitosan, poly (acrylic acid), poly (methacrylic acid), alginate, and sodium carboxymethylcellulose.[83]

Thiomers

Thiomers are polymers that have side chains containing sulfa-hydryl (thiol) groups. These sulfur containing groups can form disulfide bonds with the sulfur groups present on mucosal

glycoproteins. The reactions occur through one of two mechanisms, oxidation or thiol-disulfide exchange Figure 3.1, 3.2. Sulfur-sulfur bond energies are approximately 226 kJ/mol. These bonds are stronger than the typical non-covalent bonds found in most mucoadhesive interactions hence they are able to generate stronger mucoadhesive properties.[84]

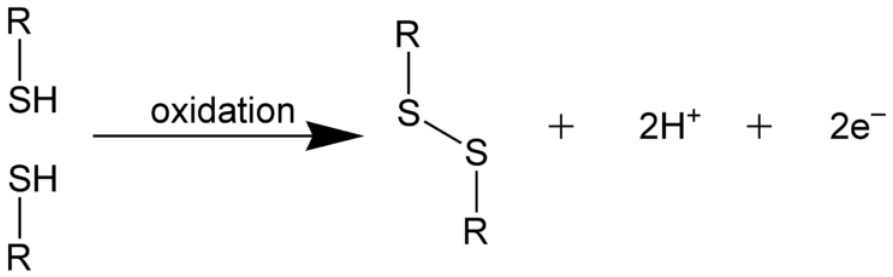


Figure 3-1. Disulfide bond formation through oxidation. Reprinted from Wikipedia.org[85]

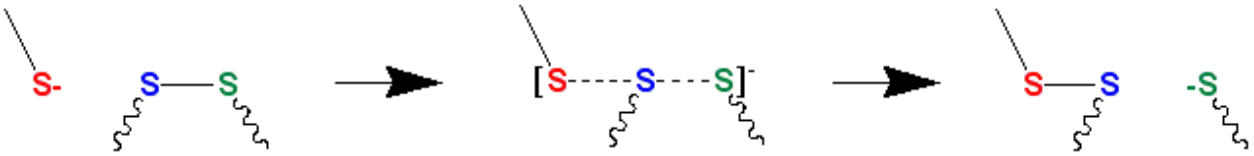


Figure 3-2. Disulfide bond formation through thiol-sulfide exchange reaction. Reprinted from Wikipedia.org[86]

To introduce these thiol groups on the polymer ligands such as cysteine were commonly used at first, then cysteine derivatives such as cysteamine and homocysteine were used for anionic polymers and newer thiol moieties such as 2-iminothiolane have been introduced for cationic polymers. The advantage of using this reagent is that reaction does not require any co-reagents to proceed and oxidation of the thiol group is minimized by the reagent. The first thiolated polymer created was chitosan modified with cysteine, followed by thiomers of alginate, poly(acrylic acid), poly(methacrylic acid), among others. However the thiolation of gelatin is a relatively unexplored field.

Thiolated Gelatin Nanoparticles

Gelatin in its natural forms is not significantly mucoadhesive. A review of the literature shows that use of gelatin modified or unmodified as a mucoadhesive substrate is very limited. There have only been a few published attempts to increase its mucoadhesiveness by thiol modification or other means. And none of these methods have been considered for use in vaccine delivery.

The objectives for this chapter were to perform a pilot study to understand the nature of particle formation on unmodified gelatin, and to determine the variables ideal for creating thiolated gelatin nanoparticles in a size range suitable for dendritic cell engulfment and to prepare and characterize the thiolated gelatin polymer and nanoparticles with varying degrees of modification. Henceforth the modified gelatin samples will denoted as 0-SH, 20-SH, 40-SH, and 80-SH. They refer to unmodified gelatin, gelatin incubated with 20 mg of iminothiolane, 40 mg of iminothiolane, and 80 mg of iminothiolane respectively.

Materials and Methods

Materials

The following chemicals were purchased from Sigma-Aldrich Company: Type B gelatin from bovine skin bloom strength 225, 2-iminothiolane (Traut's reagent), 5,5'- dithiobis(2-nitrobenzoic acid) (Ellman's reagent), potassium phosphate monobasic anhydrous, sodium phosphate dibasic heptahydrate, and albumin-fluorescein isothiocyanate conjugate. The following chemicals were purchased from Fisher Scientific: Acetone, ethanol, 2-propanol, glutaraldehyde solution 25%, L-cysteine, Ethylenediaminetetraacetic acid (EDTA). Distilled water used throughout all phases of the experiments.

Methods

Thiolated Gelatin Preparation

To prepare the conjugated gelatin, 1 gram of gelatin was added to 100 ml of DI water at 40°C and stirred on a hot plate until the gelatin appeared to be completely dissolved. The solution was cooled to room temperature under continuous stirring. A predetermined amount of 2-iminothiolane was added. Solutions were prepared for 2-iminothiolane at 20 mg, 40 mg, and 80 mg. The 2-iminothiolane was stirred into the gelatin solution for a period of 24 hours at room temperature and covered by parafilm. To remove the unreacted 2-iminothiolane, the gelatin solution was poured into dialysis membrane bags and dialyzied against 5mM HCl for a period of 24 hours. This step was followed by a repeated dialysis in 1mM HCL for an additional 24 hours. The solutions were left in liquid state and stored in the refrigerator at 4°C. The exact concentration of each solution was determined by gravimetric analysis before use.

Preparation of Gelatin Nanoparticles

20 ml of the thiolated gelatin was heated on a stir plate to 40°C until solution is evenly heated. The solution is adjusted to varying pHs (ranging from 2 – 10.5) during preliminary studies. A pH of 7.0 ± 0.05 was used for nanoparticles prepared for characterization. To prepare the unmodified gelatin control sample, 200 mg of gelatin is dispersed into DI water heated to 40°C and stirred until the gelatin is completely dissolved. The pH was adjusted using dilute HCL and NaOH. The solution is then transferred into a round bottom flask and stirred at 200 rpm. The solution is heated by a water bath to 40°C. The solvating agent was then added via a syringe dropwise into the gelatin solution until nanoprecipitation occurs, a volume of 25 ml. Immediately after all the solvent is added, 50µl of 25% glutaraldehyde is added to crosslink the particles. Crosslinking by glutaraldehyde is due to the reacts with primary amines found in

protein, predominately from the lysine contribution to form bonds. However glutaraldehyde can also react with thiols to form thioacetals (Equation 3-2).[87]

$$RSH + R'CHO \rightarrow R'CH(OH)SR \quad (3\text{-}1)$$

The solution is allowed to react at for 2 hrs, in a closed environment, under continuous heating. The resultant particles are then centrifuged by a Beckman J2-21 Centrifuge for 1 hour at 8,000 rpm. The supernatant is removed and the particles are re-dispersed by washing with a 70:30 deionized water (DI) to ethanol solution, vortexing for 2 minutes and sonicating for 2 minutes. The wash step is repeated 3 times. The final suspension of particles is stored at 4°C until further treatment or prepared for freeze-drying. The freeze-dried particles are frozen with liquid nitrogen for 12 hours. The dry particles are stored at 4°C in the freezer until further use.

To make the loaded nanoparticles to be used for the release studies, a 1% gelatin solution using unmodified and thiolated gelatin is prepared at 40°C. The pH is adjusted to 7.0 ± 0.05. Bovine Serum Albumin-fluorescein isothiocyanate (FITC-BSA) is added at 1% (w/w) to the gelatin solution. After the FITC-BSA is completely dissolved in the gelatin, the solution is transferred into a round bottom flask and heated continuously at 40°C. 25 ml of acetone is added dropwise followed by the addition of 50 µl of 25% glutaraldehyde. After 2 hours, the particles are removed from the heat and stored in the refrigerator at 4°C until washing. The same procedure is followed for washing and storing unloaded particles is used from the previous paragraph.

Analysis of Thiolation

To quantify the amount of thiol groups added per gram of gelatin a colorimetric reaction was used. 0.1 mM EDTA solution is made in a 0.1 M sodium phosphate buffer solution adjusted to a pH of 8.0. A 4mg/ml of Ellman's reagent was prepared in DI water and stirred continuously

to keep the reagent in suspension. Ellman's reagent is not easily soluble in water. To initiate the reaction, 500 µl of each modified gelatin and the unmodified control gelatin was added to 5 ml of the phosphate buffer, followed by 100 µl of the Ellman's solution. The solution was allowed to react for 15 minutes at room temperature before being analyzed by a Shimadzu UV160 U spectrophotometer. Cysteine standards were prepared in the same manner as the samples. The standards ranged in concentrations from 0.0001% to 0.1%. The concentrations were converted to -SH concentrations, and used for the calibration curve. The concentrations of thiol were extrapolated from the curve.

Characterization of Nanoparticles

Particle size of loaded and unloaded nanoparticles is determined using the Nanotrac Particle Size Analyzer, Model NPA150 (Microtrac, Inc., Montgomeryville, PA). The instrument has a measurement range between 8 nm and 6.54 µm. A photo-detector obtains data on the mean diameter when the sample particle scatters light generated by the diode laser. The number average mean diameter is used to report size and volume average and number average mean is used to determine the polydispersity index. To prepare samples for analysis, one drop of the particle suspension is dispersed into a cuvette containing approximately 3 ml of deionized water and analyzed at room temperature.

To evaluate the particle morphology, particles were examined with a Field Emission Scanning Electron Microscope. Two separate preparation methods were used to visualize particle. To prepare wet mount samples, an aluminum stub is coated with carbon black tape and a mica substrate is attached to the tape. A very dilute drop of the washed nanoparticles is placed onto the mica. The sample is dried overnight at room temperature. The sample is then sputter-coated with gold. To prepare dry mounted samples, the aluminum stub covered with carbon black tape containing the mica substrate is used. The nanoparticles are washed, frozen in liquid

nitrogen, and freeze-dried overnight. The dry particles are deposited onto the mica and then coated with gold or gold-palladium.

The Brookhaven ZetaReader was used to determine the zeta potential of the nanoparticles. The ZetaReader uses electrophoretic light scattering to measure the electrophoretic mobility of the particle. Zeta potential is calculated from the measured mobility. The particles were examined in DI water under dilute conditions. The zeta potential is used to give an estimate of the surface charge of the particle.

A Microplate Reader, Molecular Devices Corporation, was used analyze release data. SoftMax Pro was the software used to run the instrument. In vitro release studies were used to determine the release profile of the control and modified gelatin nanoparticles. The FITC-bovine serum albumin was used as the model antigen. A 1% solution was prepared by for the control and the modified gelatin and heated to 40-45°C to ensure a uniform blend then the solution is adjusted to pH 7.0 ±0.05. After cooling the solution to room temperature 2 mg (1% w/w) of FITC-BSA was added and allowed to mix for 10 minutes. The suspension was transferred to a round bottom flash, stirred at 200 rpm and heated to 40°C. The nanoparticles were formed by the dropwise addition of 25 ml of acetone. After two hours the particles were collected, centrifuged at 8,000 rpm for 1 hour, and washed until the supernatant was visibly clear. The particles were then frozen by liquid nitrogen, and freeze-dried for 14 hours. A phosphate buffer solution (PBS) was used as the releasing media for the particles, and 1.5 ml of the media was added to microcentrifuge vials. Twenty milligrams of the loaded particles were put into each of the vials and incubated at 37°C in a water bath. At 1 hour intervals, for a period of 6 hours, a 0.5 mL aliquot of the supernatant was drawn. The samples were centrifuged at 10,000 rpm for 5 minutes prior to each removal and 0.5 mL of fresh buffer is added to the vial after retrieval of the

released supernatant. The concentration of the samples was analyzed for fluorescence. The excitation wavelength of FITC-BSA is 495 nm and the emission wavelength is 510 nm. Encapsulation efficiency was determined by the following equation. The loaded nanoparticles were completely dried by freeze drying and the remaining particles weighed and the percent yield was determined.

$$EE = \frac{\text{Actual Loading}}{\text{Theoretical Loading}} \times 100 \qquad (3\text{-}2)$$

Results and Discussion

Thiolation of Gelatin

The amine group located in the ring structure of 2-iminothiolane, reacts with the primary amines of the gelatin amino acid side chain Figure 3.3. However the reactions between the iminothiolane and the lysine dominate due to the stability associated with primary amines with pKa values 9.5. Amines with pKa values near 8 or lower don't retain the thiolation and decays to a non thiol product.

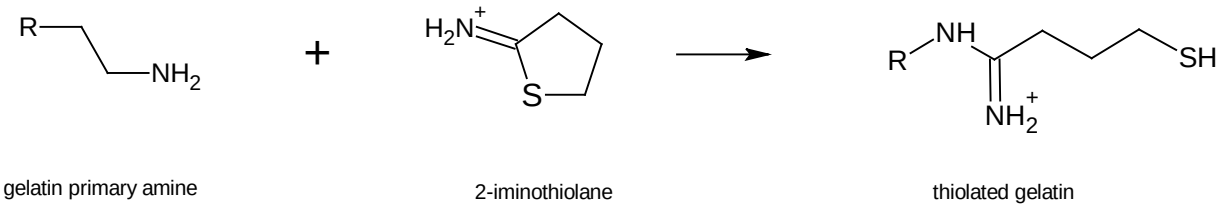


Figure 3-3. Reaction between gelatin and 2-iminothiolane

Type B gelatin has 4.4 grams of lysine per 100 grams of gelatin that can react with the iminothiolane see A-1. In this experiment 20, 40, and 80 mg of 2-iminothiolane was incubated with 1 gram of gelatin dissolved in 100 ml of DI water. 5,5'-Dithio-Bis(2-Nitrobenzoic acid), Ellman's Reagent, was used to detect how many sulfide groups were attached to the protein. The aromatic disulfide groups on Ellman's reagent react with the thiol groups on the gelatin to form a

disulfide that has an excitation wavelength at 412 nm.[88] Using a Shimadzu UV spectrophotometer the concentration of mmols of thiol per gram of gelatin in the sample was determined Figure 3-4.

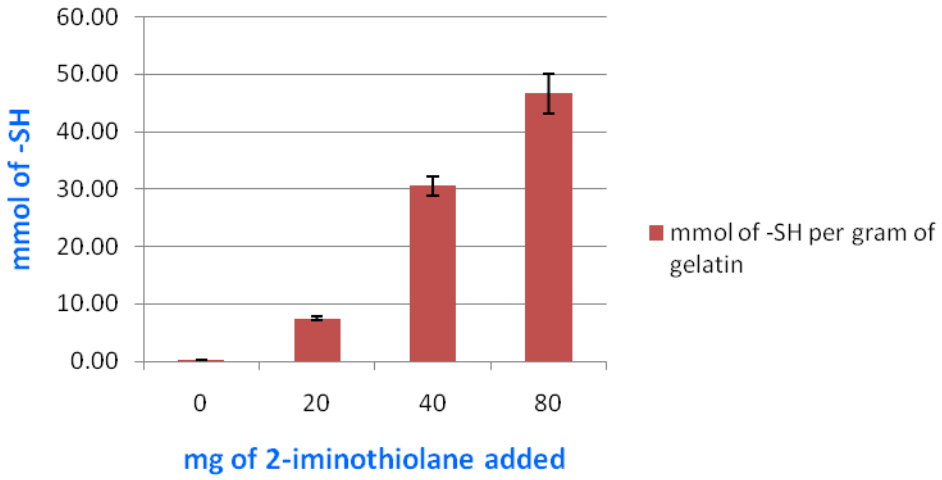


Figure 3-4. The amount of thiol groups present on gelatin. N=3

Gelatin with no thiol modification showed a value and standard deviation (SD) of 0.17 ± 0.17 mmol/g of gelatin. Gelatin incubated with 20 mg of iminothiolane had a value of 7.48 ± 0.34 mmol/g of gelatin. Gelatin incubated with 40 mg of iminothiolane had an average value of 30.53 ± 1.71 mmol/g of gelatin. And gelatin incubated with 80 mg of iminothiolane had an average value of 46.75 ± 3.50 mmol/g of gelatin. The results showed the expected increase of sulfhydryl groups present on the gelatin with amounts of iminothiolane added. However the increase was not linear. This indicates that not all of the iminothiolane present reacted with the gelatin. This excess iminothiolane was removed during the purification process, where the modified gelatin was dialyzed against mmol concentrations of HCL in DI water.

Particle Preparation

Particle formation occurs by the precipitation of gelatin nanoparticles due to the addition of the solvent to an aqueous solution. At certain water to solvent ratio dependent primarily upon pH and polymer concentration, the gelatin chains are surrounded by the solvent and begin to form dispersed spheres. This phenomenon is called desolvation and is used to form nanoparticles from proteins such as gelatin or albumin. This process is dependent upon several factors: isoelectric point of the gelatin, pH of the solution, type of solvent added, amount of solvent added, solution concentration, total volume, and temperature. These factors were evaluated in a preliminary study on gelatin particle formation. All of the particles were made at a concentration of 1% gelatin with a total volume of 20 ml, adding acetone as the solvent at a ratio of 1.25:1, while stirred at 200 rpm under constant heating at 40°C. A description of the changes at each stage of the reaction is detailed in figure 3.5. The initial gelatin solution after heating to 40 degrees is clear with low viscosity. As the acetone is added dropwise to the gelatin solution there is no change in solution appearance until the ratio of acetone to the aqueous gelatin solution is 1:1. At this point 20 ml of acetone has been added and the solution develops a slight white haze which indicates nanoparticle have begun to form. In earlier experiments there were attempts made to collect particles at this stage but recovery was relatively low. After 20 ml of acetone have been added each dropwise addition of more acetone increases the turbidity of the solution and the appearance changes from clear to translucent. After 25 ml of acetone have been added the solution the solution is whitish in appearance and a larger amount of gelatin nanoparticles can be recovered. Increasing the addition of acetone beyond this point generally results in broader size distributions.

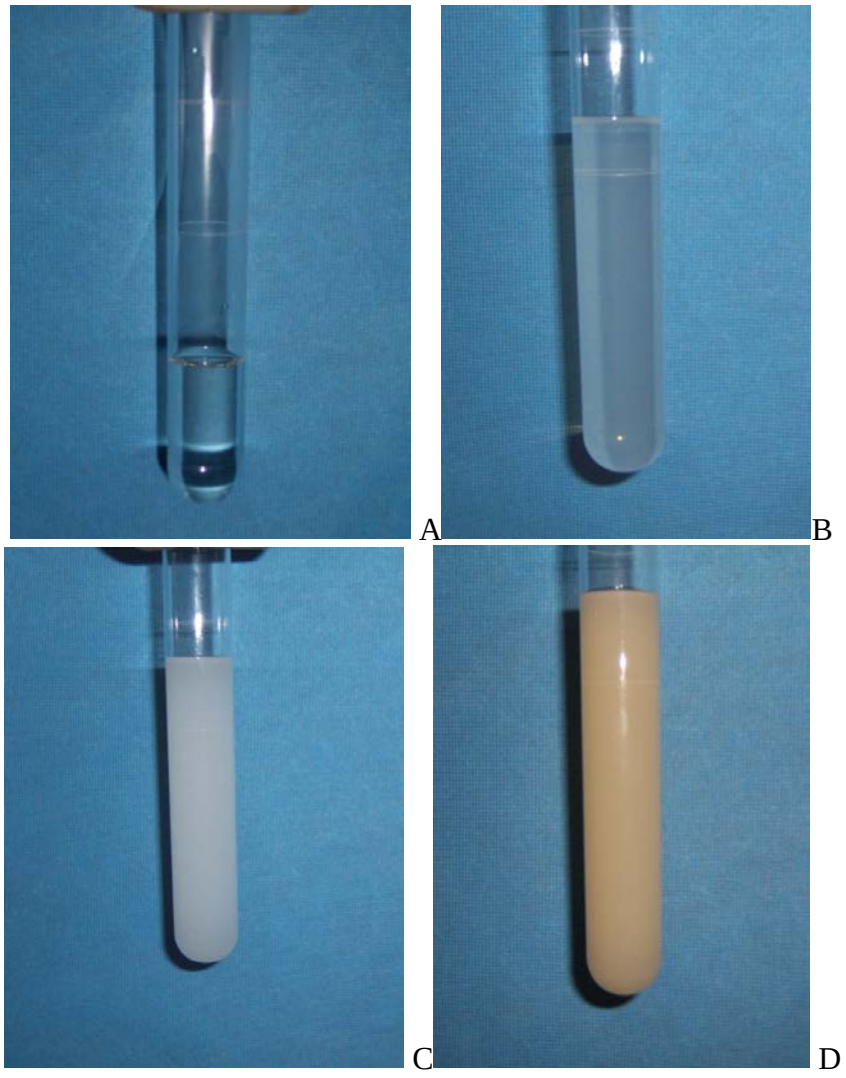


Figure 3-5. Reaction sequence of gelatin solution. A)0.1% gelatin solution. B)beginning of nanoprecipitation, 80% of solvent added. C)100% of solvent added. D) final solution after addition of crosslinker and 2 hours of heating.

Once all of the solvent had been added, 50 µl of 25% glutaraldehyde was added to crosslink and stabilize the particles. After 2 hours of heating the solution is now almost opaque and has turned light orange in color, which is similar in color to how the gelatin appears before it is dissolved in water. Without the addition of glutaraldehyde the gelatin solution would remain whitish and semi-translucent in appearance. Unmodified gelatin can form stable nanoparticles without a crosslinking agent, but attempts to form nanoparticles on the modified gelatin without

the crosslinker had limited success. The particles were largely unstable and formed very large structures or precipitated out of the solution and absorbed onto the glass of the flask and onto the surface of the centrifuge tubes used to contain them. Particle characterization was unsuccessful for the 40 mg and 80 mg of SH gelatin. Neither the Brookhaven nor the Nanotrac could fit the data to an instrument model and the results were inconclusive.

Particle Characterization

The particles were analyzed within 48 hours of preparation. They were analyzed diluted, at room temperature for the Nanotrac and the Brookhaven. The mean number average (MN) diameter for the unmodified gelatin nanoparticle was 170.8 nm the overall MN for the modified gelatin nanoparticles was approximately 275.23. There did not appear to be any statistical difference in average particle diameter among the modified gelatins Table 3-1. The size range is well within in the size tolerance for engulfment by antigen presenting cells. Dendritic cells have an optimal phagocytic range of 100 – 500 nm, and macrophages can engulf particles as large as 15 microns.[89] The polydispersity index (PDI) compared the mean number average diameter and the mean volume average diameter (MN/MV) and showed that the particles were not completely uniform in size. A PDI of 1.00 would indicate perfectly monodisperse uniform particles. The MV average was higher due to a small amount of large diameter particles. However this particle size did not represent the majority which is why the mean number average was used to represent the diameter. The zeta potential (ξ-potential) measurements indicate that the surface of the particle is negatively charged. The magnitude of the charge increases with an increase in thiolation. Zeta potential numbers < ±30 mV generally indicate a less than stable system. Unstable systems tend to aggregate and the smaller the ξ magnitude the greater the instability and aggregation. The magnitude of the ξ-potentials for the nanoparticles was between 20-30 mV. Potentials in this range is where aggregation begins to occur. This phenomenon was

observed experimentally when attempt was made to form the particles without a crosslinker, and particles with the higher thiol modifications would form initially and then destabilize. This instability is also reflected in the PDI, as the values get further from 1.00 as the degree of thiolation increases.

Table 3-1. Unloaded gelatin nanoparticles

Amount of iminothiolane (mg) per gram of gelatin	Degree of thiolation (mmol/g gelatin)	Mean diameter of nanoparticles (nm)	PDI	Zeta potential (mv)
0 mg (gelatin)	0.17 ± 0.17	170 ± 20	0.63	-21.48 ± 2.01
20 mg (SHGel)	7.48 ± 0.34	267 ± 30	0.64	-25.24 ± 1.50
40 mg (SHGel)	30.53 ± 1.71	272 ± 58	0.61	-27.30 ± 0.79
80 mg (SHGel)	46.75 ± 3.50	287 ± 54	0.55	-28.59 ± 3.02

PDI =polydispersity index. Mean ± SD (n=3)

Table 3-2. Gelatin nanoparticles loaded with FITC-BSA

Amount of iminothiolane (mg) per gram of gelatin	Degree of thiolation (mmol/g gelatin)	Mean diameter of nanoparticles (nm)	PDI	Zeta potential (mv)
0 mg (gelatin)	0.17 ± 0.17	256 ± 48	0.76	-35.42 ± 9.20
20 mg (SHGel)	7.48 ± 0.34	241 ± 6	0.77	-33.05 ± 8.49
40 mg (SHGel)	30.53 ±1.71	232 ± 54	0.77	-36.29 ± 4.39
80 mg (SHGel)	46.75 ± 3.50	260 ± 97	0.81	-39.73 ± 0.99

The addition of FITC-BSA to the solution also produced particle that were in the targeted range for uptake by dendritic cells Table 3-2. However the addition of the protein made the particle more negatively charged. FITC is an uncharged molecule so the charge effect must be a result of the BSA. Loading of the nanoparticles appeared to decrease particle size in all samples excluding the unmodified gelatin figure 3-6. This response was unexpected. The MW of the FITC-BSA compound is approximately 66kD, with the additional mass of the protein it was

expected the size would increase.[90] A possible explanation for this is due to increased internal crosslinking which can lead to decreased particle diameter.

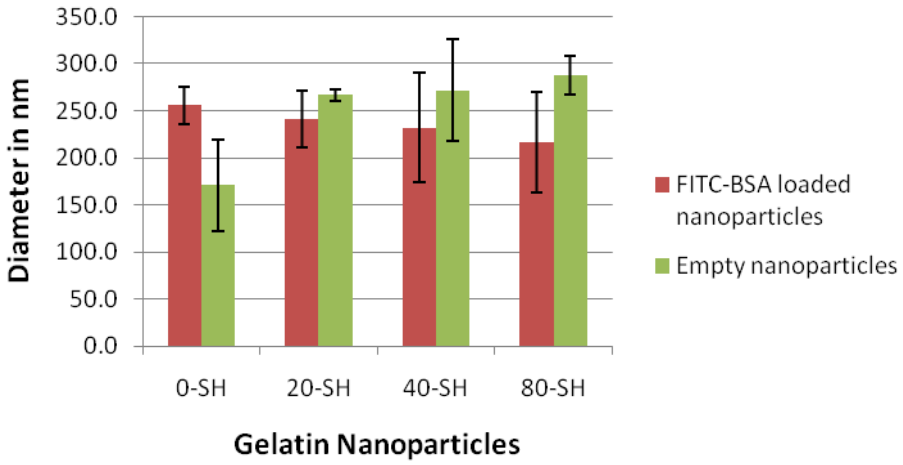


Figure 3-6. Loading effect on particle size.

Release Study

The ultimate goal of the nanoparticle is to encapsulate a subunit vaccine that is comprised of a protein. FITC-BSA was chosen as a model antigen to determine how efficient gelatin was at encapsulating a protein and at what point did the release of the protein plateau. The loaded gelatin nanoparticles encapsulated with 1 % (w/w) FITC-BSA. It is a conjugate of BSA, a globular protein that is negatively charged under neutral conditions and a fluorescent dye. At 37°C in a PBS solution at pH 7.04, the modified gelatin nanoparticles released between approximately 4.0 - 6.0% of the FITC-BSA at 6 hours into the study before the release profile began to plateau. However the unmodified gelatin nanoparticles released almost 10.0% of its contents Figure 3-7. The data shows that a great deal of the protein remains entrapped within the particle. The BSA portion of the molecule contains lysine residues that will react with glutaraldehyde in the solution that is used to crosslink the gelatin nanoparticles. Some of FITC-BSA is most likely bound to the surface and a portion of it may be crosslinked to the gelatin

internally. The gelatin particle will undergo some swelling but the crosslinked gelatin will be primarily degraded by enzymatic action and bulk erosion. Consequently a gelatin nanoparticle containing a protein antigen should not release the bulk of the encapsulated antigen by swelling.

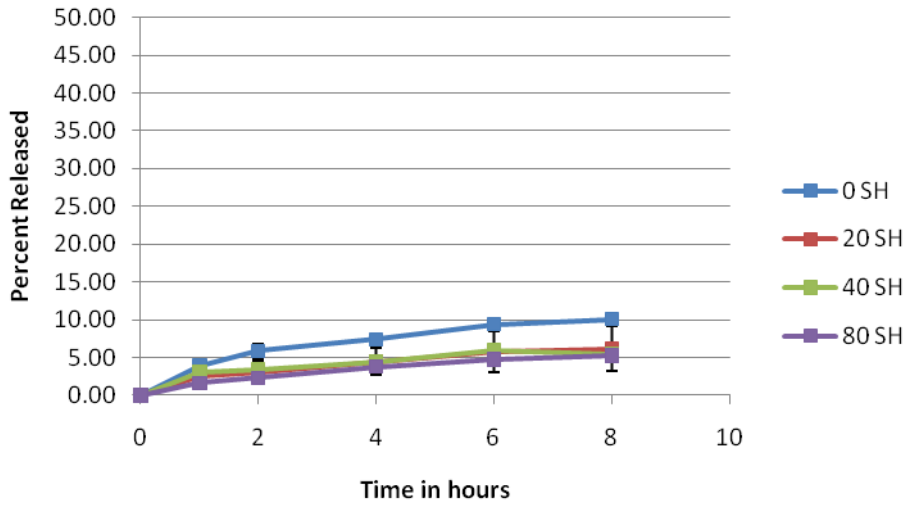


Figure 3-7. Release of FITC-BSA from gelatin nanoparticles.

The encapsulation efficiency (EE) and percent yield was determined for the loaded nanoparticles Table 3-3. An increase in EE correlated with an increase in thiolation. The hydrated volume of the BSA molecule is 125 nm^3.[91] For the unmodified gelatin nanoparticles the spheres will contain 0.7 mg of the BSA per 200 mg of the nanoparticles. The maximum protein loading occurs in the 80-SH nanoparticles, which will contain 1.64 mg of BSA per 200 mg of the nanoparticles. The percent yield was relatively low for all samples and there was no correlation between percent yield and degree of thiolation.

Table 3-3. Recovery from loaded nanoparticles.

Nanoparticles	Encapsulation Efficiency (EE) %	Percent Yield %
0 SH	35.00	27.38
20 SH	65.00	34.16
40 SH	57.50	20.05
80 SH	82.50	29.95

SEM evaluation

Several attempts were made to image the nanoparticles with a scanning electron microscope. In the first attempt the particles were taken directly from the acetone/water solution and added to a carbon coated stub and sputter coated at 1 minute with gold-palladium. The field emission SEM operated at an accelerating voltage of 12kV was unable to discern any particles from the coating. A second attempt was made also using a carbon coated stub and sputter coated with Au-Pd for 30 seconds for a thinner coating, and particles still were not visible. A third attempt was made, in which a mica substrate was mounted the carbon tape and the particles were dispersed from a very dilute aqueous solution. These particles were coated for 35 seconds with pure Au. The images obtained appeared to be of an amorphous structure and no particle were visible. A fourth attempt was made to visually the particles on a silicon substrate, coated with a thick gold coating. The particles were suspended in ethanol and a drop was added on top of the gold. The accelerating voltage was reduced to 5kV and the magnification was set at 5000X. Under these conditions dark spherical particulates were visible but details were not discernable. A final fifth attempt was made, where the nanoparticles dispersed in ethanol were deposited on a silicon wafer without coating. The accelerating voltage was at 5kV and the working distance (WD) was at 39mm. Under these conditions particles were beginning to become more visible but still of poor quality Figure 3-8. However several points were ascertained from this attempt. The first point is that on a silicon substrate gelatin does not need to be coated with gold to be visible. The second fact learned was that the beam was interacting with the gelatin and distorting the image. When a section of particles were selected to be photographed the longer the beam stayed on the image the more distorted the image became. This was particularly evident at magnifications over 1,500X. Once the beam was removed from the section the appearance of the sample returned to normal. The third fact learned is that casting the particles from an ethanol

solution left a residue surrounding the particles that cast a dark shadow. Imaging gelatin nanoparticles is possible according to the literature. However the correct substrate, coating, and dispersive media, accelerating voltage, and working distance was not discovered during the course of this study. To determine general particle morphology atomic force microscopy (AFM) and transmission electron microscopy (TEM) was used to see the particles.

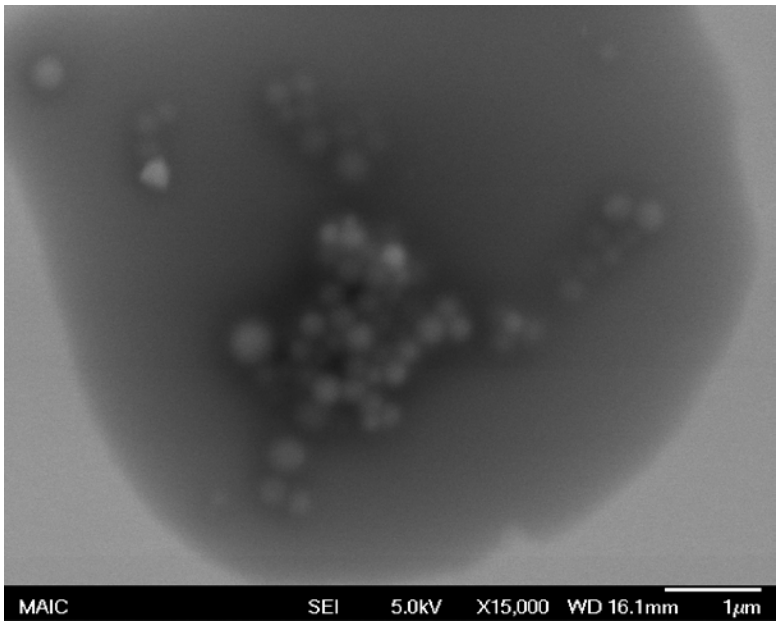


Figure 3-8. SEM micrograph of 80-SH nanoparticles

Storage Considerations

Upon centrifugation at speeds of 8000 rpm and for times of 1 hour the nanoparticles agglomerate to form a moderately condensed mass on the sides of the centrifuge tubes. Attempts to wash the particles in 100 % deionized water or a 0.5% solution of polyethylene glycol 200 MW were not successful in completely breaking up the agglomerate. Larger particles still remained even after vortexing these particles for 10 minutes followed by 10 minutes of sonication. To fully re-suspend the particles the washing step requires that the particles be

washed in 70:30 mix of DI water to ethanol and centrifuged for three times. Particle dispersion into the water/ethanol wash showed no larger particles remaining and no sedimentation after 24 hours.

To preserve the shelf-life of the particles they should be freeze-dried followed by storage at 40°C. To examine the effects of freezing rate on the gelatin nanoparticles, there were frozen in a freezer at 4°C and by liquid nitrogen at -196 °C. Complete solidification of particles in 5 ml DI by freezing took approximately 16 hours. Complete solidification in liquid nitrogen took approximately 2 minutes. The effects of the freezing solution on the nanoparticles were also examined. The particles were frozen in a 6 ml DI solution, a 6 ml 0.5% trehalose solution and a 6 ml of DI/ethanol at a 60:40 ratio. Additional studies by Zillies et al have shown that gelatin nanoparticles containing oligonucleotides frozen in excipients such as trehalose and freeze-dried can be stored to up to 10 weeks. These particles can be rehydrated in purified water and swell to sizes approximating the original volume. Those studies also showed in *in vivo* experiments that oligos retained their biological activity.[13]

Summary

Thiolated gelatin nanoparticles formed under conditions with a neutral pH do not form monodisperse particles. Forming thiolated nanoparticles under basic conditions is not a suitable environment because the thiol groups will oxidize under basic conditions. The thiol particles should be formed in a pH below their isoelectric point, in the acidic region. However at pHs below 2.5 the thiolated nanoparticles would not form at all, even though this region is optimized for discrete monodisperse gelatin nanoparticles. The addition of thiol groups changed the pI, which is essential in determining the best pH region for precipitation. If thiolated particles are to be made using the methods described in this section, formation of nanoparticles after thiolation of gelatin, then suspension conditions must be optimized for each level of modification. The

average particle diameter of both the empty and loaded nanoparticles was within the 100 – 500 nm range for optimal DC uptake. However the Nanotrec and microscopy revealed that there were particles formed in the precipitation that were ≥ 1 micron. Analysis of zeta potential showed the particles are negatively charged, ranging from -21.48 to -28.59 mV. The zeta potential of the loaded nanoparticles ranged from -35.42 to -39.73 mV. The increase in the charge on the loaded particles is thought to be due to some surface binding of the FITC-BSA to the nanoparticle. The surface of epithelial cells is also negatively charged. This will lead to charge repulsion of the particles that will act against the attraction of the thiols for the mucin proteins. The release study revealed that the gelatin nanoparticles will release a model protein antigen over a longer period of time. After 8 hours less than 15% of the contents were released before a plateau was reached for all of the gelatin nanoparticles. This release is assumed to come from initial swelling of the particle. The remaining entrapped protein will be released as the particle degrades over time due to enzymatic action. The thiol modification increases the encapsulation efficiency of a model protein antigen from 35.0% to 82.5%. However the percent yield for all of the particles was < 35%. As a vector for vaccine antigens this yield is too low for a limited antigen supply. Percent yield can be improved by improving encapsulation techniques. Fractioning the gelatin into chains with similar MW will allow the smaller MW chains to be removed, and nanoparticle formation using a homogeneous gelatin supply. Additionally all of the gelatin will precipitate under the same conditions at the same time, and the gradient of particle sizes and free gelatin found in the solution will be minimized. Several attempts were made to analyze the nanoparticles under SEM, however since no clear images were obtained SEM was not used as an analysis tool for this study. To maximize shelf-life of the particles and to minimize oxidation of the thiols it is recommended that the nanoparticles be stored frozen

under dry conditions. However the nanoparticles formed in study do not suspend readily upon rehydration. Ideally the particles would form a fine powder that would re-suspend easily. These particles formed tight pellets and particle clumps that were difficult to break apart. Changes in freezing media such as the addition of a surfactant or excipient such as trehalose made aid in stability of the individual particle upon drying.

CHAPTER 4
SURFACE ANALYSIS OF THIOL NANOPARTICLES

Introduction

The degree of thiolation onto the gelatin solution was determined by a fluorescent assay. The assay showed that with increasing amounts of iminothiolane incubated with gelatin, increasing concentrations of thiol were found. However how many of the sulfur groups are on the surface of the particle is still unknown. As the gelatin chains condense to form nanoparticles, the sulfur groups attached to short carbon chains could orient themselves in such a way that the thiol groups are facing internally into the spheres. X-Ray Photoelectron Spectroscopy (XPS) will be able to analyze the surface of the particles to determine the presence of any surface bearing thiol groups. Additionally XPS will also show if the increase of thiolation resulted in an increase of sulfur groups relative to unmodified gelatin. Atomic force microscopy will be used to examine morphology and surface properties.

Materials and Methods

Materials

The following chemicals were purchased from Sigma-Aldrich Company: Type B gelatin from bovine skin bloom strength 225. The following chemicals were purchased from Fisher Scientific: Acetone, ethanol, glutaraldehyde solution 25%. The Particle Engineering Research Center, University of Florida, provided mica film and silicon wafers. Distilled water used throughout all phases of the experiments.

Methods

Particle Synthesis

20 ml of the thiolated gelatin was heated on a stir plate to 40°C until solution is evenly heated. The solution is adjusted to pH 7.0 ± 0.05. To prepare the unmodified gelatin control

sample, 200 mg of gelatin is dispersed into DI water heated to 40°C and stirred until the gelatin is completely dissolved. The solution is then transferred into a round bottom flask and stirred at 200 rpm. The solution is heated by a water bath to 40℃. 25 ml of acetone was added via a syringe dropwise into the gelatin solution, nanoprecipitation occurred spontaneously. Immediately after all the solvent is added, 50µl of 25% glutaraldehyde is added to crosslink the particles. The solution is allowed to react at for 2 hrs, in a closed environment, under continuous heating. The resultant particles are then centrifuged by a Beckman J2-21 Centrifuge for 30 minutes at 7,000 rpm. The supernatant is removed and the particles are re-dispersed by washing with a 50:50 (DI) to ethanol solution, vortexing for 2 minutes and sonicating for 2 minutes. The wash step is repeated 3 times. The final suspension of particles is washed and stored in 100% ethanol at 4℃.

Analysis of surface chemistry

A PHI 5100 ESCA System (XPS/ESCA, Perkin-Elmer) was used to determine the chemical composition on the surface of the particle. The sample surface is bombarded with x-rays and the binding energy of the ejected photoelectrons is analyzed to give information about the elements present on the surface and their relative amounts.

To prepare the samples a 10 mm x 10 mm section of silicon wafer is cleaned with ethanol. One drop of each of the concentrated particle solution is deposited onto separate sections. The wafer is then dried overnight to remove all volatiles.

AFM analysis

Atomic force microscopy obtains data on the particle by scanning the surface of the particle with a cantilever and tip that is deflected by interatomic forces. A MFP-3D atomic force microscope, Asylum Research, Santa Barbara, CA was used to analyze morphology and topography. The cantilever was an Olympus AC240 TS, Olympus Corporation. The tip used on

the cantilever was composed of silicon with an aluminum backside and has a radius of 7nm. The cantilever was operated in Non-contact (AC) mode. The software Igor Pro 6.1 was the interface used to process and analyze the data.

To prepare the samples a 1 square inch piece of mica film was glued to a microscopic glass slide. The top layer of the mica film was removed with a piece of clear tape to expose a clean surface. One drop of the prepared nanoparticle was diluted in a microcentrifuge vial with 1.5 ml of ethanol. A drop of the diluted sample was placed on the mica film and allowed to cover the surface. Excess moisture was wicked away using a kimwipe and the sample was dried completely with blown air.

Results and Discussion

XPS Analysis

The XPS was used to perform a comparative evaluation of the unmodified gelatin nanoparticles and the highest modified samples, 80-SH nanoparticles before proceeding with the 20- and 40-SH. In XPS analysis, low energy x-rays hit the surface of a sample causing ejection of core electrons from atoms present in the material as a function of their binding energy Table 4-1. The atoms that are found in gelatin are C, H, N, O, and S; however H will not show on XPS. Their presence and respective amounts should be displayed in the chemical spectra.

Table 4-1. Binding energies of elements in sample.

Atomic Species	Orbital	Binding Energy
Carbon (C)	1s	284.2
Oxygen (O)	1s	543.1
Nitrogen (N)	1s	409.9
Sulfur (S)	2p	163.6
Silicon (S)	2p	99.82

The sulfur content on the surface was expected to be low in comparison to the carbon, oxygen, and nitrogen that constitute the majority of gelatin atoms. Sulfur appearing on the unmodified gelatin is due to the amino acid methionine present in gelatin. Of the 18 amino acids

that compose Type B gelatin, methionine is present at 3.9 units per 1000 residues or 0.6 g per 100 g of protein. It contains only one sulfur atom in its molecular formula, $C_5H_{11}NO_2S$. Therefore sulfur is not expected to be found in significant concentrations. The sulfur peak for both samples is barely discernable above the noise level. And it appears there was no significant difference in surface S content between the unmodified gelatin and the higher modified gelatin, Figure 4-2 and 4-3. This result was unexpected because the colorimetric assay showed that unmodified gelatin has a sulfur content of 0.17 ± 0.17 mmol/g of gelatin and the 80 mg incubated gelatin had a sulfur content of 46.75 ± 3.50 mmol/g of gelatin. However the XPS results indicate the sulfur present in the gelatin is not located on the surface. Two conclusions can be drawn from this. One is that the thiol conjugated to the gelatin in solution was no longer present upon formation of the nanoparticles. This explanation seems unlikely due to other analysis indicating the thiol end groups are affecting nanoparticle properties. The most likely explanation is that the sulfur groups are present but located inside the nanoparticle.

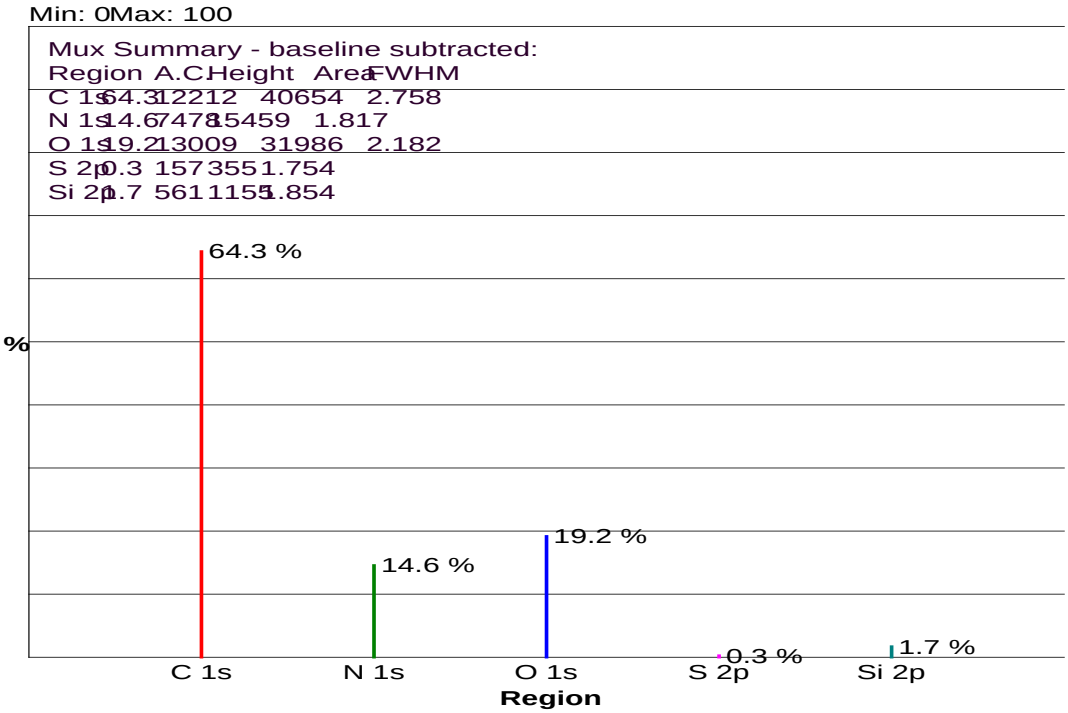


Figure 4-1. XPS multiplex spectra of unmodified gelatin.

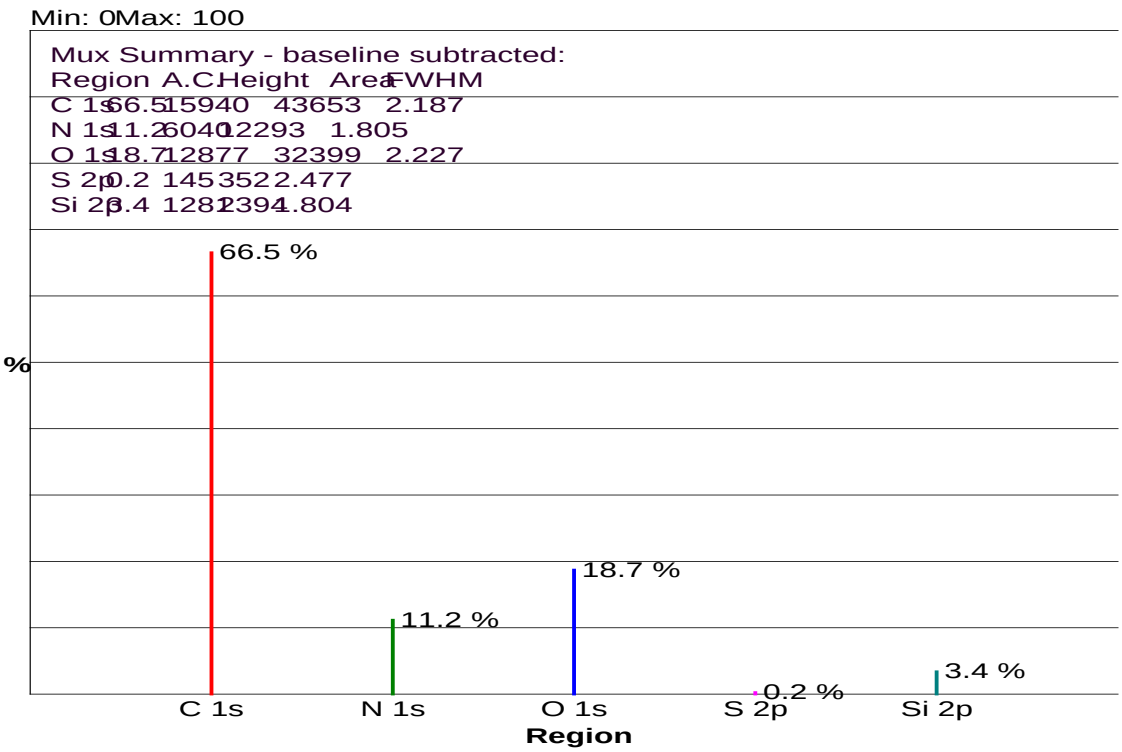


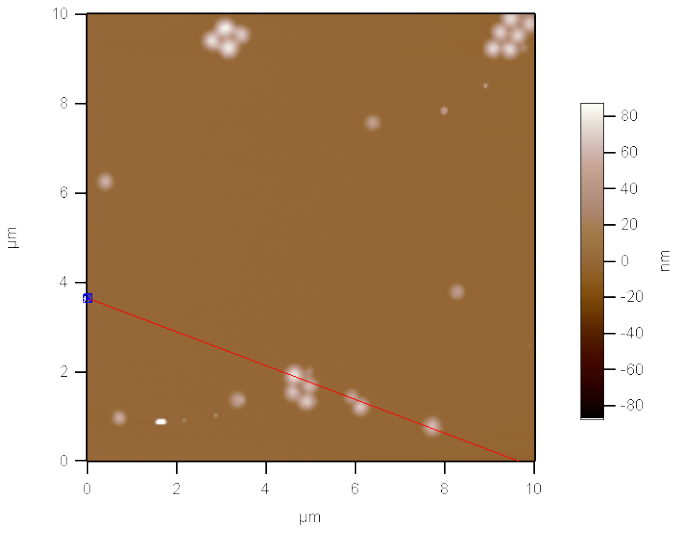
Figure 4-2. XPS multiplex spectra of 80-SH gelatin.

However an unexpected result of the scan was the decrease in atomic amounts of nitrogen the in the 80-SH sample relative to the unmodified sample. This decrease in nitrogen thought to be due to the iminothiolane reacting with the primary amines on lysine to form the thiol end group. Lysine and hydroxylysine are present at 4.4 g per 100 g of protein and 0.8 g per 100 g of protein respectively. The iminothiolane reacted with the amine groups on the lysine and hydroxylysine to form end chain thiol groups. Subsequently during particle formation the chain bearing the nitrogen molecule within and the sulfur group on the end is orientating to face the inside of the particle resulting in a reduction of surface levels of nitrogen.

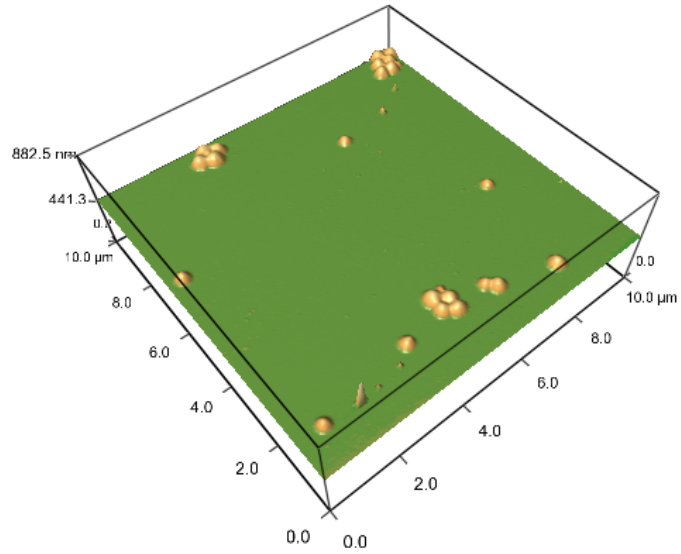
AFM Analysis

Using the AFM in image mode showed particles that were spherical in shape. There was some aggregation of the particles that seemed to increase with thiolation. However the most significant finding was that particles were round in two dimensions but appeared flattened in the

direction of the plane. Particles that are perfectly spherical and perfectly rigid would register a height that was relatively equivalent to its diameter the tip of the probe; which was 7nm. The unmodified gelatin showed the smallest peaks even after several particles were examined, Figure 4-3.

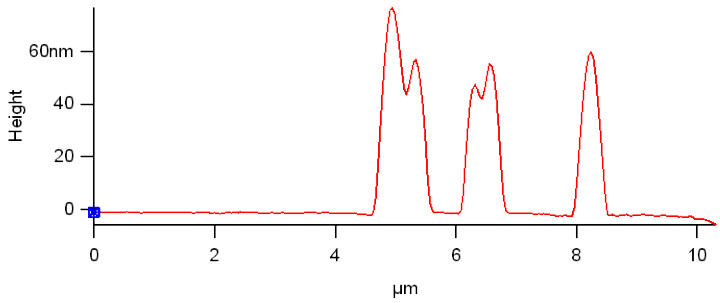


A



B

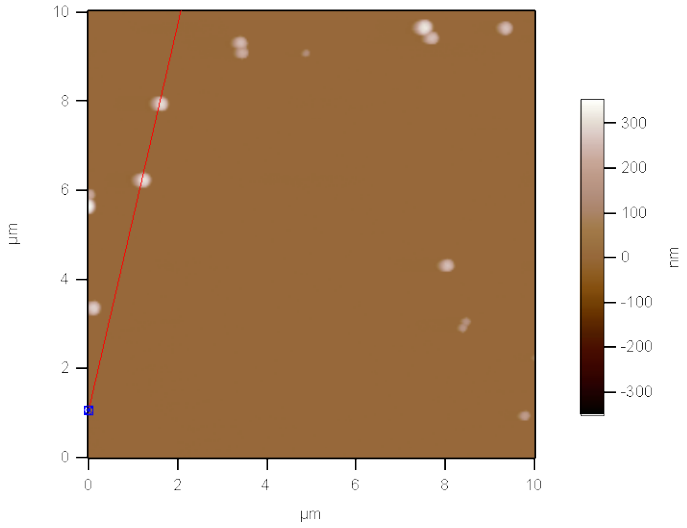
Figure 4-3. Image and topography of unmodified gelatin (0-SH). A)Two-dimensional image showing size and morphology. B)Topographical map of surface C)Height of three selected particles from figure A.



C

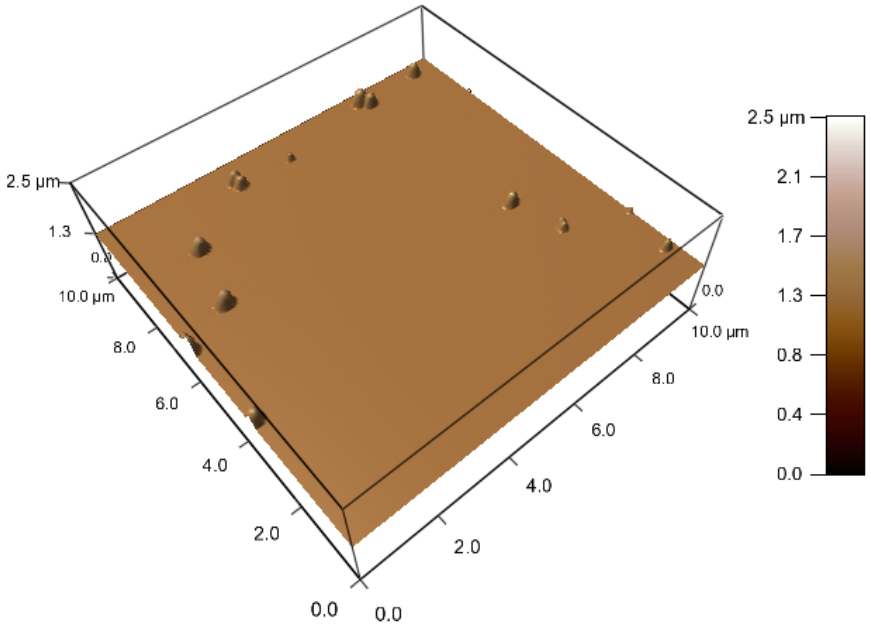
Figure 4-3. Continued

Like the unmodified gelatin, the 20-SH nanoparticles showed very spherical dispersed particles. The 20-SH nanoparticles have a mean average number diameter of 266.6 ± 29.82. In the sample selected there were several particles much smaller than average figure 4-2. The particles appeared less agglomerated in this sample but this could be due to unequal dilution factors and not as a result of surface chemistry. The particle in the 20-SH also showed particles that are much taller than 0-SH nanoparticles. Several of the particles selected had heights that were over 250 nm, Figure 4-4. There were no particles this high in the 0-SH sample.

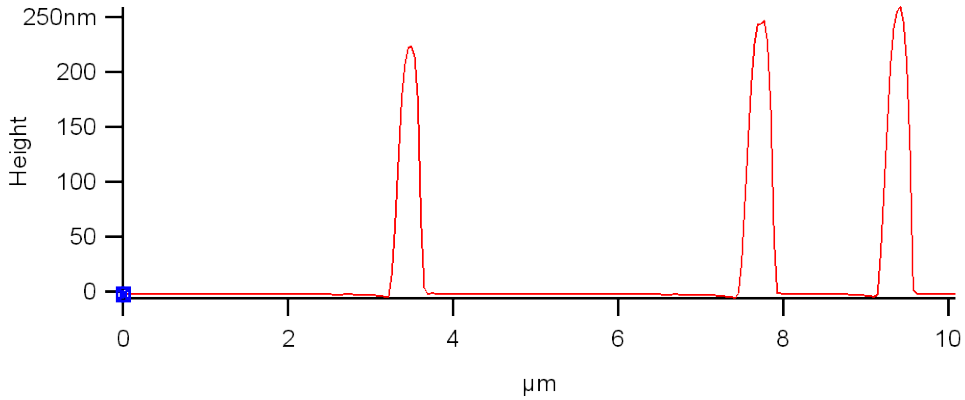


A

Figure 4-4. Image and topography of the 20-SH gelatin. A)Two-dimensional image showing size and morphology. B)Topographical map of surface C)Height of three selected particles from figure A.



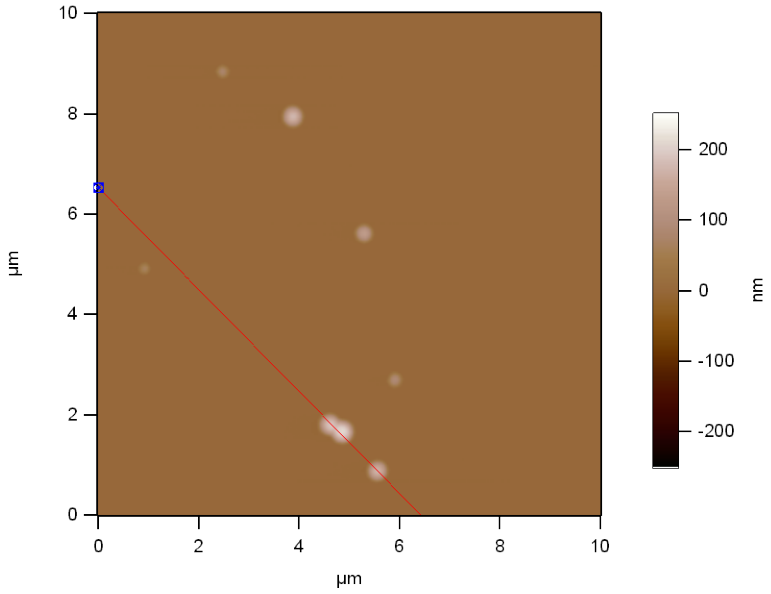
B



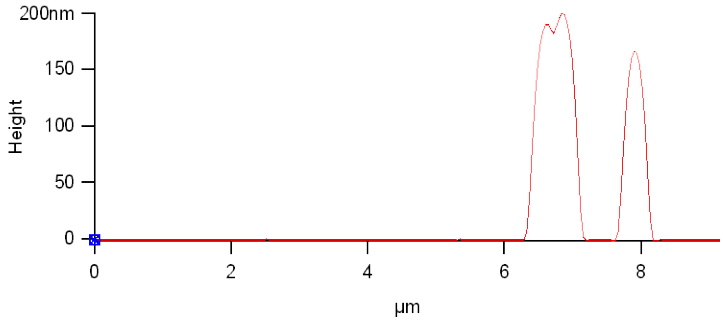
C

Figure 4-4. Continued

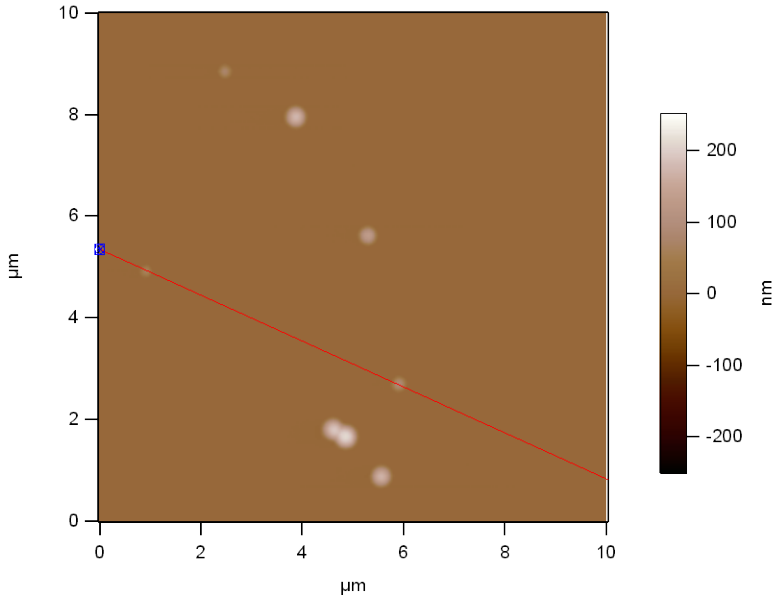
The 40-SH samples were over diluted so several images were taken to get a better representation of size and topography. Particle analysis data shows that particles have a mean number average diameter of 271.8 ± 58.37. These particles appear to be spherical and also taller than the unmodified gelatin. The height extrapolated from several particles imaged, showed particles ranging from 50 nm to 200 nm Figure 4.3.



A

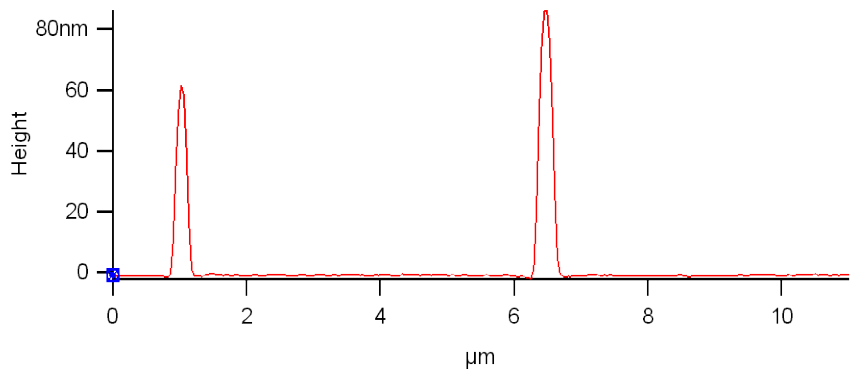


B

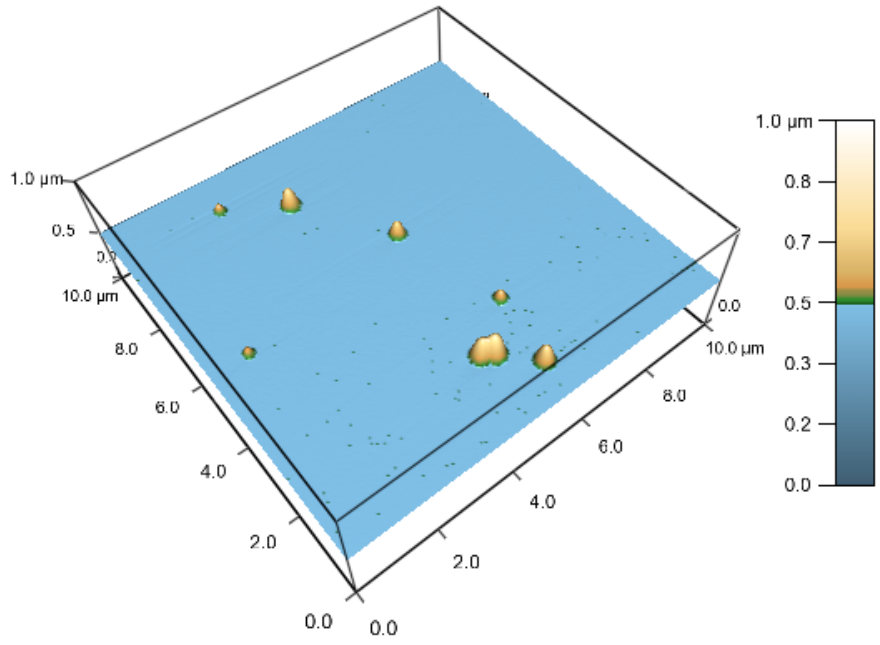


C

Figure 4-5. Image and topography of the 40-SH gelatin. A)Two-dimensional image showing size and morphology. B)Plot of particle height for particles selected C)Same 2-D image different particles highlighted. D)Plot of particle height for new particles. E)Topographical map of surface.



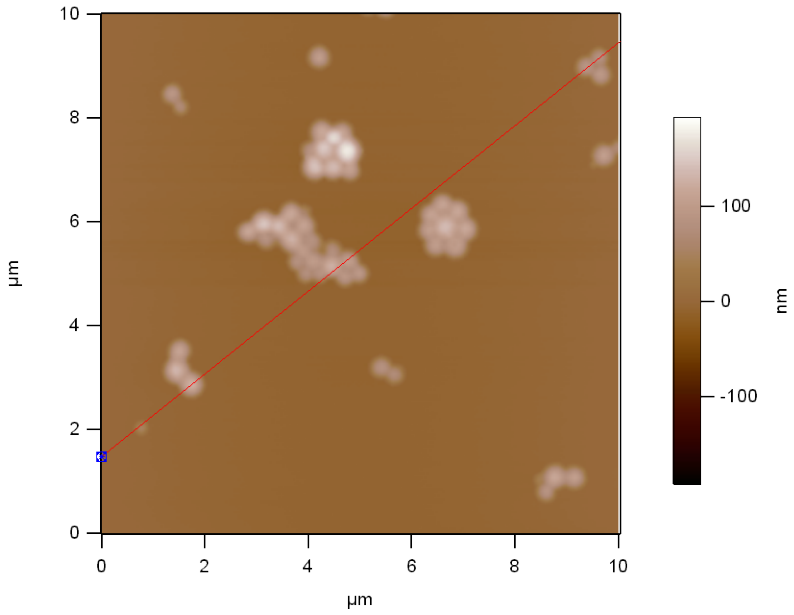
D



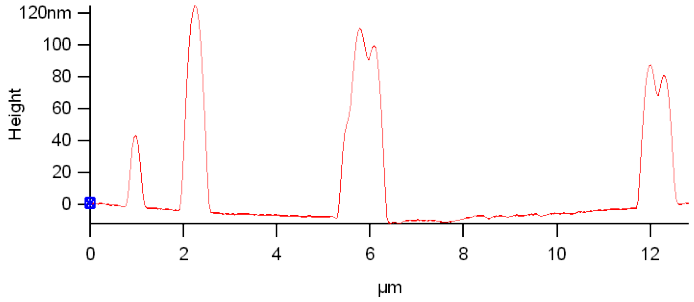
E

Figure 4-5. Continued

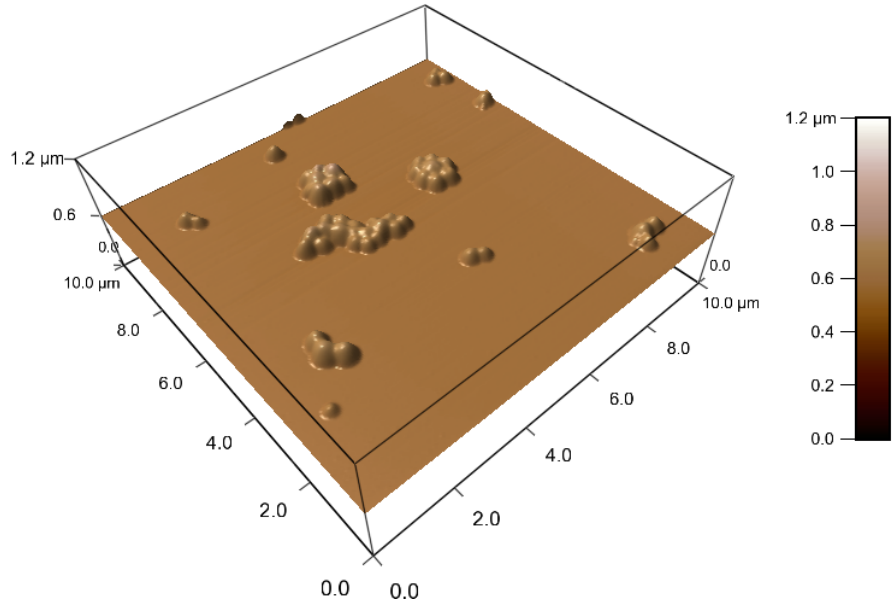
The 80-SH visually appeared to be the most aggregated. These particles have a mean number diameter of 287.3 ± 53.49 nm. The spherical particles appeared to form clusters of particles upon viewing figure 4-4. These particles were prepared in the same manner as the other samples but they seemed to have increased aggregation. This may be attributed to the thiol chains forming disulfide bridges with each other. But XPS analysis did not show high levels of sulfur on the surface so this explanation seems unlikely.



A



B



C

Figure 4-6. Image and topography of the 80-SH gelatin. A)Two-dimensional image showing size and morphology. B)Topographical map of surface C)Height of three selected particles from figure A.

Summary

The colorimetric assay on the spectrophotometer showed that the number of thiol groups increased with the increasing incubation levels of iminothiolane. However when the nanoparticle was examined for the presence of sulfur on the surface the XPS revealed that there was no increase in sulfur concentration. One possibility was that the sulfur added was too small relative to overall number of other atoms. A second possibility is that the thiols have been converted to thioacetals by the glutaraldehyde; however the reaction does not result in a loss of sulfur molecules so the sulfur atoms should still be present on the surface. A more likely possibility is that the thiol groups on the end of the aliphatic chains oriented themselves during nanoparticle formation such that the sulfur groups are facing the inside of the sphere. If the thiol groups are internalized, mucoadhesive tests may not show any significant difference between any of the modified gelatin nanoparticles and none of the particles may exhibit any mucoadhesiveness. A way to test this hypothesis would be to make the gelatin nanoparticles before thiolation followed by incubation with iminothiolane. Then perform an XPS analysis on the pre- and post- iminothiolane incubated gelatin nanoparticles to determine if there is an appreciable difference. The AFM studies showed that the particles are spherical with a range in sizes and some aggregation. This was also observed using TEM but not clearly discerned on SEM. AFM topography maps also showed that the particles are somewhat flat relative to their diameter. This effect was noticeably apparent on the unmodified gelatin nanoparticles. The collapsed state of the particles may be due to how the particles were dried and prepared for analysis. But there is also the possibility that the particles have a low modulus and are partially deformable. In addition, since the modified nanoparticles showed higher peaks than the unmodified gelatin, it is conceivable that the thiol groups and their chain additions add rigidity to

the particles and increase their modulus. Further analysis using the AFM in tapping mode to apply a compressive load could determine mechanical properties of the particles.

CHAPTER 5
IN VITRO STUDY NANOPARTICLE MUCOADHESIVENESS

Introduction

The objective of this chapter is to evaluate the interactions between the nanoparticles and mucus producing nasal epithelial cells (RPMI 2650). Nanoparticles come in contact with antigen presenting cells (APC) by penetration of the epithelial cell surface that is generating the mucosal layer. The penetration can occur by paracellular transport, passive diffusion, carrier-mediated transport, or endocytosis Figure 5-1. It is important to analyze the mucus nanoaparticle interaction, but how epithelial cells respond when in contact with the nanoparticles is also critical. The interaction will be evaluated in three ways. The first analysis will determine how nanoparticles associate with epithelial cells and whether the increased thiolation has any increase on this effect. The mechanism of cell association could be through cell uptake or membrane binding. The FITC-BSA loaded nanoparticles will be incubated against nasal epithelial cells for a period of 24 hours. Confocal microscopy will be used to determine if the nanoparticles are present on the cell membrane or inside the cell. The second analysis will be a direct measure of the adhesion of mucus to the nanoparticles. Mucus produced from the nasal epithelial cells will be harvested from several flasks incubating mucus producing cells. The nanoparticles will be suspended in a solution of cell media containing a quantity of the mucus. After a brief amount of time the solution will be centrifuged at a rate to separate the mucus from the solution. Fluorescence of the solution will be measured before and after centrifugation, and the percent removed will give a quantitative estimation of mucus adhesion.

Mechanisms of Mucoadhesion

How materials bind to mucus is not completely understood. Under physiological conditions mucus is negatively charged and extremely hydrophilic. Adherence is believed to be

caused by several factors including, chemical bonding, electrostatic interactions, hydrophobic interactions, or physical chain entanglements acting independently or in consort.[93] These factors influence how the mucus-material interaction behave in the two stages of adhesion; the contact stage which is the initial binding and the wetting stage which describes stronger and more prolonged fixation. The factors that are due predominately to environmental factors such as pH, swelling, and contact time are described by four prevailing theories: electronic theory, adsorption theory, wetting theory, and diffusion theory. There are also factors inherent to the polymer they may fit with one or more of these theories, but have they have not been fully investigated. These are aspects related directly to polymer properties such as functional groups, charge, molecular weight, chain flexibility, and concentration.[80, 94]

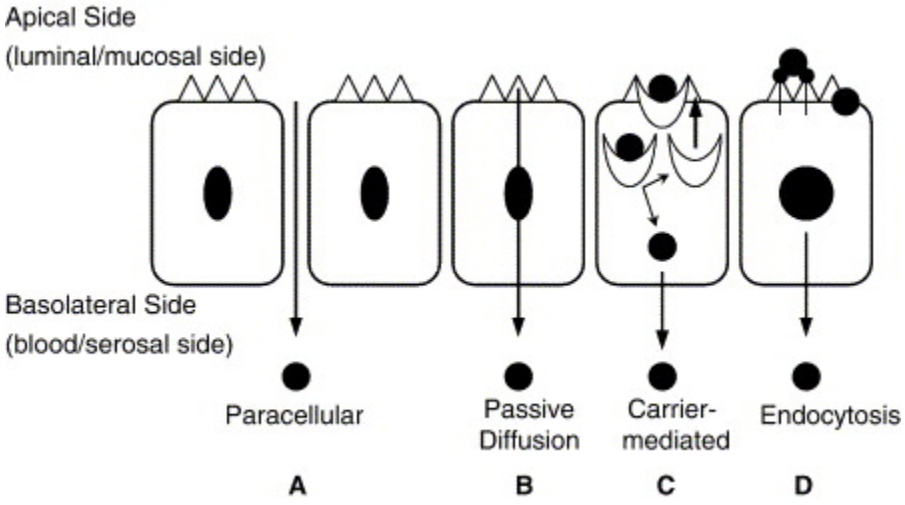


Figure 5-1 Transport of nanoparticles through epithelial layer.[92]

Several studies performed over the last two decades have investigated a variety of polymers for their bioadhesiveness and potential for mucosal drug delivery.[81] Due to these investigations, several features have been identified as key for mucoadhesiveness. Interpenetration and chain entanglement have been shown to be a strong influence. High molecular weights, greater than 100,000, have shown increased adhesion due entanglements but

diffusion is hampered by long chains. Additionally how these chains are arranged spatially affects the adhesive strength. Polymers that have a helical confirmation may have lower adhesion strength than linear polymers due to shielding of groups that can interact with mucins. Consequently there is no definitive best molecular weight, it is dependent upon each polymer system and an exact range cannot be given. Another significant factor in the adhesion process is due to hydrogen bonding. Polymers bearing carboxyl (COOH), hydroxyl (OH), amide (NH_2) and other hydrophilic functional groups are able to form networks with the mucin glycoproteins. Additionally the physiological conditions of how and at when these functional groups dissociate affects pH and charge.

Characterization Methods

There is no definitive analytical method for how the mucoadhesiveness of a material is measured. The manner in which mucoadhesiveness is quantified depends on the techniques used, and the values vary between references. Most of the standardized data comes from *in vitro* testing, but *in vivo* and *ex vivo* testing can give reliable preliminary data on how a material will respond in contact with mucin. Currently there are cited accepted methods used to determine mucoadhesiveness directly or indirectly. They are based on properties such as viscosity, tensile strength, surface energy, electrical conductance, fluorescence and contact time.[94] Rheology is a commonly used indirect method for estimating adhesiveness. The viscosity of a mucin solution, polymer solution, and mucin/polymer blend is measured and the molecular interaction is derived from a linear equation. This interaction is given a value that is used to indicate a level of mucoadhesiveness. However this method is not recommended solely to determine adhesiveness because of variations in polymer and mucus concentration, mucus type used, and rheometric systems.[95] Another common indirect method uses surface energy to estimate adhesiveness. The contact angle is measured between a thin polymer film and drops of distilled

water and mucus solution. Smaller contact angles indicated better wetting which correlated with stronger bioadhesive interactions.[96]

Direct methods for measuring adhesiveness usually require that the polymer come in contact with a cell or tissue sample with a mucosal surface. Adhesion assays use tensile strength, shear strength, or peel strength to determine the detachment force between a mucoadhesive tissue section and a polymer film. The test can be performed in wet or dry environments. And the force can be measured by several tensile devices: tensiometer, Instron, spring tension gauge, and spring balance. The measured value is usually given as maximum detachment force relative in units of mN/cm^2.[95] A simpler direct method for evaluating the adherence of polymers to the mucosal surface is the flow assay. Excised tissue is placed in a flow cell, with the mucosa surface exposed to a polymer formulation for a fixed time, temperature, and moisture level. Then air or liquid washes the surface at a constant flow rate and the adhesiveness of the polymer is determined by either the retention amount of the polymer or the detachment time of the particle. Of all the methods reviewed the majority of them are not suitable for measuring the mucoadhesiveness of particles only the cell flow assay and AFM force measurements generate data that is directly relevant to particle adhesion as opposed to polymer adhesion.

Nasal Drug Absorption Model

When evaluating the adhesive force in nasal drug delivery, *in vivo* and *in vitro* animal models are commonly used. Animals that have been evaluated for these studies include rat, rabbit, dog, sheep, cows, and monkeys. The problems with using animals for *in vivo* testing are in the difficulty in obtaining the animals, the cost associated and ethical issues. The *in vitro* models are primarily excised tissue models. They are simpler, less expensive, and give good data on nasal permeation and metabolism. The excised tissue is taken from rabbits, cows, sheep,

and dogs. However neither model can completely account for the differences between human and animal anatomy and physiology.

To study adhesion at the nasal mucosal epithelial surface, an in vitro model using cultured nasal cells is best suited for the application. This model has shown validity in pharmacokinetic research and can be cultured to produce a reasonable mimicking of nasal epithelium. Currently the only cell type established for this model is the human nasal epithelial cell. In vitro these cells can grow cilia, form tight junctions, and most importantly secrete mucus.

Materials and Methods

Materials

The following chemicals were purchased from Sigma-Aldrich Company: Type B gelatin from bovine skin bloom strength 225, 2-iminothiolane (Traut's reagent), Fetal Bovine Serum (FBS), L-glutamine. The following chemicals were purchased from Fisher Scientific: Acetone, ethanol, 2-propanol, 25 % glutaraldehyde solution, microscope glass slides, clear 75 X 25. The following chemicals and supplies were purchased from ATCC: RPMI 2650 (human nasal epithelial cells), Eagle's Minimum Essential Medium (MEM), trypan blue. The following chemicals were purchased from Mediatech, Inc: Penicillin-Streptomycin 100X, Trypsin-EDTA 1X. The following item was purchased from MP Biomedicals: 0.4% Trypan Blue. The following items were purchased from Invitrogen: CellMask Orange plasma membrane stain 5mg/ml in DMSO, Hoechst 33342, trihydrochloride, trihydrate –Fluoropure grade. The following items were purchased from Corning: 75 cm^2 rectangular canted neck cell culture flask with vent cap, Transwell 3402 membrane plates.

Methods

Culture of Nasal Epithelial Cells

The media for the RPMI 2650 cells was prepared by adding 450 ml of Eagles MEM to a sterile bottle-top filter, to this 45 ml of FBS was added followed by 5 ml of Penicillin-Streptomycin. RPMI 2650 cells were stored in liquid nitrogen (N_2) immediately upon arrival. To prepare cells for culture the cells were thawed at 37°C in a water bath. The cells were transferred to a centrifuge tube where 10 ml of prepared media was added dropwise slowly to the cells. The cells were then centrifuged for 3 minutes at 1000 rpm. The supernatant was drawn off then discarded. To the pellet of cells 12.5 ml of the prepared media was added slowly. The cells were mixed gently with 2 ml of the media by pipetting. A 50 µl aliquot of the cells is mixed with 50 µl of trypan blue to determine total cell population. A 10 µl sample is loaded into the hemocytometer and the total living cell count is taken. 12.5 ml of prepared media was added to a 75 ml cell culture flask. No greater than 10^5 cells are added to one 75 ml cell culture flask. The flask was placed in an incubator at 37°C and 5% CO_2. After 48 hours the media was suctioned out and the surface was rinsed with PBS. The cells were checked for growth in viability under the microscope. The media was replaced with 15 ml of fresh media and continued to incubate. The removal and replacement of the media was performed every 48 hours. The adherent cells were removed with 3 ml of trypsin, washed with media, and pelleted by centrifuge. After the cell count was taken the cell were distributed for use. The cells used in this study were passed 5-6 times before use.

Cell Fixation for Confocal Microscopy

Of the epithelial cells, 20 µl was placed into the center of a glass bottom culture dish. Approximately 1.5 ml of prepared media was added to the cells and incubated for 24 hours at 37°C and 5% CO_2. To 2 ml of the media for each culture, 50 µl of the nanoparticles containing

FITC-BSA was added. After 24 hours the culture dishes containing the cells was removed from the incubator and the old media was removed and discarded. The media containing the nanoparticles was added to the cells dropwise. The cells were returned to the incubator for an additional 24 hours. After this time, all plates were removed and the media was discarded. Half of the plates were stained with a solution of CellMask Orange and Trihydrochloride. The duplicate plates were stained only with trihydrochloride trihydrate.

Preparation of Gelatin Nanoparticles

20 ml of the thiolated gelatin was heated on a stir plate to 40°C until solution is evenly heated. The solution is adjusted to pH 7.0 ± 0.05. To prepare the unmodified gelatin control sample, 200 mg of gelatin is dispersed into DI water heated to 40°C and stirred until the gelatin is completely dissolved. The solution is then transferred into a round bottom flask and stirred at 200 rpm. The solution is heated by a water bath to 40°C. Twenty-five ml of acetone are then added via a syringe dropwise into the gelatin solution until nanoprecipitation occurs. Immediately after all the solvent is added, 50µl of 25% glutaraldehyde is added to crosslink the particles. The solution is allowed to react at for 2 hrs, in a closed environment, under continuous heating. The resultant particles are then centrifuged by a Beckman J2-21 Centrifuge for 1 hour at 8,000 rpm. The supernatant is removed and the particles are re-dispersed by washing with a 70:30 deionized water (DI) to ethanol solution, vortexing for 2 minutes and sonicating for 2 minutes. The wash step is repeated 3 times. The final suspension of particles is stored at 4°C until further analysis.

Mucus Adhesion Assay

Three 75 cm^2 cell flasks plated at 2.4 x 10^5 cells/ml were tyrpsinized and washed with a 15 ml of media per flask. After 30 minutes the mucus and cells detached from the surface. The cells and mucus were centrifuged at 3000 rpm for 3 minutes. The mucus is then removed from

the cells and washed with 10 ml of PBS and re-centrifuged. The mucus plug is diluted with 5 ml of PBS and 1 ml of the mucus solution is added to centrifuge tubes for each particle modification type. The nanoparticles centrifuge tubes were suspended in 5 ml of PBS. An aliquot was taken from the mucus/particle solution for each modified gelatin nanoparticle. The particles and mucus solution were incubated for 20 minutes at 37°C. The suspension was then centrifuged again at 3000 rpm for 3 minutes and a second aliquot was taken from each sample. The aliquots were then analyzed for fluorescence.

Results and Discussion

Confocal Analysis

The fluorescent images taken under the confocal microscope showed little to no uptake or association with the cell membrane or within the cell Figure 4-1. The cell membrane is stained with the CellMask and is fluorescing bright orange; the cell nucleus is stained with the trihydrochloride trihydrate to appear blue. Any FITC-BSA nanoparticles would appear bright green within the field. The highest thiol modified gelatin nanoparticles showed a few green fluorescent particles under the microscope but it was not substantially different from the other samples and the particles were not captured digitally when photographed. These results may indicate several things. One is that the thiol groups of the gelatin nanoparticles are not on the surface and these results would corroborate the XPS study the showed no increased sulfur concentration on the surface. Second is that thiolation has no effect on epithelial cell association. And third is that the results are possibly a result of a failure of experimental design. Too few nanoparticles in the suspension or nanoparticles that are not sufficiently fluorescent will not be image properly.

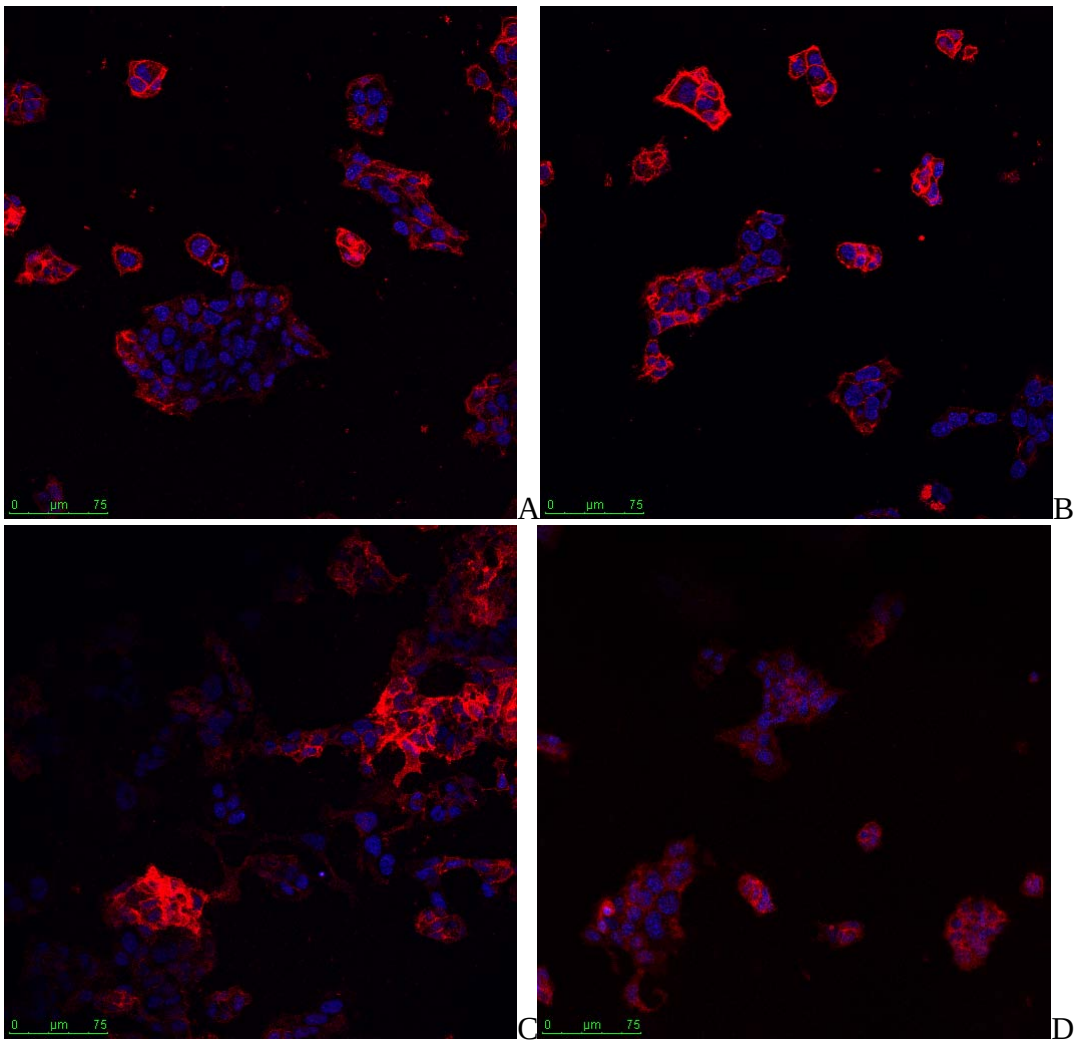


Figure 5-2. Epithelial cells stained for fluorescence. A)Cells incubated with the 0-SH nanoparticles B)Cells incubated with the 20-SH C)Cells incubated with the 40-SH D)Cell incubated with the 80-SH

Mucus Adhesion Analysis

The purpose of this assay was to determine if the thiol modifications on the gelatin nanoparticle showed an increased affinity for mucus binding. The absorbance of the nanoparticles and mucus were measured pre and post incubation and centrifugation. Low centrifugations speeds of 3000 rpm and short spin times (3 minutes) are insufficient to remove the nanoparticles from the suspension. Therefore it was expected that the gelatin entrapped within the mucus would be removed from the solution with the gelatin, and the change in

fluorescent reading would indicate mucus-particle interactions. The data showed no net decrease. The 0-SH, 20-SH, 40-SH actually showed very small increases in fluorescence Figure 5-3. This increase is thought to be caused by a variation in the results or elution of the FITC-BSA. There were some flaws in the mucus adhesion test such as standardizing the exact amount of mucus between samples, attaching the FITC to the surface of the nanoparticle as opposed to encapsulating a FITC containing protein, and optimizing incubation time. But two facts can be ascertained from this are at short contact times gelatin shows no particular affinity for mucus and an increase in thiolation had no effect on the mucoadhesiveness of the particle.

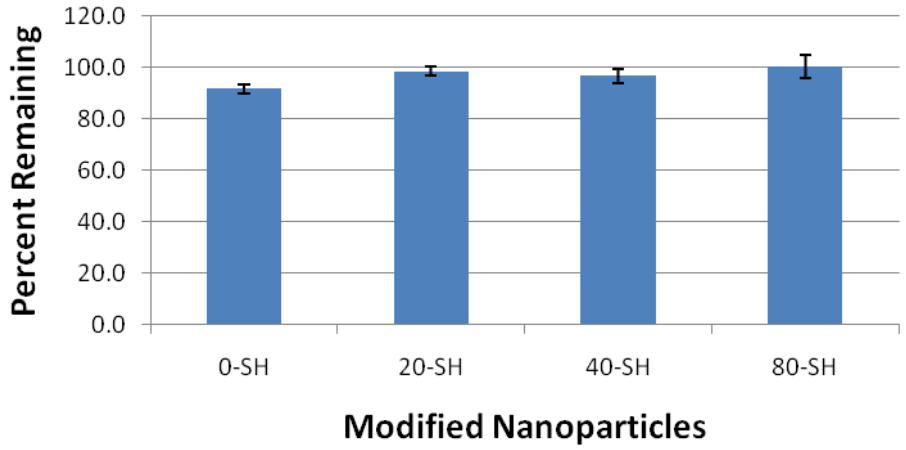


Figure 5-3. Analysis of the adherence of gelatin nanoparticles to mucus. N=2

Summary

Nanoparticles loaded with FITC-BSA was incubated with epithelial cells for 24 hours and examined under confocal microscopy. Analysis of the cells did not show any particles that were internalized inside the cell or adhering to the surface. It is possible that the incubation period was not long enough for the cells to interact with the nanoparticle and the uptake should be studied over a period of 7 days with analysis performed every 24 hours to determine when or if the uptake occurs. The loaded nanoparticles were also incubated against mucus isolated from the cells and diluted with PBS. Initial fluorescence measurements were taken before incubation and

final measurements were taken after incubation with the mucus for 20 minutes at 37°C. The mucus-particle suspension was centrifuged to remove the mucus. Particles adhering to the mucus would be entrapped and removed with the mucus. Free nanoparticles would remain in the solution as measured by fluorescence. However the results revealed no net change in fluorescence. One possible reason for the failure of this experiment is that the extracted mucus was diluted beyond the consistency of mucus produced in the nose and the final diluent did not posses and even distribution of mucosal contents, namely mucin proteins. Additionally incubation time with the mucus could be increased but the 20 minute incubation time is already beyond the normal mucus clearance time of 10-15 minutes.[97] The in vitro test in this study could not conclusively measure the interaction between gelatin nanoparticles and epithelial cells or the effect of thiolation on these interactions

CHAPTER 6
CONCLUSIONS AND FUTURE WORK

Conclusions

Several new pieces of information were uncovered in this research that had not previously been investigated. The most significant findings are: gelatin nanoparticles prepared by thiolating the gelatin instead of the particle do not show significant increase in sulfur content, and thiolated gelatin nanoparticles do no show affinity to nasal epithelial cells.

The conditions used to form unmodified gelatin nanoparticles are not suitable to form uniform, monodisperse thiolated gelatin nanoparticles. Particle formation is largely dependent upon the isoelectric point. With the addition of the thiol groups the pI of gelatin appeared to have changed. Additionally thiolated nanoparticles formed at pHs below 7.0 minimize the oxidation of their thiol groups. However at extremely acidic conditions, pH'≤ 3 .0, thiolated gelatin nanoparticles will not form using acetone even at 2:1 ratios of solvent to solution, twice the normal solvating amount. Although unmodified gelatin will easily form monodisperse nanoparticles in the 200 micron range under these conditions.

The thiolation of gelatin increases the encapsulation efficiency of protein inside the nanoparticle. The unmodified gelatin nanoparticle had an encapsulation efficiency of 35% by increasing the thiol groups encapsulation was increased to 82.50%. The protein is most likely crosslinked to the gelatin and will not elute until the particle is degraded by enzymatic action. However for all particles the percent yield was < 35%. The amount of lost product and payload could be cost prohibitive for encapsulating a limited amount of antigen. The percent yield could be improved fractioning the gelatin before particle formation to obtain a more homogeneous sample.

It was proven that thiol groups were attached to the gelatin polymer at concentrations of 7.48, 30.53, and 46.75 mmol per gram of gelatin. It did not appear that those groups were present on the surface of the gelatin nanoparticle. XPS scans revealed approximately 0.3% S content. The assumption was made that the gelatin was present in the sample but not on the surface but there is also the possibility that the thiols were converted to thioacetals by reaction with the glutaraldehyde. However this does not result in a decrease in sulfur atoms.

The *in vitro* analysis showed that neither the gelatin nanoparticles nor thiolated gelatin nanoparticles showed any particular affinity to epithelial cells. The particles did not adhere to the cell membrane or appear to penetrate the cell membrane. However this was an inconclusive study. The failure to show an association could be a result of a failure in experimental design and not a true measure of interaction.

The research presented in this dissertation showed the effect of thiolation on particle formation, morphology, charge, release kinetics, and encapsulation. The data also indicated that the addition of thiol groups did not generate an increase in mucoadhesiveness. However there can be no conclusive statements made about the effect of thiol groups on mucoadhesiveness because it was not proven that the sulfhydryl groups present in the gelatin were available for disulfide bonding with the sulfur present in the mucin glycoproteins.

Future Work

The objective of the research presented in this dissertation was: to study the formation of thiolated gelatin nanoparticles, to synthesize nanoparticles suitable for delivering a protein antigen to a nasal environment, to define and characterize the size, encapsulation potential and surface chemistry of the nanoparticles capable of engulfment by antigen presenting cells, and to study the mucoadhesive interactions between the nanoparticles and the mucus coated surface of

nasal epithelial cells. However there are several pertinent studies that should be considered for future work.

1. **Optimizing nanoparticle formation for the exact modification.** As the thiolation increased the properties of the gelatin change. Nanoparticles should ideally be formed from a purified gelatin solution to ensure the precipitation conditions are optimized for a consistent product. Then this step should be followed by the incubation the formed particles with 2-iminothiolane to ensure the thiol is present on the surface

2. **Determination of isoelectric point of thiolated gelatin.** The pI of a protein plays a significant role in determining how and where the nanoparticles will form under what pH conditions. The pI of gelatin is reported as 4.7-5.2, and a 1.5% solution at 25°C the pH is 5.0 -7.5. Experimentally the pH of a 1.0% solution of gelatin at 25°C was found to be 5.35 ± 0.14, of the 20-SH to be 2.97 ± 0.31, of the 40-SH to be 2.92 ± 0.19, and of the 80-SH to be 3.06 ± 0.55. The changes in solution pH indicated that addition of sulfur groups had changed the pI. However to what extent was unknown. To accurately determine the new isoelectric points a type of 2-D gel electrophoresis called isoelectric focusing (IEF) should be performed. The point where the protein is stationary in an electric field as it goes through a pH gradient is defined as the pI.

3. **Synthesis and characterization of gelatin nanoparticles using alternative forms of gelatin.** The nanoparticles created in this study were derived from type B porcine gelatin. The various types of gelatin differ in amino acid compositions and pI. These differences would affect the conditions the particle is formed, the degree of thiolation, degree of crosslinking, surface charge and antigen loading.

4. **Analysis of mechanical properties.** The AFM analysis revealed that gelatin nanoparticles lay flat against the surface of the substrate. The unmodified gelatin did not have any peaks over 80 nm, the modified gelatin nanoparticles had peaks ranging from 60 nm to 350 nm. It appears that unmodified gelatin particles are more deformable and this discrepancy may be due to changes in elastic modulus. Young's modulus of the material can be determined by using AFM in tapping mode.

5. **Stability and storage study.** In the literature unmodified gelatin nanoparticles containing plasmid DNA have shown that they can retain their stability up to four weeks and in vivo effectiveness. The affects of freeze drying of thiol modified nanoparticles has not been determined. The morphology of the particles, loss of antigen, how well they resuspend can altered by the freeze drying process. Additionally there are storage considerations that need to be examined. Nanoparticles are to be evaluated under a range of temperatures, wet and dry conditions, and solutions should be evaluated to determine the degradation of the polymer, degradation of the antigen, diffusion of the antigen, maximum shelf-life and sterility.

6. ***In vitro* study on particle uptake by macrophages and dendritic cells.** The ability and efficiency of antigen presenting cells to engulf the nanoparticles via phagocytosis or pinocytosis needs to be evaluated. For macrophage uptake, murine macrophage cells are

commonly used. They should be incubated with fluorescently labeled nanoparticles and then lysed to determine cellular uptake. For DC uptake, the cells are typically cultured monocytes in fresh blood or bone marrow. The cells should be incubated with labeled nanoparticles. Flow cytometry and fluorescent microscopy should be used to determine quantitative and qualitative data.

7. ***In vivo* animal study on the immunogenicity of the antigen-adjuvant complex.** The ultimate goal of the nanoparticle as a vaccine adjuvant is to increase the immune response to the antigen. The protocol for this is described several places in the literature. An in vivo model using C57-BL/6 mice is suitable for this person. The animal would be inoculated with the particles containing the antigen and blood samples will be taken and antibody responses will be determined using an ELISA assay. The assay will determine if IgG antibody titer levels have increased.

APPENDIX A
PRELIMINARY STUDY: EVALUTAION OF FACTORS FOR GELATIN PARTICLE FORMATION

Introduction

There are several factors that affect gelatin particle formation: gelatin concentration, solvent amount, solvent type, solution volume, pH, temperature, stir speed, and crosslinker concentration. The goal of this chapter is to determine which variables have the greatest effect and develop a protocol for making reproducible nanoparticles in the target range of $\geq$ 500 nm.

Gelatin

What sets gelatin apart from this group is widespread availability, low cost of use, and ability to encapsulate a number of different drugs, proteins, and DNA. Since 2005, over 300 thousand tons of gelatin are produced world-wide.[68] It has Food Drug Administration (FDA) approval as food, cosmetic, and medical device.[98] In the pharmaceutical field 90% of pharmacy grade gelatin is used to make hard or soft shell capsules.[68] Gelatins as nano- and microspheres have gained the most interest and related research. Gelatin microspheres and nanoparticles can be delivered topically, orally, or intravenously. They can be dispersed in solutions and ointments, or in the framework of other hydrogel materials. Gelatin microspheres containing antibiotics, anti-fungal agents, growth factors, probiotics, plasmids, oligonucleotides, and drugs have been synthesized.[11, 71, 99-102] Since the process of making gelatin microspheres is generally done under mild conditions, most therapeutic agents can be preloaded into the aqueous solution. However for those molecules that may be damaged by either the temperatures used or the crosslinking chemicals, a method of post-loading has been developed. Briefly, freeze-dried gelatin particles are in a solution containing the agent and as the particle swells, the agent will diffuse into the particle if the pore size permits.[69] The use of gelatin nanoparticles is a more recent development. The first gelatin nanoparticles were fabricated in

the late 1970s, and by the 1990s they were being used to encapsulate a number of drugs.[103, 104] To date gelatin nanoparticles have been used to encapsulate chemotherapy drugs, anti-HIV drugs, plasmid DNA, magnetite, [105-108] .Thus far the research has been primarily focused on achieving particle sizes increasingly smaller. Smaller particle sizes are desirable because of the ability to permeate cell membranes and to transport through intercellular and paracellular pathways. However particle sizes that are in the upper nano-range and are considered submicron moreso than nanoparticles, has not be thoroughly investigated. The particles have the benefit of greater loading capacities of nanoparticles, yet still retain the ability to be phagocyzed by cells such as macrophages.

Gelatin is a linear protein that is typically only soluble in water. The isoelectric point (pI) of gelatin determines its behavior and is dependent on its extraction process. Gelatin with a pI of 4.7-5.2 is identified as typeB, and gelatin with a pI of 7.0 – 9.0 is called typeA It is further identified by its source: bovine, porcine, or fish. Gelatin is typically only soluble in aqueous solutions. The molecular weight of gelatin is indicated by its Bloom number. For these experiments a medium bloom strength which correlates to an average molar mass between 40,000 – 50,000.

Particle Formation

Gelatin particles have been fabricated at the micro- and nano- level to deliver DNA, protein, drugs, and vaccines. Particle formation and morphology is determined by a number of experimental variables. Depending on how the processing method used, gelatin particle size can range from the lower nano level, <100 nm, to large beads >1000 microns. But typically no matter what the size, it is preferred to produce particles within a narrow size range free of aggregates to create uniform, reproducible particles with minimal loss of material. In this chapter, the objective is to determine the feasibility of creating genipin crosslinked submicron particles in the

range of 750 nm – 1µm and to study the effect of processing variables on the size, morphology, and distribution of gelatin micro- and nanoparticles.

Emulsion

To form particles in the microsphere range, the most common technique is the water-in-oil emulsion. This method was investigated initially to determine if it was possible to create particle below the 2 micron range that had been cited in the literature. In this method, the aqueous phase for a gelatin emulsion is generally distilled water or a buffered phosphate or citrate solution. The oils most commonly used are cottonseed oil, corn oil, or olive oil. The particles produced by this technique tend not to be monodisperse and range in size from 6µm to over a 1000µm depending on experimental parameters. The variables that effect particle size are polymer type, polymer size, polymer concentration, water-to-oil ratio, stir speed, surfactants, degree of cross-linking, emulsion time, temperature, and pH. The benefits of using this procedure are that the technique is fairly rapid, easy to replicate, inexpensive, and environmentally safe. The drawbacks are that a narrow size range may not be possible, and particles below the sub-micron range are difficult to achieve. One of the most significant reasons for such large variation in particle size is because the gelatin bloom level used contains a large range of molar molecular weight.

Desolvation

An alternative to the water-in-oil emulsion is a process called two-step desolvation. Desolvation is the use of solvents to separate out fragments of gelatin at different molecular weights. The first successful nanoparticles created using this technique was in the 1970s.[109, 110] However the particles formed aggregates and the procedure was determined to be too labor intensive and skill dependent.[79] Optimization of the desolvation method into a double desolvation have streamlined the procedure to make it more practical in application.[79, 111, 112] After the initial precipitation of gelatin, the smaller fragments are decanted off and

discarded and the remaining gelatin is re-suspended in solution. This method results in a narrower distribution in molecular weight.

Crosslinking Methods

There are a number of different methods both chemical and physical that can be used to crosslink gelatin depending on how they were produced and the intended use. Physical crosslink methods that have been reported are thermal, microwave irradiation and γ-ray irradiation.[113, 114] In thermal crosslinking and cross-linking by irradiation is theorized to happen due to a condensation reaction between the amino groups and carboxylic acid groups. In this study a chemical crosslinker was chosen. Glutaraldehyde is one of the most commonly used crosslinkers. It is soluble in both water and organic solvents. One of its advantages is that it has been widely studied and reasonable in cost. It can be used for both water-in-oil emulsions and desolvation, which so far has been shown to be used exclusively. The potential drawback is that it has been shown to be toxic. Other chemical crosslinkers that were considered were D,L-glyceraldehyde, diisocyanates, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), but they also have toxicity concerns. EDC is particularly useful for the fact that it is zero length crosslinker that will not add a significant increase to the size of the formed microsphere.[99] A chemical crosslinker called genipin with minimal toxicity was considered but the cost was prohibitive for use. The other more commonly used biocompatible crosslinker is genipin, the compound selected for preparing the particles in this section and described previously.

Glutaraldehyde

Glutaraldehyde is a 5 carbon dialdehyde containing a formyl functional group on each end figure 3-1. It reacts with the primary amine groups found in proteins, particularly lysine via a Schiff Base reaction. However it can also react with tyrosine, histidine, and cysteine. Because of its bifunctionality it is used as a tissue fixative agent in a number of biological applications.

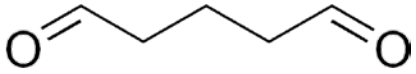


Figure A-1. Structure of glutaraldehyde

The greatest concerns with glutaraldehyde are related to its toxicity. In vitro experiments have shown damage to bacterial DNA. To prepare these particles, both a water-in-oil emulsion and a two-step desolvation method will be employed.

Materials and Methods

Materials

Gelatin (Type B, 325 Bloom), Tween 85, Span 80 were supplied by Sigma-Aldrich, St. Louis, MO, USA. Glutaraldehyde 50% (w/w), Glutaraldehyde 25% (w/w), acetone, ethanol, 2-propanol and cottonseed oil were supplied by Fisher Scientific, Pittsuburg, PA, USA. Genipin was supplied by Wako Chemicals USA, Inc, Richmond, VA, USA. Corn oil was purchased locally

Preparation of Microparticles by Water-in-Oil Emulsion

The basic procedure to make gelatin microspheres is as follows. Distilled water was heated to 60℃ and gelatin was added into the water, stirred at low speeds. Varying concentrations (1%, 3%, 10%, and 20% w/v) were used to form the particles. The solution is allowed to stir until the protein is completely dissolved and the solution is clear. Cottonseed oil is heated to 70℃, and a surfactant is added to the oil. Following the gelatin is added dropwise using a 22G needle. An overhead 2-blade stirrer is set to 1000 rpm, and the emulsion is allowed to mix for. After 20 minutes an ice bath is added to cool the emulsion to≈ 5℃, mixing is allowed to continue for an additional 15 minutes. A volume of chilled acetone equal to the volume of the emulsion is added, mixing occurs for an additional 20 minutes. The formed microspheres are collected by vacuum filtration, and washed 3 times with chilled acetone. The

particles were allowed to dry under vacuum for 24 hours at room temperature. There were several modifications to this procedure to decrease particle size.

The most significant modification is to gelatin solution concentration and the addition of a surfactant. 30 ml of DI water was heated to 60°C and 3 grams of gelatin was added and stirred until dissolved. 0.6 ml of Span 80 was added to 60 ml of cottonseed oil and heated to 60°C. The heated oil was added to the gelatin solution and the stir speed was set to 1000 rpm, and the solution mixed for 10 minutes. The mixture was rapidly cooled to 5°C with the use of an ice bath and stirred for 10 more minutes to consolidate the particles. 90 ml of chilled acetone to the mixture and stirred for 15 more minutes to further harden the particles. The formed particles were filtered and wash with 25 ml of chilled acetone for a total of 3X to remove all traces of the oil. The particles were collected and vacuum dry for at least 2 days. No crosslinker was added. The particles were examined under light microscopy Figure A-2. The above results were repeated and the concentration of the gelatin solution was decreased from 10 to 5%. Particles made from this procedure were also analyzed for a swelling response in DI.

Preparation of Sub-Micron Particles by Double Desolvation

In a procedure adapted from Azarmi et al[111], Gelatin nanoparticles are prepared by dissolving gelatin (1.25g to 3.25g) in 25 mL of distilled water at 45°C. 25 mL of a solvent (acetone, ethanol, 2-propanol) is added. After complete precipitation, the supernatant is removed and 25 mL of distilled water is added to the precipitate. Dilute hydrochloric acid or sodium hydroxide is added to the solution to adjust the pH between 2 and 12. A second desolvation step initiates the nanoparticle formation. 75 mL of a solvent corresponding with the first desolvation is added drop-wise to the solution. After all the solvent is added, the crosslinker is added. Varying amounts of 50mM Genipin in 60% (v/v) ethanol are added to the solution. Additionally 250 µL of 25% glutaraldehyde is also added to a control solution. The solution is allowed to stir

for 12 hours at 600 rpm. The particles are removed by ultrafiltration by an Amicon XM300 membrane, and washed with a 1% w/v Tween 20 solution. The particles are then lyophilized in 5% trehalose and stored at 5°C until further use.

Size and Distribution Characterization

A Coulter LS230 is used to measure both the nano- and microparticles. To prevent swelling and agglomeration, isopropanol is used as the mobile phase in place of distilled water.

Microscopy Characterization

A Zeiss optical microscope was used to observe the microspheres. The spheres were imaged with a 40X objective is used to verify particle formation, level of aggregation, and swelling under aqueous conditions. For the nanoparticle evaluation, a Hitachi 7600 Transmission electron microscope was used and the data was analyzed using AMT Imaging software. The particles were prepared from suspension by staining them with a 2% aqueous solution of uranyl acetate.

Results and Discussion

Emulsion

Particles formed using a 10% solution of gelatin in a 1% (w/w) of Span 80 in cottonseed oil, were in the 20 – 50 micron range Figure A-2. They appeared mostly dispersed but were one order of magnitude too large to be phagocyzed by macrophages and two orders of magnitude too large for optimal dendritic cell uptake. Decreasing the concentration by ½ showed the formation of many smaller particles that were intermixed with larger particles in the 10-30 micron range Figure A-3.

Particles prepared from the 5% gelatin solution were also evaluated for swelling capacity in an aqueous solution. Uncrosslinked gelatin microspheres increased their volume in a linear fashion by a 100% within 1 hour Figure A-4.

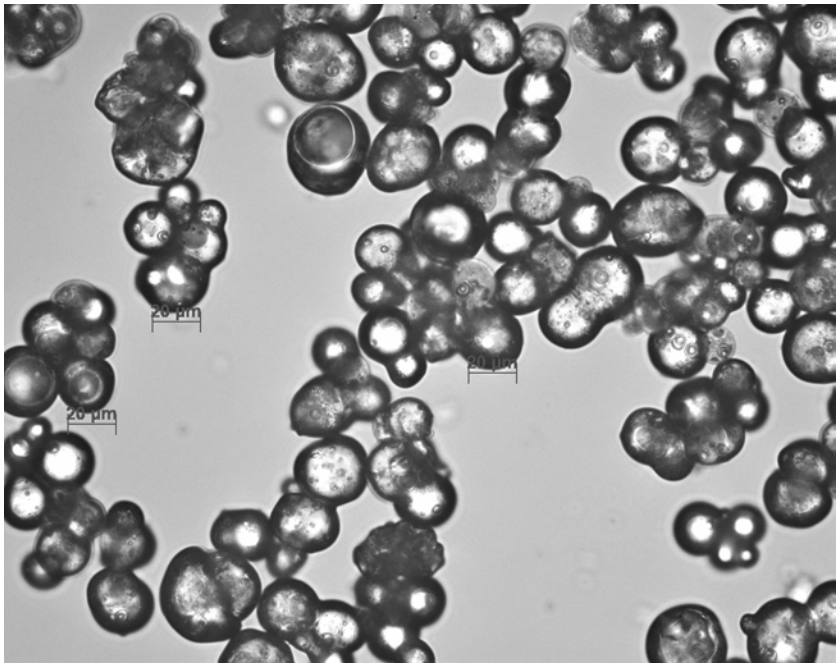


Figure A-2. Gelatin microparticles from a 10% solution formed from water-in-oil emulsion. Magnification is 40X

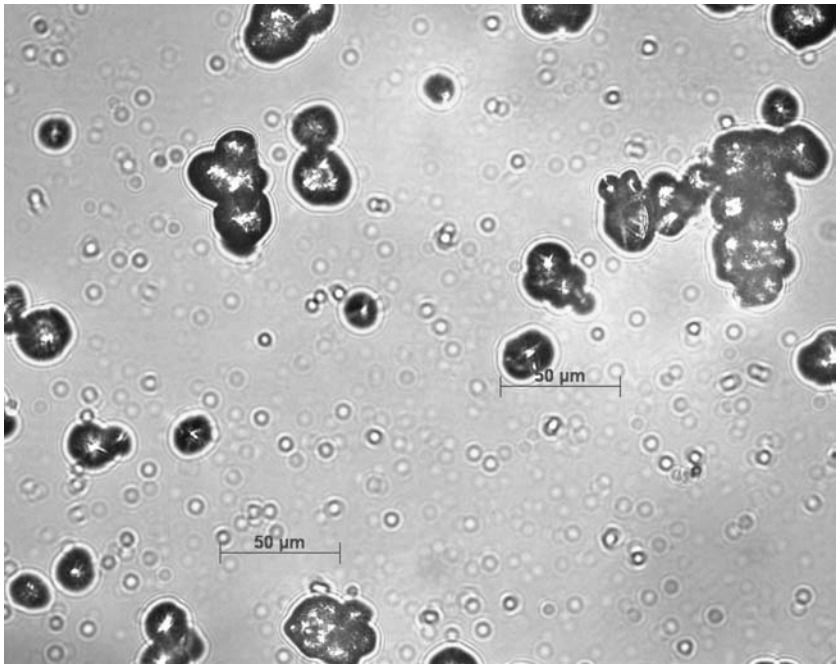


Figure A-3 Gelatin microparticles from a 5% solution formed from water-in-oil emulsion. Magnification is 40X

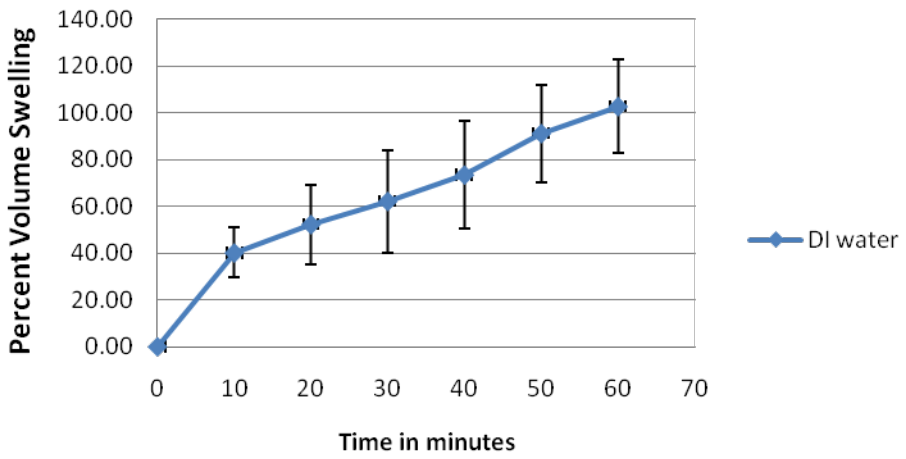


Figure A-4. Swelling of uncrosslinked gelatin particle in water.

The particle sizes obtained with the water-in-oil emulsion showed a significant decrease in particle going from a 30% solution to a 20% solution figure 3-1, but the following decreases in concentration from 20% to 10% to 5% did not show a substantial decrease in particle size. A portion of this effect can be attributed to agglomeration of smaller particles at lower concentrations. There is also some additional error associated with how the particles are washed and collected from the emulsion. The particles formed in the lower concentrations appear to undergo a caking effect that forms a semi-stable product when collected under vacuum filtration. When centrifugation is used a pellet forms in less than 5 minutes of centrifuging that resist manual and mechanical dispersion efforts.

Desolvation

After several attempts at decreasing particle size with modifications to the emulsion method, it was apparent that nanoparticle formation was unlikely through these means. In my first attempt at using the double desolvation method I created spherical disperse particles that were 50 microns in size Figure A-5. These particles were not any smaller than the particle prepared using the water-in-oil emulsion but they were more dispersed.

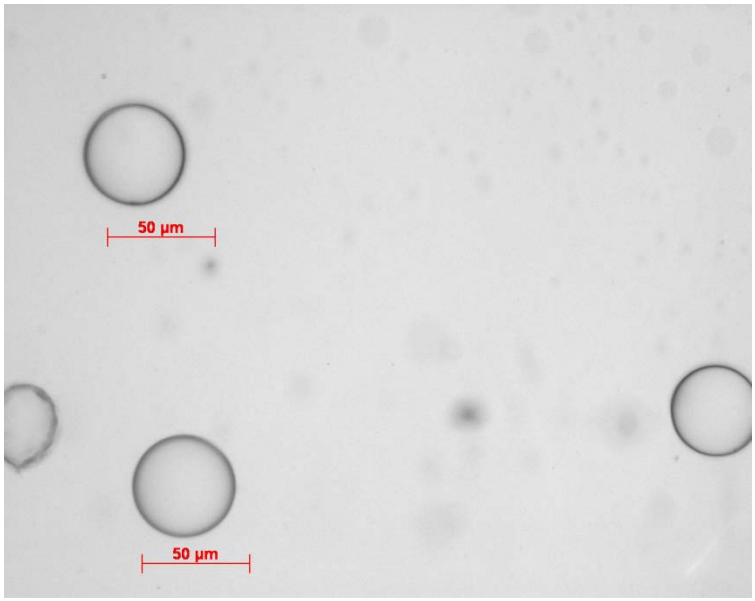


Figure A-5. Gelatin microspheres formed by desolvation. Magnification 40X

From a review of the literature I ascertained that it is necessary to keep your solvent at a constant volume throughout the experiment. During the initial stages of the precipitation when the particle is first forming this is critical. In my first experimentation with desolvation, I created the particles in an open system under heating. This allowed the solvent to evaporate from the solution changing my experimental conditions within minutes. My second attempt was much more successful. The particles were made in a round bottom flask to ensure adequate mixing. The flask was sealed with a stopped to prevent solvent loss. Later experiments used a condensing tube to keep the acetone within the flask. A nanotrec analysis of the particles revealed that the number average diameter (MN) was 65.50 and the volume average diameter was 81.40. Analysis on the coulter showed similar results MN= 75 nm and MV = 96 nm. Attempts made to image the particles under SEM were unsuccessful.

The next phase of the desolvation was to evaluate which parameters had the greatest effect on particle formation. An experiment was designed to scan through a number of conditions

many of the conditions were only run twice per sample. The results gave information on trends but it was not a statistical significance study therefore no error bars were used.

The first variable examine was the effect of pH on particle size, Figure A-6. The isoelectric point of Type B gelatin is 4.7 - 5.2. Therefore to be able to precipitate gelatin in the form of nanoparticles as opposed to a solid mass, the pH of the solution must be moved away from the isoelectric point. In the literature it is suggested that the most extreme points for this to occur are at a pH of 2.5 and 12. At a pH of the mean diameter was 194.2 nm, at a maximum pH of 11 the diameter was 259.7 nm. Since both pHs represent maximum charge extremes, it seems that the sizes should be more similar. However the literature also shows a slight size increase for particles made in a basic solution.

The next variable examined was to determine how the amount of solvent (acetone) added affected the properties of the particle, Figure A-7. As solvent is added to the solution the solvent molecules began to displace the water molecules surrounding the protein and at a critical point the protein precipitates out as a particulate. There is an upper and lower limited associated with how much solvent can be added to form nanoparticles, this limit is dependent upon polymer concentration, temperature, and pH. With the experimental conditions I used my particles appeared to decrease in size relative to the increase of acetone used. I haven't found a literature citation with the exact experimental conditions and outcome but the values are well within range.

The next variable examined was that of temperature, Figure A-8. According to the literature, as temperature increases the polymer tend to be less condensed which lead to larger particle formation. The minimum temperature that can be used is near 40°C, because temps lower than that can cause premature gelation. The maximum temperature than can be used is >80°C to avoid degradation of the polymer. From the data, we can see that particle average

diameter increased by over 100nm from 40°C to 60°C. These results are consistent with the literature.

The final variable examined was that of glutaraldehyde concentration on particle size, Figure A-9. According to an article published by there is no statistically significant variance with concentration at amounts between 100 µl-500 µl.[111] My data also showed similar results. The explanation for this is that even at the lowest concentration the polymer was at maximum crosslinking concentration.

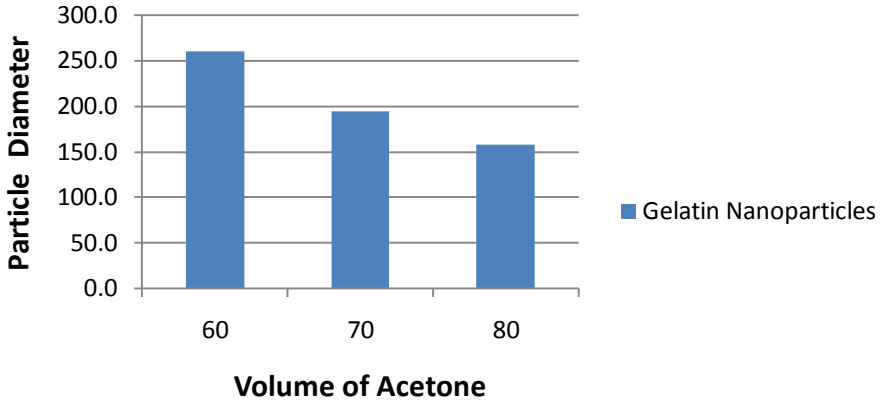


Figure A-6 Effects of acetone on particle size. Temperature was 50°C, pH was 2.5, glutaraldehyde concentration was 250 µl. N=1

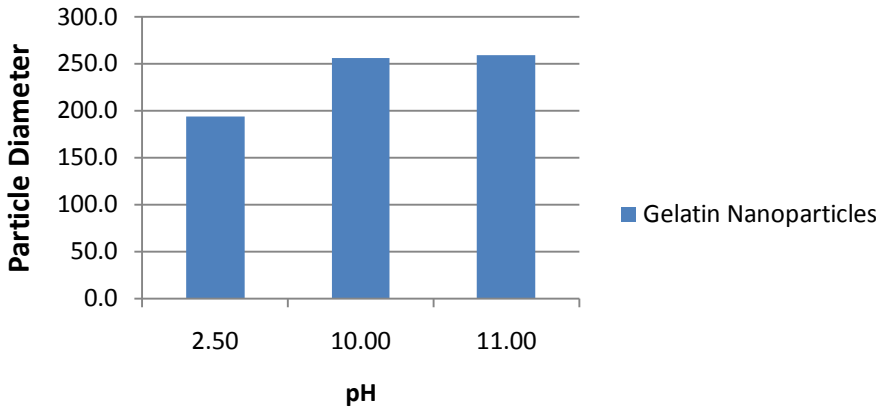


Figure A-7. Effect of pH on particle size. Temperature was 50°C, acetone volume was 60ml, glutaraldehyde concentration was 250 µl. N=1

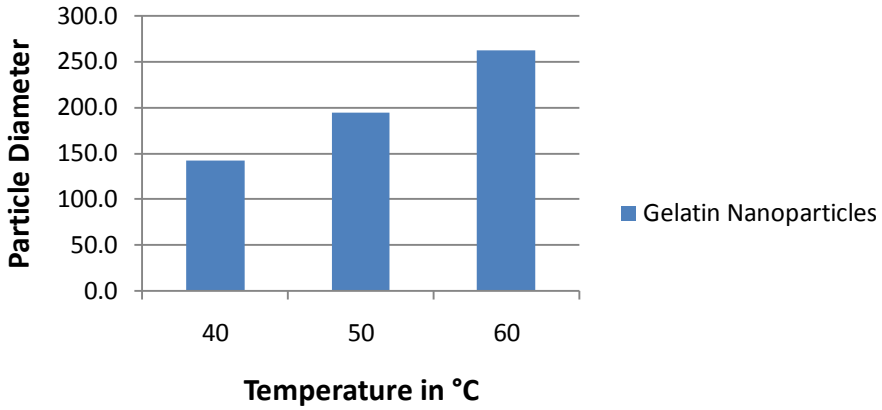


Figure A-8. Effect of temperature on particle size. Acetone volume was 60ml, glutaraldehyde concentration was 250 µl, pH was 2.5. N=1

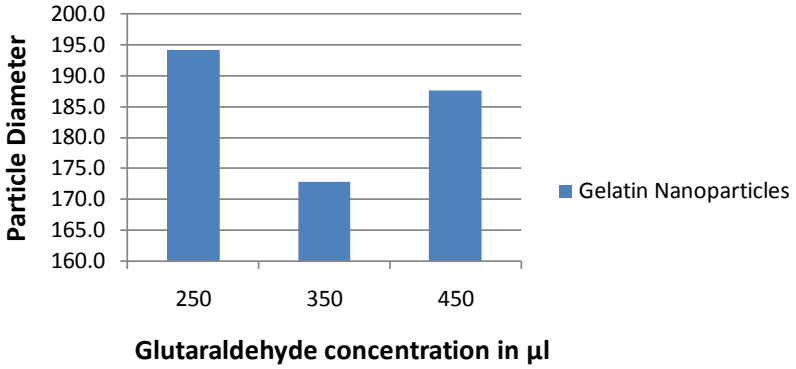


Figure A-9. Effect of crosslinker concentration on particle size. Temperature was 50°C, acetone volume was 60ml, pH was 2.5. N=1

Particles from the first series, 60 ml of solvent at 50°C, with 250 µl of glutaraldehyde were imaged using TEM. The particles appeared spherical dispersed and ≥ 300 nm, Figure A -10.

Summary

These preliminary experiments provided a focal point on the parameters that would lead to nanoparticle formation. However a key error made in this rough screening study is that the variables were studied independently. The conditions are not stationary and many of the variables that are used to create nanoparticles at one range will act entirely differently depending on the environment. The two variables that seemed to be the most interlocked are pH and solvent volume. A factorial design of variables would have been much more helpful in

determining what conditions to use for gelatin, however the thiolated gelatin would need a separate factorial study because there are fundamental differences between the two types.

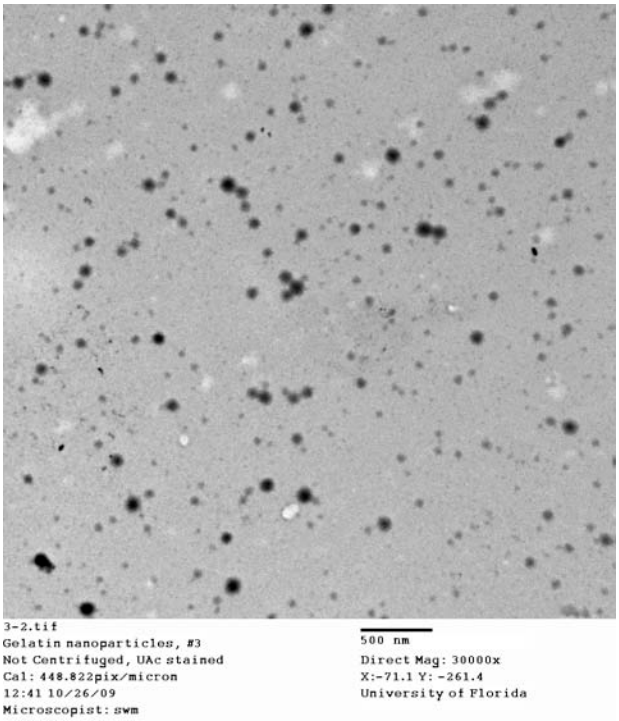


Figure A-10 TEM image of nanoparticles made by desolvation. Magnification 30K

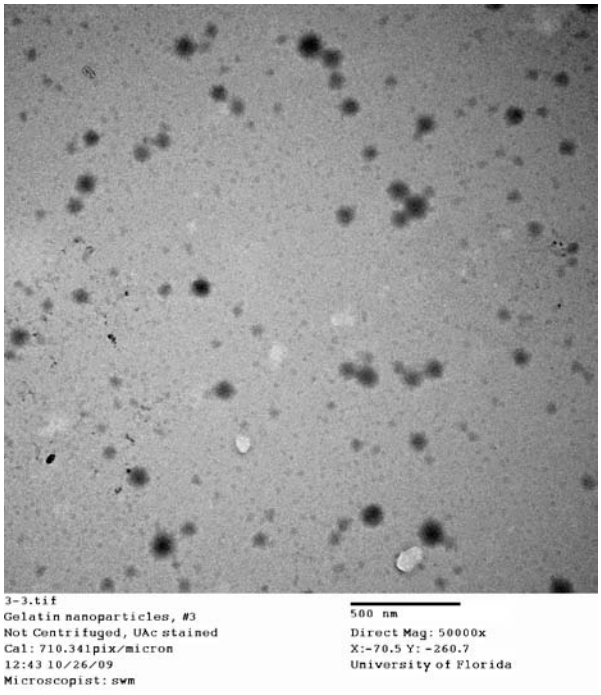


Figure A-11 TEM image of nanoparticles made by desolvation. Magnification at 50K.

APPENDIX B
AMINO ACID IN GELATIN

Table B-1. Amino acid properties of gelatin

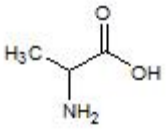
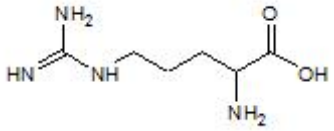
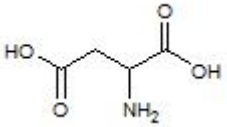
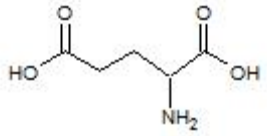
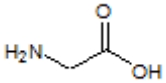
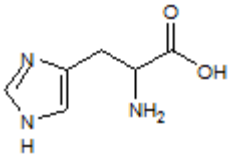
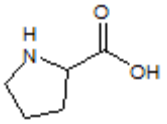
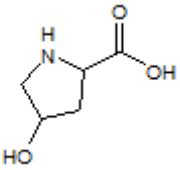
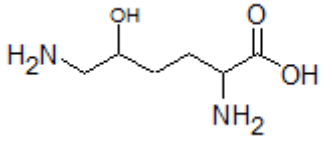
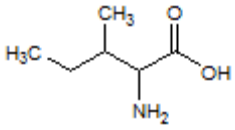
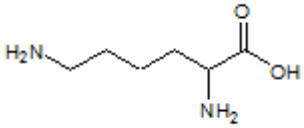
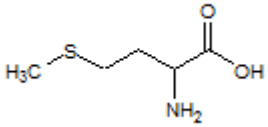
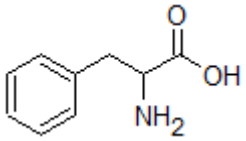
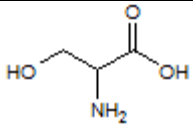
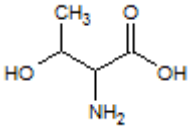
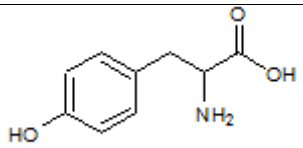
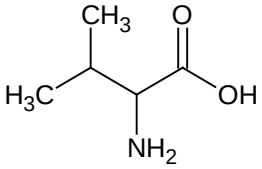
Amino Acid	g amino acid per 100 g of pure protein	pKa of charged side chains at pH 7.4	Structures
Alanine	11.3	***	
Arginine	9.0	12.10	
Aspartic Acid	6.7	3.71	
Glutamic Acid	11.6	4.15	
Glycine	27.2	***	
Histidine	0.7	6.04	
Proline	15.5	***	
Hydroxyproline	13.3	***	

Table B-1. Continued

Hydroxylysine	0.8	***	
Isoleucine	1.6	***	
Lysine	4.4	10.67	
Methionine	0.6	***	
Phenylalanine	2.5	***	
Serine	3.7	***	
Theronine	2.4	***	
Tyrosine	0.2	10.10	
Valine	2.8	***	

*** There were no charged side chains

APPENDIX C
AFM COMPARATIVE ANALYSIS

During the AFM analysis, a particle from the least modified and most modified gelatin samples were selected to determine an approximation of the surface roughness and charge distribution over the surface. However since only a single particle from both samples was imaged, they could not be considered a true representation of how the bulk of the particles appear. A scan of both the unmodified (0-SH) and most modified (80-SH) showed a rough surface that did not appear to be dependent on thiol modification, Figure C-1, C-2. Phase images were also taken of the same particles to determine if the particles exhibited homogeneity on their surface. The 0-SH particle showed regions where the charge distribution was unequal, Figure C-3. The charges on the 80-SH were relatively consistent over the surface of the entire particle.

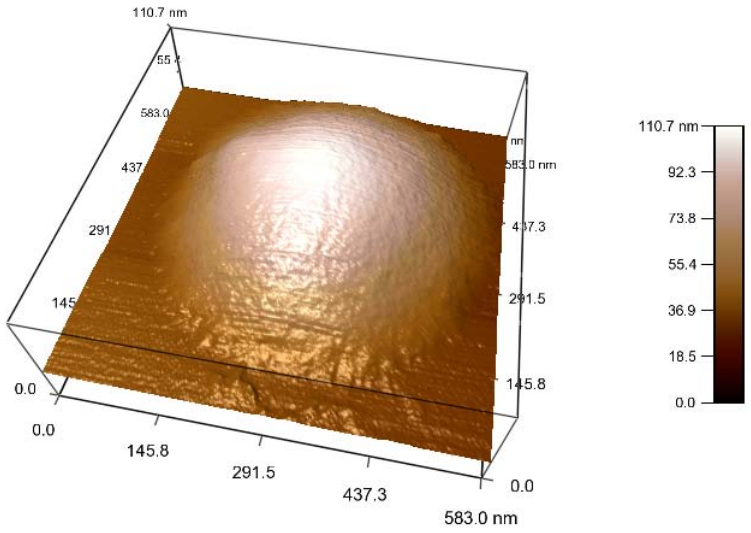


Figure C-1. Surface roughness of 0-SH sample

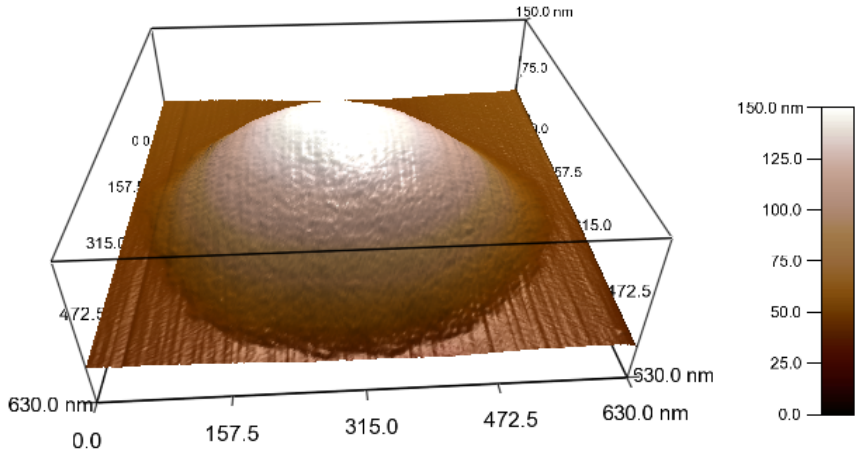


Figure C-2. Surface roughness of 80-SH sample

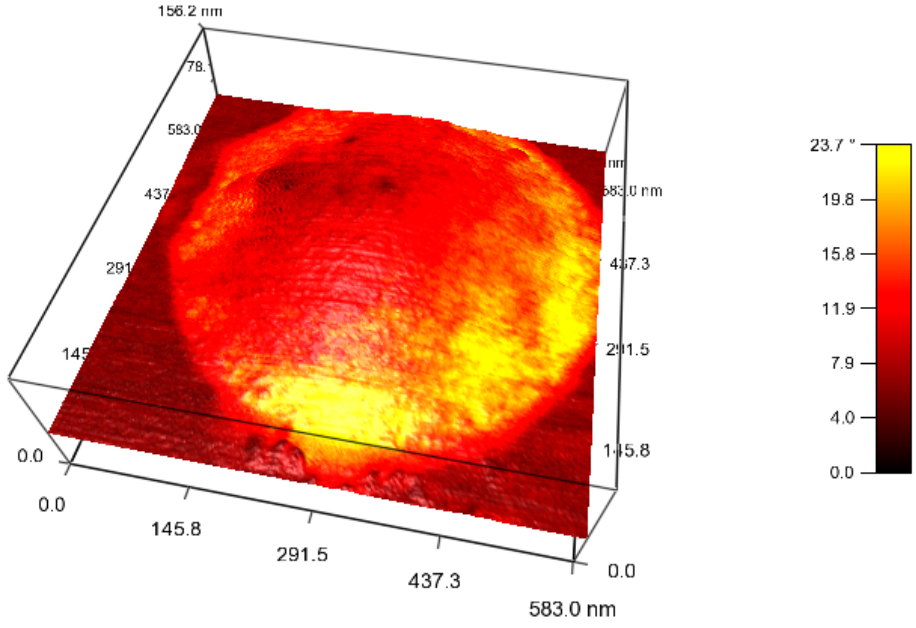


Figure C-3. Phase contrast image of 0-SH showing heterogeneity.

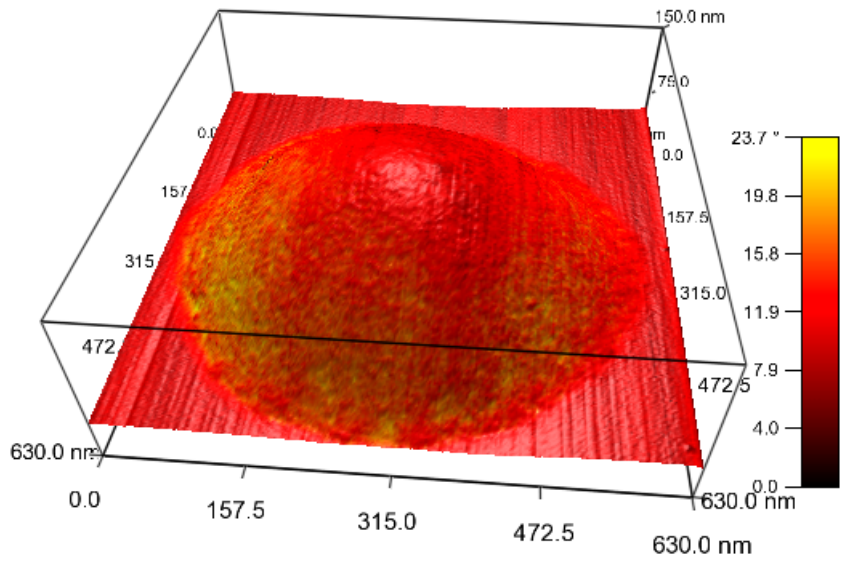


Figure C-4. Phase contrast image showing homogeneity of 80-SH

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BIOGRAPHICAL SKETCH

I was born in Tallahassee, FL the oldest of three girls to Freddie and Shirley Washington. I attended high school at Leon High where I first discovered my passion for science in Mr. Steverson's marine biology class. Upon graduation I attended Florida State University and majored in biochemistry. During my tenure at Florida State, I enhanced my academic career by obtaining employment as a research assistant in both a biochemistry lab and a chemical engineering lab. In my final year I joined the Alpha Chi Sigma chemistry fraternity, where I formed life-long friendships. In 1998, I graduated with my B.S. in biochemistry and shortly began work at the Florida Department of Environmental Protection, Bureau of Labs. After a two year stay, I returned to school as a graduate student to study Materials Science and Engineering at the University of Florida. While there I desired to increase my involvement with the various professional societies in my discipline. I subsequently joined, the Society for Biomaterials, the Materials Research Society, and the National Society of Black Engineers. Through the Materials Research Society I have participated in their apprentice reporter program, and have written several articles for the monthly bulletin. In 2003, I received my Master of Science degree in Materials Science. This accomplishment helped me to obtain internships at Regeneration Technologies, Inc and Vistakon. In 2009, I expect to receive my PhD in Materials Science and Engineering.