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β-Glucan Particles as a Delivery System in Peptide Vaccine Development

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ABSTRACT

The successful delivery of vaccine antigens such as peptides and proteins to stimulate CD4 and CD8 T cell immunity could provide a prevention or treatment of infectious diseases and malignant disorders. This project tested different strategies of peptide delivery to phagocytic cells using β-glucan particles (GPs), and assayed the *in vitro* immune-stimulatory capabilities. GPs are hollow microparticles derived from Baker's yeast that can be used to encapsulate or bind peptide payloads to ensure delivery to target cells. Various synthetic strategies were evaluated to identify an effective delivery method for *in vitro* testing using a T-cell proliferation assay.

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BACKGROUND

Role of Vaccines in Human Health

Significant progress has been made over the past several years in the areas of virology, immunology, and the development of vaccines. Vaccines have been a critical part of the fight against various diseases both in the developed and the developing world. The introduction of vaccines into medical practice has had an outstanding impact on human health, drastically diminishing or eradicating the incidence and morbidity of a large number of infectious diseases such as smallpox, polio, and diphtheria. Currently, three main types of vaccines are used in humans: live-attenuated vaccines composed of a virus or bacterium that is less pathogenic than the real pathogen, inactivated vaccines that are heat- or chemically-inactivated particles of the pathogen, or subunit vaccines that are made of components of the pathogen. In the process of attenuation, an infectious agent is altered so that is less virulent, while retaining its ability to interact with the host organism and stimulate an immune response (Badgett et al., 2002). Live attenuated vaccines are often able to induce strong, long-lasting immunity. An example of successful live attenuated vaccines that are delivered by injection is a vaccine for measles, mumps, rubella and the varicella zoster virus (Knuf et al., 2008). There are also a number of mucosally delivered live vaccines, including oral vaccines against poliomyelitis (Bonnett and Dutta, 2008), cholera, and typhoid fever (Levine, 2000). Live attenuated vaccines have a number of advantages over the others, such as the effective stimulation of cellular, humoral, and mucosal immunity, and their low cost. Despite these

advantages, there are some downsides including storage limitations and possible mutations of the organism, which often leads to negative effects. For cases in which live attenuated vaccines have serious adverse effects, further refinement of the vaccine can sometimes be provided by developing an inactivated formulation for immunization by killing the disease-causing agent with chemicals, heat or radiation. Most inactivated vaccines, however, stimulate a weaker immune response than do attenuated vaccines. The comparison study on live attenuated versus inactivated influenza vaccines showed that the live vaccines are more efficient and safer, when administered to infants and young children of 12 to 59 months of age (Belshe et al., 2007). In contrast, a live attenuated oral cholera vaccine showed no significant protection in a large field trial in Indonesia. These results demonstrate that inactivated vaccines can elicit some degree of immunity and provide protection from mucosal pathogens (Richie et al., 2000).

Recent advances in molecular biology and genetics gave rise to the ability to produce subunit vaccines. Subunit vaccines include only the antigens that best stimulate the immune system, lessening the chance of adverse reactions to the vaccine. The first recombinant subunit vaccine against Hepatitis B was developed in 1986 in *Saccharomyces cerevisiae* by isolating and transferring the gene encoding the Hepatitis B surface antigen into a non-pathogenic organism (yeast). The recombinant subunit vaccine was then produced by the yeast, and could be purified for vaccine purposes and designed for delivery (Valenzuela et al., 1982). Today, recombinant subunit vaccines are being investigated in the development of new vaccines or more effective vaccines with less adverse effects.

The Immune System

The immune system of multicellular organisms plays an essential role in protecting the host against infection, and consists of two components: innate and adaptive immunity. Innate immunity is triggered by infection to protect the host organism by destroying the invading microbes and viruses, while adaptive immunity develops with exposure to various antigens, building a defense that is specific to that antigen.

Innate Immunity

Innate immunity is the first-line of the host-defense, and is mediated by leukocytes, macrophages and dendritic cells, which engulf and kill the microbes. Initially, the innate immune system response was thought to be non-specific, relying on a very limited number of receptors that recognize the structure of microbes. However, a study done in *Drosophila* showed that protein *Toll* was critical for defending fruit flies against fungal infections (Lemaitre et al., 1996), and this led to the identification of at least 10 similar proteins, named toll-like receptors (TLRs) that recognize and respond to infection by enabling innate response and also inducing an adaptive response (Takeda et al., 2003). Pathogens are "betrayed" to the innate immune system by pathogen-associated molecular patterns (PAMPs) that are vital for their own homeostasis (Janeway, 1989). Innnate immune cells can recognize PAMPs through TLRs expressed on the cell surface, which leads to activation of T cells. The innate immune system provides an immediate defence

against the infection, and is considered as a temporary system until adaptive immunity is induced (Akira, 2011).

Adaptive Immunity

In some cases, the innate immune system is unable to fight the infection, so the activation of an adaptive immune system is required. Adaptive immunity recognizes peptide antigens using antigen receptors expressed on the surface of B and T lymphocytes. These cells are able to rearrange their immunoglobulin and cell receptors genes to generate a vast variety of different antigen receptors. Adaptive immunity is further divided into humoral and cellular immunity.

Cell-mediated immunity is responsible for the eradication of intracellular pathogens and is driven by T lymphocytes. T cells target pathogen-infected cells through peptide-bound MHC class I molecules expressed on the cell surface, inducing apoptosis through perforin- and granzyme (granule enzyme) -mediated cytolysis (Lieberman, 2003). Humoral immunity (antibody production) is involved in the destruction of microbes present in the blood or fluid by generating antibodies produced by B cells. Antibodies bind to pathogens, blocking infection by mediating phagocytosis or by helping activate the complement pathways. CD4+ helper T cells play a role in humoral immunity by driving B cell maturation to antibody secreting plasma cells (Kutteh and Mestecky, 1994). Components of humoral immunity include soluble pattern recognition molecules (PRMs) that recognize PAMPs and initiate the immune response in coordination with cellular immunity.

Vaccine Development

Subunit Vaccines

The goal of vaccination is to generate a strong immune response, providing a long-term protection against infection. Many of the vaccines developed during the last two decades are based on the subunit approach. Different strategies of generating subunit vaccines were developed such as crude extracts, proteins, MAPs, and peptides.

Multiple Antigenic Peptides (MAPs)

The method of multimerization of peptides, known as the multiple antigenic peptide (MAP) system, was developed by Tam to improve the immunogenicity of subunit peptide vaccines (Tam, 1988). In this MAP system, multiple copies of antigenic peptides are simultaneously bound to Lys-based dendrimer and were used to induce higher amounts of antibody production. However, additional components such as adjuvants were required to achieve the effects. In a study done by Kumar, multiple T-cell and B-cell epitopes from *Plasmodium falciparum* were synthesized as the constituents of MAP, and showed induced antibody and cellular responses in mice (Mahajan et al., 2010). The antibodies generated by this immunization significantly reduced the growth of blood stage parasites in erythrocyte cultures.

Crude Extract Vaccines

Lopez et al. (2007) developed a strategy to generate bioactive compounds through the chemical diversification of inactive crude product mixtures, which gave rise to the development of crude extract subunit vaccines (Lopez et al., 2007). An example of such vaccine is the use of *Pasteurella* lipoprotein E (PlpE) as a subunit vaccine to protect animals from diseases caused by *P. multocida*. The vaccine is comprised of lipoprotein PlpE as an antigen and adjuvant (Chang et al., 2011). This chemical diversification approach provides a great potential for developing a practical vaccine to assist with the control of human diseases.

Protein and Peptide Vaccines

Proteins and peptides are increasingly recognized as excellent prospects for the development of new vaccines. Peptides, identified as immunogenic epitopes, can elicit a strong immune response when delivered together with a carrier (Simard et al., 1997) or an adjuvant (Hsu et al., 1996). Peptide and protein therapy allows delivery in very low doses due to their specificity. However, various obstacles to their successful delivery hamper their therapeutic potential and clinical application. Recent studies in the fields of liposomes, microparticle, and polymer conjugation strategies allowed the development of novel peptide delivery systems and their validation *in vivo*.

Peptide Vaccine Delivery

Microparticles

Particles between 1 and 1000 μm are classified as microparticles. Various studies were done to design microparticle formulations for peptide and protein delivery. In a study for diabetes treatment, insulin-loaded microparticles were formulated for oral insulin delivery, and its effectiveness was tested through the reduction of initial blood glucose levels in diabetic rabbits. The blood glucose tests showed a 70% glucose reduction in rabbits treated with sodium alginate microparticles (Builders et al., 2008). In another study, Marazuela et al. evaluated whether intranasal administration of synthesized peptide containing poly-(lactide-co-glycolide) (PLGA) microparticles were able to prevent mice from allergic sensitivity to the whole protein. It was reported that PLGA microparticles had an initial burst release of 74% peptide followed by a slow release over several weeks, and the release decreased the allergic sensitivity (Marazuela et al., 2008). The studies show the effectiveness of microparticles as a potential drug delivery system.

Liposomes

Liposomes are synthetic microscopic vesicles composed of lipid membranes surrounding discrete aqueous compartments (Weissman and Sessa, 1968). Due to their flexibility in size, composition, and charge, various types of liposome formulations have been developed for enzyme delivery. They have also been used for delivering anti-cancer drugs targeting tumor tissues (Ryman and Tyrrell, 1980).

However, peptides coupled to liposomes usually yield products that are chemically and physically ill defined. To overcome this problem, Gyongyossy-Issa et al. reported the use of disulfide linkages on the surface of the liposome to attach a peptide representing one-half of a ligand-receptor pair to achieve target specificity (Gyongyossy-Issa et al., 1998).

Polymer Conjugation

Renal filtration and excretion are responsible for the rapid clearance of serum proteins with low molecular weights from the organism. For vaccines, this issue can be prevented by conjugating the biomolecules with water-soluble polymers, which results in molecules of higher molecular weights. Another advantage of peptide-polymer binding is that polymers increase the resistance against enzymatic degradation and lower the immunogenicity.

Several polymers have been used for protein conjugation. Currently, poly (ethylene glycol) (PEG) is one of the most widely used polymers for the modification of peptides and proteins. This polymer is inexpensive, has low toxicity, and has been approved for use by drug regulatory agencies (Roberts et al., 2002). The first Food and Drug Administration (FDA) approved PEGylated pharmaceutical (PEG-ADA) appeared in 1990 for the treatment of severe combined immunodeficiency disease (SCID). Since then, several more PEGylated products have received approval from the FDA, testifying to the safety and applicability of this polymer conjugation for the treatment of severe diseases (Levy et al., 1988). Chitosan and chitosan derivatives have been extensively used in the delivery of therapeutic proteins due to their

absorption-enhancing properties. In many studies, it has been demonstrated that chitosan-based formulations significantly enhanced the absorption of proteins (Calvo et al., 1997; Vila et al., 2004). Other alternative polymers that have been successfully used for protein conjugation include PLL (poly-L-lysine) and PEI (polyethyleneimine). PLL and PEI have been widely used as non-viral vectors for gene delivery, favored due to the biodegradability of these polypeptides and their accessibility within a broad molecular weight range. However PEI shows far superior delivery efficiency due to its high charge density. In the study done by Erbacher et al. (1999), RGD peptides were covalently bound to PEI to achieve specific cell adhesion enhancing cellular uptake (Erbacher et. al, 1999). Polymer conjugation methods can be further explored with regard to the chemistry of binding, as well as the development of polymers with enhanced stability, improved cellular uptake, and minimal adverse effects.

β-Glucan Particles as an Adjuvant and Drug Delivery System

Unlike live attenuated vaccines, both inactivated and subunit vaccines require the addition of an adjuvant to be effective. Adjuvants are used to enhance the immune response against antigens and to increase immunological memory and improve protection through the stimulation of immune responses. One of the key features of an efficient adjuvant formulation is the activation of innate immunity by PAMPs, which leads to protecting, long-lasting immunity against the infection. β-glucan particles (GPs) are hollow, porous, 2-4 μm spherical particles extracted from *Saccharomyces cerevisiae* (Baker's yeast) cell walls. In order to prepare β-glucan

particles, yeast cell walls are treated so that the cell wall components and the soluble proteins within the yeast cell are removed (Yan et al., 2005). GPs are composed of β-1-3-D-glucan and residual chitin. The porous and hollow structure of glucan particles allows encapsulation of payload molecules within the particles and provides the capability of delivering the drug into cells. β-glucan cell surface receptors dectin-1 and Complement Receptor-3 (or CP3) allow targeted delivery of the drug into cells such as macrophages and dendritic cells (Brown and Gordon, 2001). β-glucan is considered a PAMP that acts independently of the Toll receptor pathways, and has been referred to as a "biological response modifier", known for its ability to stimulate the immune response (Chihara, 1992). The ability of glucans to deliver drugs to macrophages and dendritic cells, as well as their ability to enhance the immune response, makes glucan particles an appealing option to develop formulations comprised of a peptide core inside the glucan particles as potential drugs. The use of GPs for the delivery of macromolecules, such as DNA, siRNAs, and protein has been successfully demonstrated both *in vitro* and *in vivo* (Soto and Ostroff, 2008; Aouadi et al., 2009). Different methods of trapping macromolecules within GPs have been tested, including polyplex formation or layer-by-layer synthetic method, nanoparticle and GP surface derivatization.

Macromolecule Loading in Cores

The strategy of loading macromolecules in GP cores includes the physical entrapment of the payload drug such as Rifampicin (Rif) within the glucan particles using an alginate or chitosan hydrogel to slow down the release of the drug. The

hydrogels are high-water content materials that are prepared from cross-linked, biodegradable, biocompatible, and non-toxic polymers. The use of GPs for encapsulation of Rif was demonstrated by Ostroff and proved to delay Rif release from the GPs over a 24 - 72 h period (Soto et al., 2010).

Macromolecule Loading in Layers

This method entails the encapsulation of therapeutic molecules in a layer-by-layer (LbL)-assembled GP cores. These can accommodate high payloads, and are compatible with targeting and controlled release of the drug. The LbL approach includes the assembly of different components through electrostatic interactions. An efficient microparticle-encapsulated nanoplex-based DNA delivery system was developed by Ostroff (Soto and Ostroff, 2008). Nanoparticulate cores composed of anionic and cationic polymers were constructed within GPs, followed by the binding of DNA to it, and coated by a second cationic polymer layer to protect the DNA. Polyethylimine (PEI) was used as the trapping polymer layers in the original study to demonstrate the use of GP for DNA delivery. Currently, research is being conducted to find polymers with equal or better efficiency due to the cytotoxicity of PEI.

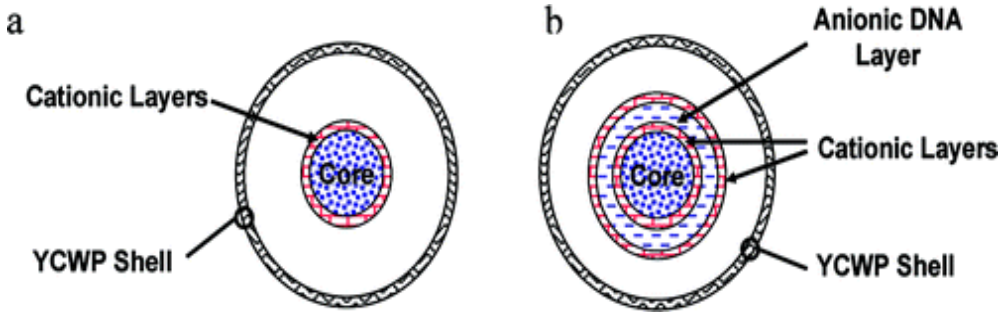

Figure 1: Schematic representation of a) a nanoparticulate core constructed within porous, hollow GP, and b) adsorption of DNA onto an encapsulated nanoparticulate core (Soto and Ostroff, 2008).

Macromolecules Loading in Nanoparticles

Nanoparticles can act as drug carriers for different water insoluble molecules. The development of nanomaterial for delivering such drugs has received significant interest in the past decade. The ideal drug delivery system has to be able to target to specific cells, and to control the drug release. The ability to selectively target phagocytic cells makes the glucan particles a potential drug delivery system for nanoparticles.

Loading Nanoparticles Inside GPs

Because of the ease of synthesis and ability to entrap therapeutic drugs, mesoporous silica nanoparticles (MSNs) have been used as a model system to load nanoparticles inside the GPs. MSNs containing the anti-cancer drug Dox (Doxorubicin) were electrostatically bound to GPs derivatized with PEI (polyethyleneimine) and were successfully delivered to the cells (Soto et al., 2011).

Loading Nanoparticles Outside GPs

In addition to the use of the hollow cavity of GPs for drug encapsulation, the surface of the GPs offers another option for drug binding. Chemical derivatization of the GP surface has been studied to introduce targeting ligands to increase cell tropism of the particles, and derivatization of polymers for covalent and non-covalent binding of payload drugs. Cationic GPs are synthesized by functionalization with branched PEI, and fluorescent particles of larger sizes are electrostatically bound to the PEI-GP surface. The studies demonstrated successful targeted delivery of Dox-MSN-PEI-GPs to cells inhibiting the growth of phagocytic cells (Soto et al., 2011).

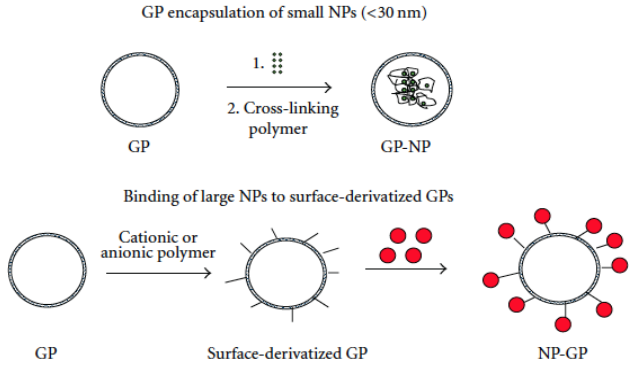

Figure 2: Schematic depiction of several glucan particle/nanoparticle synthesis methods (Soto et al., 2011).

Other Binding Methods

The GP surface can also be derivatized with molecules that are used as universal acceptors for the attachment of ligands for specific chemical reactions. For instance GPs derivatized with biotin can bind ligands through biotin-avidin or biotin-streptavidin interactions.

GP Surface Modification by Derivatization with SATA

Another approach of GP surface modification is its derivatization with SATA (N-succinimidyl S-acetylthioacetate). SATA is a reagent used to introduce sulfhydryl groups into variety of amine-containing proteins and peptides (Duncan et al., 1983). SATA contains an N-hydroxysuccinimide (NHS) ester, which reacts with amine group by nucleophilic attack, resulting in a stable covalent bond formation. Several advantages of using SATA for introducing sulfhydryl groups into proteins and peptides include the ease of storage, reaction with primary amines, and the addition of protected sulfhydryl, which is then deprotected by hydroxylamine.

PROJECT PURPOSE

Vaccination is one of the most important and cost-effective methods of preventing infectious diseases. Previous experiments using β-glucan particles (GPs) have shown success in using this technology as a delivery vehicle for macromolecules (i.e. DNA, siRNA, and proteins), and there is an interest to extend its use to delivery of peptides. The main purpose of this project was to test different strategies for peptide delivery using β-glucan particles. Different methods were tested, such as building core complexes inside the GPs, adding trapping polymers, GP surface derivatization with polymers, and SATA for better binding of the peptide to GPs. The methods were compared for successful loading and release of the peptide, and for *in vitro* stimulation of T cell replication. The optimum methods will eventually be tested for *in vivo* immunogenicity.

MATERIALS AND METHODS

1. Materials

Reagents:

Glucan particles were prepared from Fleishmans Baker's yeast (AB Mauri Food Inc, Chesterfield, MO) according to previously published procedures (Soto and Ostroff, 2008). C(Npys)-OVA-323-339-amide peptide (ISQAVHAAHAEINEAGR-amide, 90.12% purity) was purchased from 21 Century Biochemicals (Marlborough, MA). Ovalbumin (OVA) from chicken egg white, Ribonucleic acid from torula yeast Type VI (tRNA), chitosan from crab shells, Poly-L-Lysine-hydrobromide (PLL), 1,3-diaminopropane (DAP), 25 kDa branched polyethylenimine (PEI), hydroxylamine hydroclochloride, ethylenediamine-tetraacetic acid (EDTA), ethanol, L-glutathione (GSH), acetic acid and glycine were purchased from Sigma-Aldrich (Allenstown, PA), and used as received. Materials for cell tissue culture experiments were purchased from Gibco Scientific (Grand Island, NY). SATA (N-Succinimidyl S-Acetylthioacetate) was purchased from Pierce Chemicals (Rockford, IL).

Equipment:

The Virtis lyophilizer was from Virtis Company (Gardiner, NY), the 96-well Costar plates with UV transparent flat-bottom were from Corning Inc, (Corning, NY), the Safire Tecan microplate reader was from Tecan Group (Mannendorf, Switzerland).

2. Methods

2.1 Preparation of GPs

Yeast glucan particles (YGPs) were prepared from Baker's yeast (*S. cerevisae*) by a series of alkaline and acidic extraction steps. The yeast cells were collected by centrifugation, washed in water, suspended in 1M NaOH, and heated at 90˚C for 1 h. The centrifugation and alkali extraction were repeated, the particles were then suspended in water at pH 4.5, and heated at 75˚C for 1h, followed by washes of the particles with water (three times), isopropanol (four times) and acetone (two times). YGPs were then dried and resuspended in 0.9% saline, sonicated, counted on a hematocytometer, aliquoted, and dried to obtain 5 mg/ml YGP samples.

2.2 Synthesis of Cationic GPs (PEI-GP, CN-GP)

GPs (5 mg) were resuspended in 10 mL of water by homogenization. Potassium periodate (0.4 mL of a 1 mg/mL solution) was added and the mixture stirred in the dark at room temperature for at least six hours. Oxidized GP samples were washed three times with water, and used immediately for reductive amination synthesis. Cationic polymers (PEIs and CN) and water were added to the oxidized GP samples (1 μmol PEI/mg GP), and the particles were resuspended and mixed at room temperature overnight. The aminated samples were reduced with sodium borohydride (0.1 g), and incubated at room temperature for 48 hours. The reduced samples were washed with water. Tris buffer (5 mL, pH 7.5, 0.05 M) was added, and the sample was incubated for 30 minutes. The samples were washed with water,

resuspended in 70% ethanol, and stored overnight at −20°C for sterilization, then aseptically washed three times with 0.9% saline, resuspended in 0.9% saline. The particles were counted with a hematocytometer, and the particle suspensions were diluted to a concentration of 1×10^8 particles/mL and stored at −20°C.

2.3 Loading of HA Flu Peptide/Ova Core Components Inside the GPs

Solutions of Ova with a concentration of 25 mg/ml and solution of Alexa HA Flu peptide with a concentration of 100 μg/ml were prepared. The empty GP samples (5mg) were loaded with Alexa HA peptide (4 μl of 25 μg/μl peptide), then incubated for 30 min on ice to allow peptide penetration and lyophilized. The samples were then treated with water (3 μl water push) and lyophilized. After this step, 5 μl of tRNA were added to the samples and they were incubated for 30 min. Another step of water push was performed, and the samples were left for vapor hydration overnight. Finally 10 μl of octanoic acid were added to the samples to enrobe the core inside the GPs. The samples then were washed three times with saline, and the fluorescence of the supernatants was measured to assess peptide-binding % to the GP-protein/tRNA core inside the GPs.

2.4 OTII Peptide-Albumin/tRNA Core Loading and Lipid Enrobement of GPs

Prior to loading inside glucan particles, solutions of BSA were prepared in water with a concentration of 25 mg/ml, and a solution of FL-OTII peptide was

prepared in water with a concentration of 10 µg/ml. First, 20µl of the FL OTII solution was added to 5 mg of empty GP samples. These samples were incubated for 30 min on ice and lyophilized. The samples were treated with 15 µl water (water push), mixed and lyophilized. Next, 20 µl of the BSA solution was added to the dry FL-OTII peptide-loaded GPs and processed as described above. After this step 15 µl of tRNA was added, mixed and incubated for 30 min. The samples then were treated with 10 µl of octanoic acid and incubated overnight to lipid enrobe the peptide-BSA-tRNA core inside GPs. Control samples were also set up using peptide solution but without adding trapping polymer. In addition a FL-OTII mixture was prepared and used as a tracer to estimate OT-II incorporated in GPs. The particles were collected through centrifugation, and the fluorescence of the supernatants was measured to evaluate % loading efficiency of the peptide.

2.5 Synthesis of GP-CN-SATA cores

5 mg of SATA was dissolved in 25 µl of DMSO, and 20 µl of SATA in DMSO solution were mixed with 80 µl of PBS. 5 µl of SATA/PBS solution were added to 5 mg GP and CN-GP samples, then they were mixed and incubated for two hours at room temperature. The samples were washed, centrifuged and lyophilized.

2.6 Derivatization of GP-SATA-Surface

The GP surface can be derivatized with different molecules used as ligands for binding specific functional groups. Different cationic polymers such as chitosan or PEI have been added to the surface of GPs, establishing electrostatic or covalent

payload binding mechanisms. The surface of cationic GPs was derivatized with Chitosan-GP (CN-GP) and Polyethyleneimine-GP (PEI-GP) to provide a binding group for further derivatizaton with N-Succinimidyl S-Acetylthioacetate (SATA). SATA is a sulfhydryl-containing modification reagent that reacts with primary amines (-NH2), which are present in the side chain of antibodies and proteins. SATA creates protected sulfhydryl groups that are then treated with hydroxylamine to yield sulfhydryl groups for conjugation reactions. CN-GP and PEI GP samples were available in Dr. Ostroff's lab.

2.6.1 Synthesis of Surface Derivatized SATA-GPs

Previously synthesized CN-GPs, PEI-GPs, and unmodified GPs were labeled with SATA. GPs (5 mg samples) were resuspended in 100 µl of PBS and 100 µl of SATA in DMSO solution (20 mg in 600 µl DMSO) were added. The samples were cup sonicated, and incubated for two hours at room temperature. The samples were centrifuged and washed three times with water. The GPs then were resuspended in 1 ml water, aliquoted into tubes (40µl), and lyophilized to obtain 0.2 mg SATA labeled GP samples.

Particles (5mg)	µl PBS	µl SATA in DMSO
CN-GP	100	100
25kPEI-GP	100	100
GP	100	100
PLL-GP	100	100
DAP-GP	100	100

Table 1: Synthesis of SATA Surface-Derivatized GPs.

2.6.2 Characterization of SATA Modified GPs (Ellman's Assay)

To measure the sulfhydryl derivatization of GPs, the Ellman's assay was used. The Ellman's method for assaying thiols introduced by reaction of SATA with cationic GPs is based on the reaction of thiols with the chromogenic DTNB (5,5′-dithiobis-2-nitrobenzoate) whereby formation of the yellow dianion of 5-thio-2-nitrobenzoic acid (TNB) is measured. SATA surface-derivatized GPs (0.2 mg samples) were resuspended in 5 µl of deacetylation solution to allow deprotection of SATA. 0.5 M hydroxylamine hydrochloride, 25mM EDTA in PBS deacetylation solution was prepared by dissolving 0.0439 g hydroxylamine hydrochloride (0.626 mmol) and 0.0119 tetrasodium salt of EDTA (0.031 mmol) in 1 ml of PBS. The samples were sonicated and incubated for two hours. After the incubation period, the samples were washed three times with water. Ellman's Reagent solution was prepared by dissolving 4 mg of Ellman's Reagent in 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. 5 µl of Ellman's Reagent solution were added to the deprotected GP samples and incubated for 15 min at room temperature. 195 µl of

sodium phosphate buffer were added to the samples, then the samples were centrifuged and transferred to a 96-well plate to measure absorbance at 412 nm.

2.6.3 Peptide Binding to SATA modified GPs

Two peptides containing cysteine residues were used for binding to SATA modified GPs. One peptide containing a fluorescent tag (fluorescein) was used as a model compound to optimize the reaction conditions for binding to SATA modified GPs. A second peptide containing an Ova amino acid sequence was used to produce samples with biological activity.

2.6.4 f-Cys Peptide

f-Cys peptide containing a fluorescent tag (fluorescein) was tested for binding to SATA-GPs. Flow cytometry (FACS) analysis and fluorescent microscopy were used to evaluate the binding of the peptide to SATA-GPs, while glutathione (GSH) and pH assays were used to indirectly measure the peptide binding and release from SATA-GPs.

2.6.5 f-Cys peptide Binding

5 µl of 0.5 M hydroxylamine hydrochloride, 25 mM EDTA in PBS deacetylation solution were added to GP samples (0.2 mg) to allow deprotection of SATA. The samples were sonicated and incubated for two hours. After the incubation period, the samples were washed three times with water and then reacted with 6 µl of f-cys peptide (0.5 mM, 0.3 x 10^{-5} mmol) overnight in the dark. 4

µl of PBS were added to bring the volume of the solution to 10 µl. After the incubation period, the samples were resuspended in 190 µl of PBS, centrifuged (10,000 rpm for 5 minutes) and the supernatants were transferred to a transparent 96-well plate, leaving the pellet in the centrifuge tube. The fluorescence of the unbound peptide was measured. The pellet was washed three times with 1 ml of PBS and resuspended in 500 µl. Particles were counted with a hematocytometer, and the particle concentration was adjusted to 1 x 10^8 particles/ml with PBS. Samples were characterized for peptide binding capacity by indirect measurement of peptide bound, and released after incubation of samples in glutathione, flow cytometry and fluorescence microscopy.

2.6.6 f-Cys Peptide Binding and Release Assay

50 µl of the synthesized GP samples at 1 x 10^8 particles/ml concentration were added to microfuge tubes. The samples were mixed with 150 µl of 0.15 M glutathione solution, and were incubated in dark room overnight. After the incubation period, the samples were centrifuged and the supernatants were transferred to a 96-well plate. The fluorescence of f-Cys peptide was measured in the glutathione fractions to evaluate the % of f-Cys peptide released. Furthermore, the GP samples were resuspended in PBS (pH 7), sodium acetate buffer (pH 5), or 0.2M glycine-HCl buffers to evaluate the release of f-Cys peptide at different pH conditions.

2.6.7 Flow Cytometry (FACS)

FACS measurements were obtained with a Becton Dickinson FACSCalibur instrument (BD, Franklin Lakes, NJ). A particle count of the GP samples was performed to bring the concentration of GPs to 1 x 10^8/ml for the FACS analysis. An unmodified GP sample was used as the negative control, while fluorescently labeled GPs were used as the positive control. The particles were analyzed with a FL4 laser at 605 nm by collecting an average of 15000 measurements.

2.6.8 Microscopy

The synthesized GP particles and unmodified GP were transferred to slides and examined under a fluorescence microscope at 100x magnification. Furthermore, the f-Cys SATA-GPs (1 x 10^8 particles/ml) were used in cell uptake experiment. 190 µl of 0.9% sterile saline and 10 µl of GPs were mixed and delivered to 3T3-D1 and DC2.4 phagocytic cells in DMEM + 10% fetal calf serum + 1%penicillin + streptomycin + glutamine (250µl). The cells were plated in 24-well plates containing 1 x 10^7 cells/well. The samples were transferred to the labeled wells and incubated for one hour at 37˚C and 5% CO_2. The cells were washed with PBS and fixed with 1% formalin. The cells were examined for evidence of GP mediated delivery of f-Cys peptide under a fluorescent microscope at 20x.

2.7 NPYS OVA Peptide

After the conditions were optimized using f-Cys peptide, NPYS OVA peptide was tested to evaluate binding to SATA GPs and to assess the effects of delivering

NPYS OVA to cells. NPYS selectively reacts with free thiol groups forming disulfide bonds, thus preventing peptide S-S dimers and providing a colored reaction product to measure peptide conjugation efficiency.

2.7.1 NPYS peptide Binding

The deacetylation of SATA-GP, SATA-PEI-GP and SATA-CN-GP-samples was performed as indicated in section 2.1.1. The deprotected SATA-GP samples were reacted with 5 µl of 2.5 mM NPYS Ova peptide ($1.25*10^{-5}$ mmol) and were incubated overnight. 5 µl of water were added to obtain a total volume of 10 µl. After the incubation period, the samples were resuspended in 190 µl of water, centrifuged (10,000 rpm for 5 minutes) and the supernatants were transferred to a transparent 96-well plate, leaving the pellet in the centrifuge tube. The absorbance of the released Py group in the supernatants was read at 312 nm allowing an estimation of the amount of peptide bound to SATA. The pellet was then washed three times with 1 ml of water, and resusupended in 500 µl of water.

2.7.2 Biological Assay-OTII T Cell Proliferation

Purified T cells (10^5 cells/well) were incubated with mitomycin C-treated BMDCs (10^6 cells/well) and the indicated stimuli for 4 days in round-bottom 96-well plates in 200 µl R10 medium. [^{3}H]-thymidine (1 µCi/well; PerkinElmer, Boston, MA) was added for the last 24 h of incubation. Each condition was studied in triplicate. Cells were collected on filter paper (Wallac, Turku, Finland) using a

harvester (Tomtec, Hamden, CT), and [^{3}H]-thymidine incorporation was measured with a beta counter (Wallac 1450 Microbeta).

RESULTS

The purpose of this project was to test different strategies for building core complexes inside glucan particles (GPs), adding trapping polymers, and derivatizing the GP surface with Chitosan or Polyethyleneimine to provide a binding group for further derivatizaton with N-Succinimidyl S-Acetylthioacetate (SATA). The various methods were compared for successful loading and release of the loaded peptide.

1. OT-II Peptide Loading Inside GPs

The first set of experiments involved testing the loading efficiency and capacity for a fluorescently labeled test peptide, OT-II. OT-II is an ovalbumin (OVA) (323-339) peptide consisting of an ISQAVHAAHAEINEAGR amino acid sequence which produces a distinct T cell response. The OT-II peptide was loaded inside the GP and coated with tRNA to assess the peptide binding capacity to GPs. GP OTII peptide/tRNA cores were enrobed with octanoic acid (OA) for retention of the peptide inside GPs. After lipid enrobement, the peptide-loading efficiency was evaluated by measuring fluorescence (**Figure 3**). The results for the OT-II peptide coated with tRNA and enrobed with OA showed a 65% binding efficiency (right histobar), while samples with no tRNA (left histobar) or lipid enrobement (middle histobar) bound 61% and 48% of peptide, respectively. These results indicate that OT-II peptide/tRNA core enrobed with octanoic acid is the most efficient loading method.

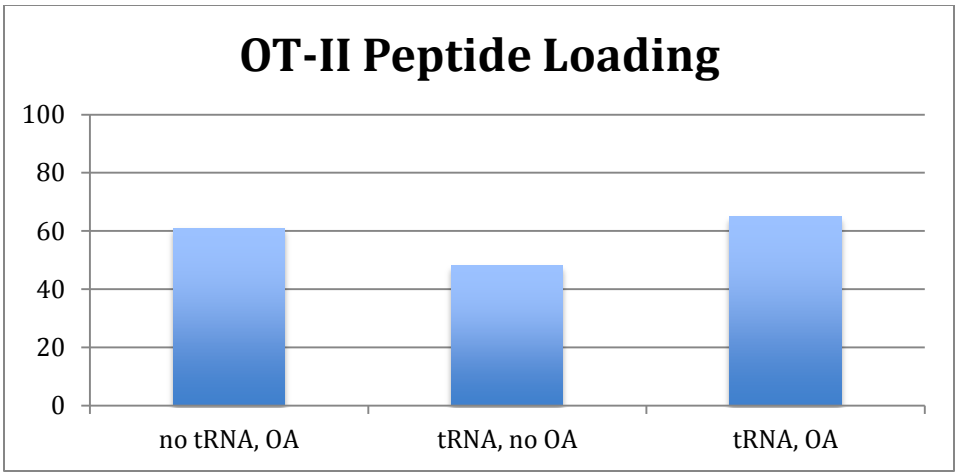

Figure 3: OT-II Peptide Binding Capacity.

2. HA Peptide and Ova Formulations Inside GPs

2.1 HA Peptide binding assay

Fluorescently labeled influenza hemagglutinin HA peptide (fl-HA) and Ovalbumin (Ova) formulations were loaded inside the GPs and enrobed with octanoic acid to evaluate the loading efficiency. After the loaded GPs were enrobed with lipid, the fluorescence of the GPs was measured to evaluate peptide-loading capacity (**Figure 4**). The lipid-enrobed GPs showed 48% loading efficiency, while the GPs with no enrobement resulted in 6% loading.

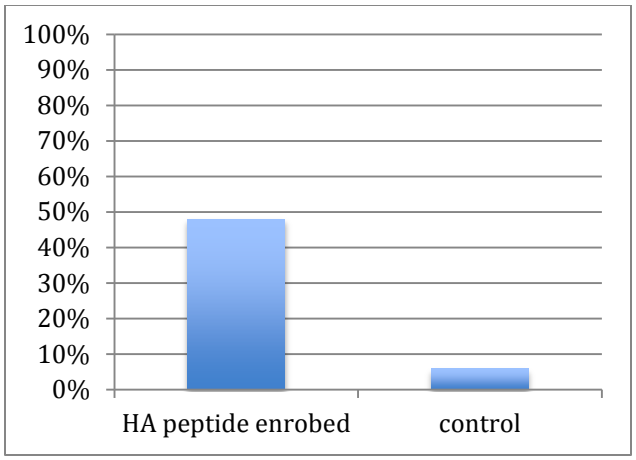

Figure 4: Lipid Enrobement of the HA Peptide Inside GPs.

2.2 Microscopy

Further assessment of the fl-HA peptide encapsulation inside GPs was measured using fluorescent microscopy. The GP particles were transferred to slides and examined using a fluorescent microscope at 100x. The data (**Figure 5**) shows fluorescent GPs (left panel), confirming that the peptide was entrapped inside the lipid-enrobed particles, while the unenrobed GPs (right panel) showed no fluorescence.

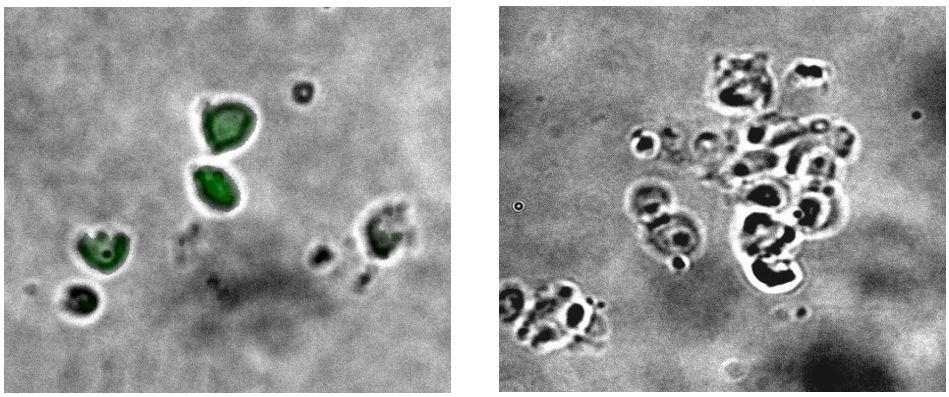

Figure 5: Fluorescent Microscopic Images of HA Peptide Formulation Inside GPs.

3. SATA Inside GP Core

SATA is a reagent for introducing protected sulfhydryls into different molecules such as proteins, peptides, and other amine-containing molecules. Sulfhydryl groups are introduced in a protected form to be stored for longer period until treated with hydroxylamine to expose the reactive sulfhydryl on SATA-modified molecules for final conjugation reactions. SATA contains an N-hydroxysuccinimide (NHS) ester, which forms a covalent amide bond with primary amines. The SATA was introduced as a part of the GP-Chitosan (GP-CN) core for functionalization with f-cys peptide via sulfhydryl linkages. However, no evidence of SATA binding to GP-CN core was observed by Ellman's method (data not shown).

4. SATA Surface-Derivatized GP

SATA was used to introduce sulfhydryl groups to the surface of cationic GPs to be available for immediate reaction with amine groups. The synthesized SATA-GPs were evaluated for fluorescent peptide binding and release and cell uptake.

4.1 Ellman's Assay

Ellman's Assay was used to determine the quantity of sulfhydryl groups (SH) introduced by reaction of SATA with cationic GPs. **Table 2** summarizes the calculated concentrations (mmol SH/mg GP) of sulfhydryl groups present in the unmodified and synthesized GPs, which shows that the amount of free thiols introduced by SATA is higher in modified GPs, with SATA-PEI-GP yielding the highest concentration.

Table 2: Concentration of Free SH Groups in the GPs.

	SATA-GP	SATA-PEI-GP	SATA-CN-GP	SATA-PLL-GP	SATA-DAP-GP	GP
Concentration (mmol SH/mg GP)	$1.14*10^{-5}$	$0.82*10^{-3}$	$2.25*10^{-5}$	$1.55*10^{--4}$	$1.78*10^{-5}$	0

4.2 Cys-Peptide Binding Capacity

0.5 mM f-Cys peptide was reacted with the synthesized SATA-GP, SATA-CN-GP and SATA-PEI-GP formulations, and the % peptide binding was measured using a binding assay (**Figure 6**). The % binding is the ratio of peptide concentration obtained from fluorescence emission measurements divided by the input of peptide concentration and multiplied by 100.

[Measured unbound peptide]/[input peptide concentration]*100 = % unbound peptide
100% - % unbound peptide = % bound peptide

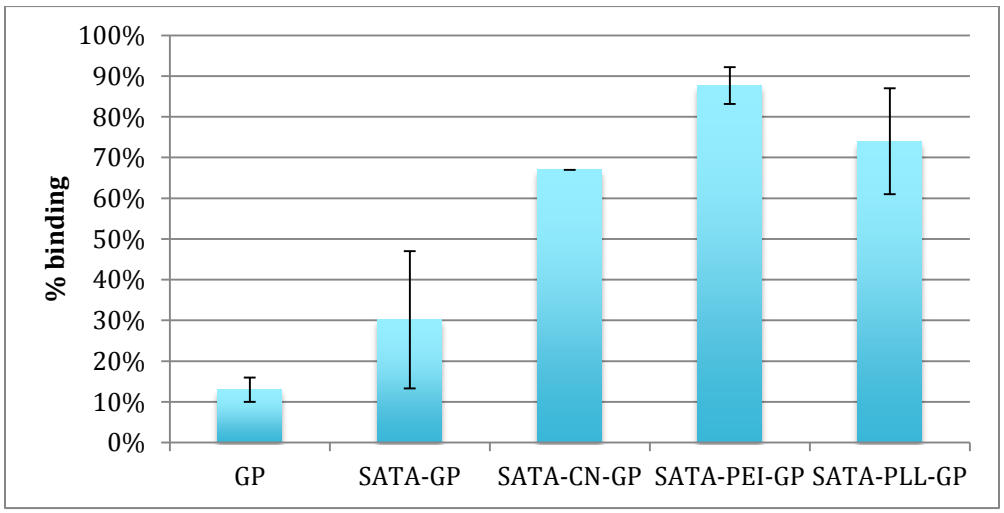

Figure 6: Percent Peptide Binding of the Synthesized SATA-GP, SATA-CN-GP, SATA-PEI GP, SATA-PLL-GP and the Unmodified GP.

The results indicate that the highest percent peptide binding resulted from the SATA-PEI procedure.

4.3 Flow Cytometry (FACS)

Fluorescence activated cell sorting (FACS) (flow cytometry) was also used to measure the level of fluorescent peptide bound to the synthesized GPs. The results shown below reveal that peptide is bound to the particles with SATA-derivatized surface (**Figure 7**). SATA-PEI-GP, SATA-PLL-GP, SATA-CN-GP showed more peptide % binding than SATA-GP, while no peptide was bound to the unmodified GP.

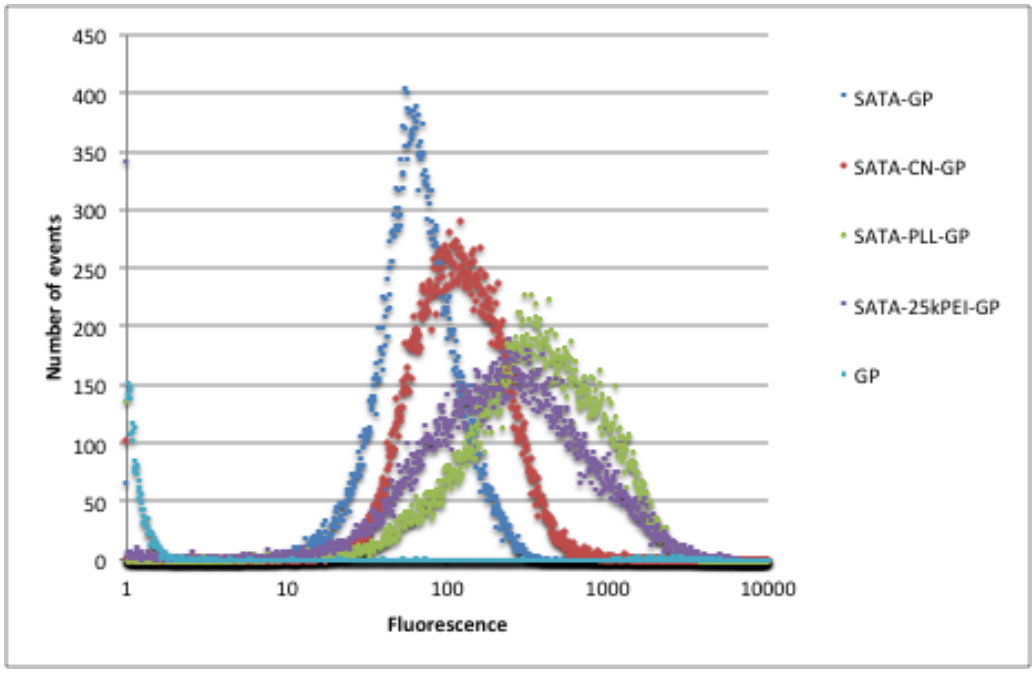

Figure 7: FACS Results for the SATA-Labeled GPs and the Unmodified GPs.

The particles were transferred to slides and examined using a fluorescent microscope at 100x (**Figure 8).** The results show clear evidence of fluorescent peptide binding to the synthesized GPs, while the unmodified GP showed no traces of bound peptide.

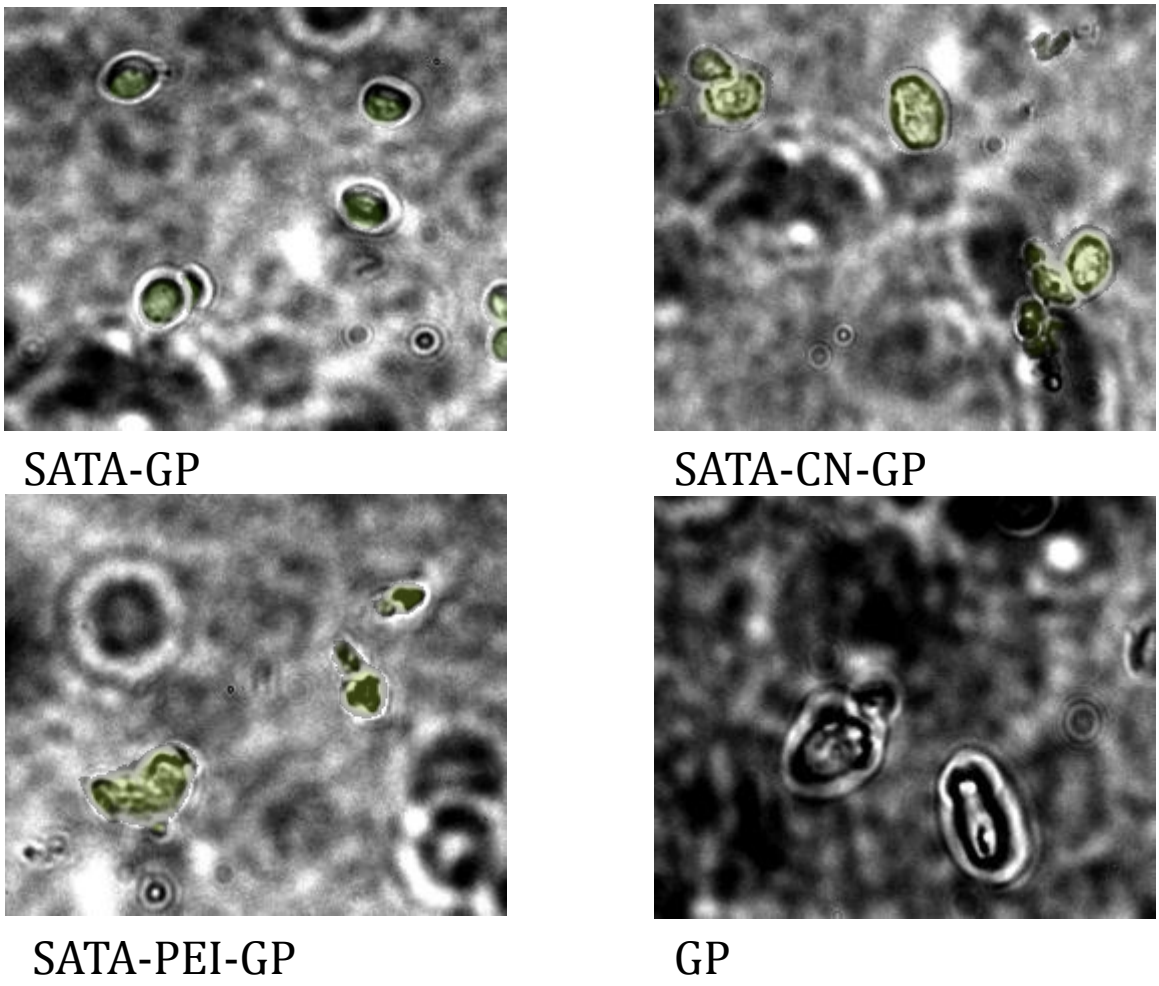

Figure 8: Fluorescent Microscopy of f-Cys Peptide Binding to the Synthesized SATA-GP, SATA-CN-GP, SATA-PEI-GP and the Unmodified GP.

4.4 Binding and Release Assay

The next phase of the project involved testing the amount of loaded peptide released from various GPs. When testing the release of peptide from the

synthesized SATA-GPs, both SATA-CN-GP and SATA-GP were used. These synthesized GP samples were treated with 0.15 M glutathione solution and glycine buffer (pH 3) to quantify the amount of bound peptide released from SATA-GPs (**Figure 9**). The results showed that most of the peptide under these conditions was released from all GP samples in 3 h.

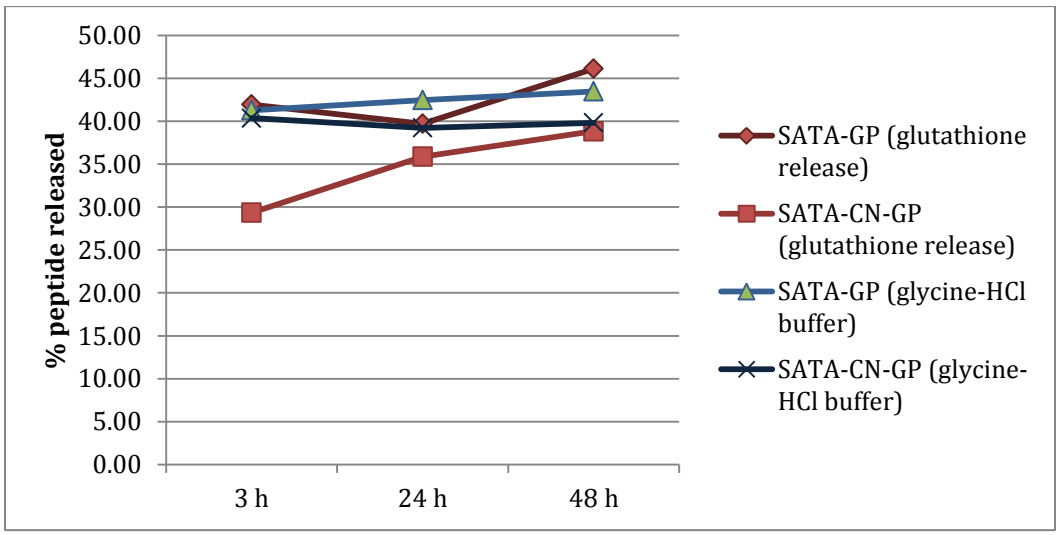

Figure 9: Glutathione and 0.2M Glycine-HCl Buffer-Mediated Release of Peptide from SATA-GPs Over 48 hours at 37°C.

4.5 Flow Cytometry

Flow cytometry measurements were used to determine the difference between the samples before and glutathione reactions. SATA-GP and SATA-CN-GP flow cytometry results are shown in **Figures 10** and **11**, and indicate that the samples treated with glutathione (red) are less fluorescent than the originally synthesized GPs (blue) indicating peptide release from the GP.

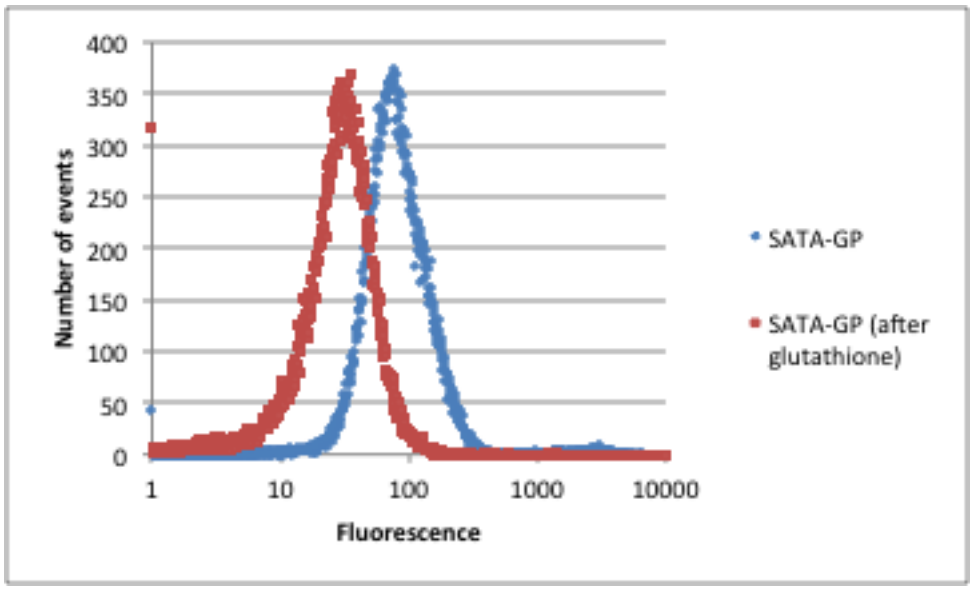

Figure 10: FACS Results for SATA-GP Before and After Glutathione Release.

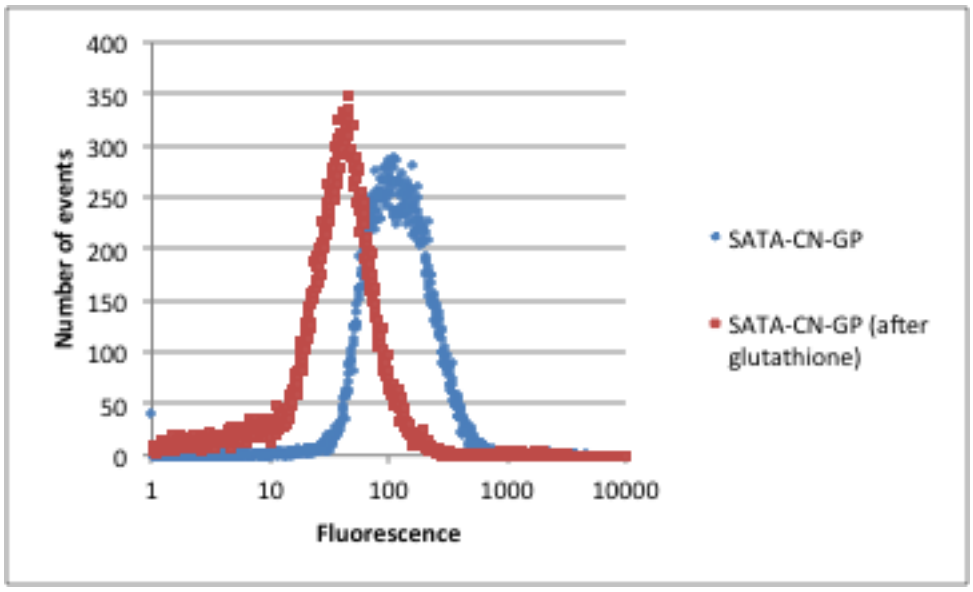

Figure 11: FACS Results for SATA-Chitosan-GP
Before and After Glutathione Release.

5. 3T3-D1 and DC2.4 Uptake of f-cys Peptide-Labeled GPs

The GP particles were delivered *in vitro* to phagocytic 3T3-D1 and DC2.4 cells to assay the uptake of fluorescent peptide-SATA GPs. The cells were incubated with the synthesized GPs for 1 to 3 hours at 37˚C under CO_2 to allow efficient particle

uptake. The cells were fixed and observed under fluorescent microscope at 20x magnification (**Figures 12** and **13**). The synthesized GPs successfully delivered fluorescent peptide into both 3T3-D1 and DC2.4 cells, however only SATA-PEI-GP and SATA-CN-GP showed evidence of efficient peptide binding to the GPs.

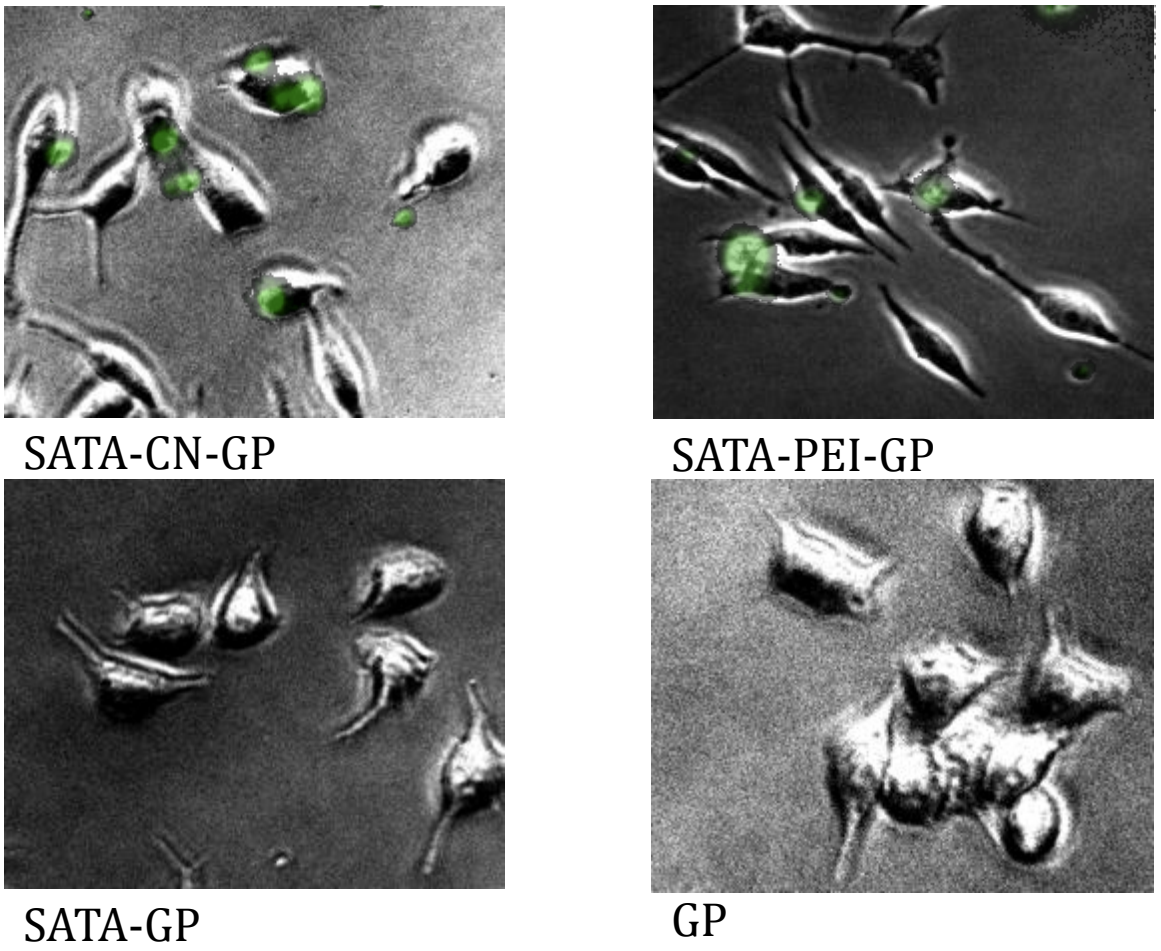

Figure 12: Uptake of Fluorescent Peptide-Labeled GPs into Phagocytic DC2.4 Cells.

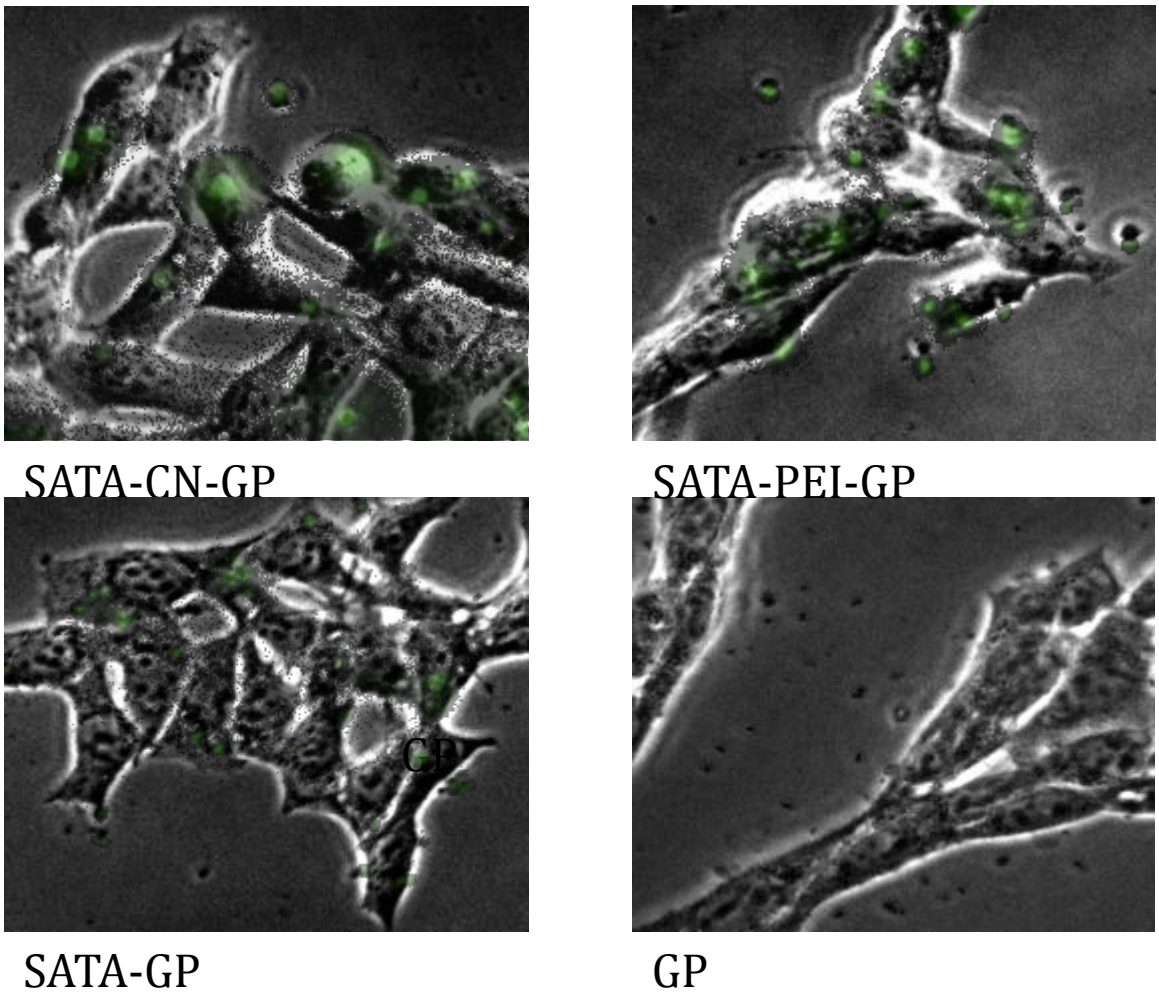

Figure 13: Uptake of Fluorescent Peptide-Labeled GPs into Phagocytic 3T3-D1-Cells.

6. NPYS peptide binding

After the conditions for f-cys peptide binding to GPs were optimized, the NPYS-Ova peptide binding to GPs was tested. The synthesized SATA-PEI-GP, SATA-CN-GP, and the unmodified GP were labeled with NPYS-Ova peptide, and the percent of binding was quantified (**Figure 14**). The NYPS molecules cleave upon reaction of the peptide with deprotected SATA allowing measurement of the absorbance that directly correlates with bound peptide concentration. The results show that the

SATA-PEI-GP (red) and SATA-CN-GP (green) samples have significantly higher peptide-binding than control GP (blue). The SATA-CN-GP % binding was 3x higher and SATA-PEI-GP was 10x higher than the control GP.

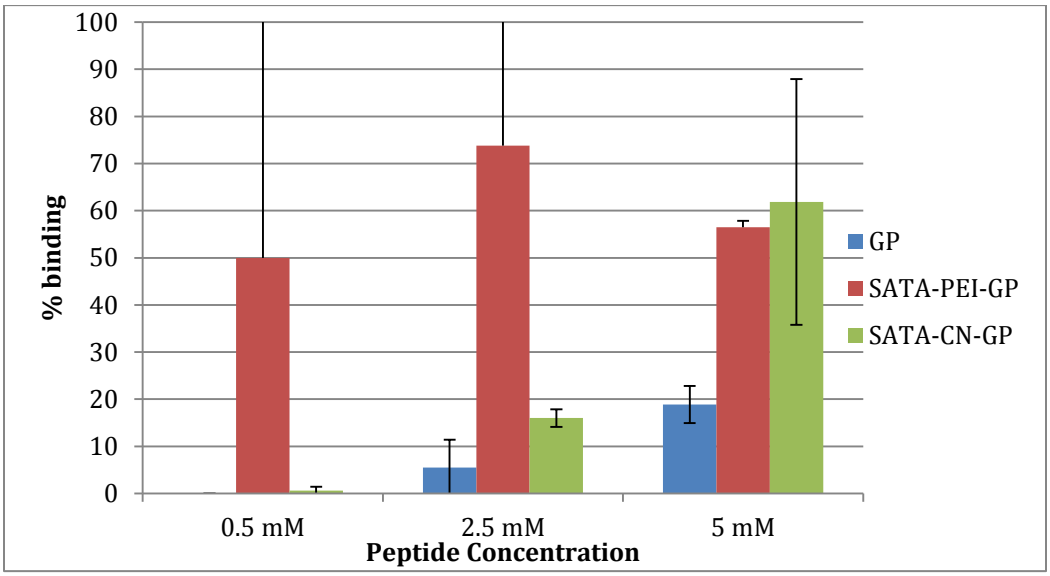

Figure 14: The Percent Binding of NPYS-Ova Peptide at Different Peptide Concentrations.

7. Biological Assay: OTII T Cell Proliferation

In order to demonstrate whether NPYS peptide-encapsulated GPs can be effectively delivered to T cells, an *in vitro* [^{3}H]-thymidine T-cell proliferation assay was performed (**Figure 15**). $CD4^+$ T cells obtained from OT-II mice which recognize the OVA peptide were stimulated *in vitro* with GP - C(Npys)-OVA peptide. The results indicate that polymer surface-derivatized GP-Npys-OVA led to an enhanced $CD4^+$ T cell proliferation, compared to Npys-Ova-GPs (~50% difference). These results reveal that the Npys peptide was delivered with GPs and was efficiently processed and presented by DCs.

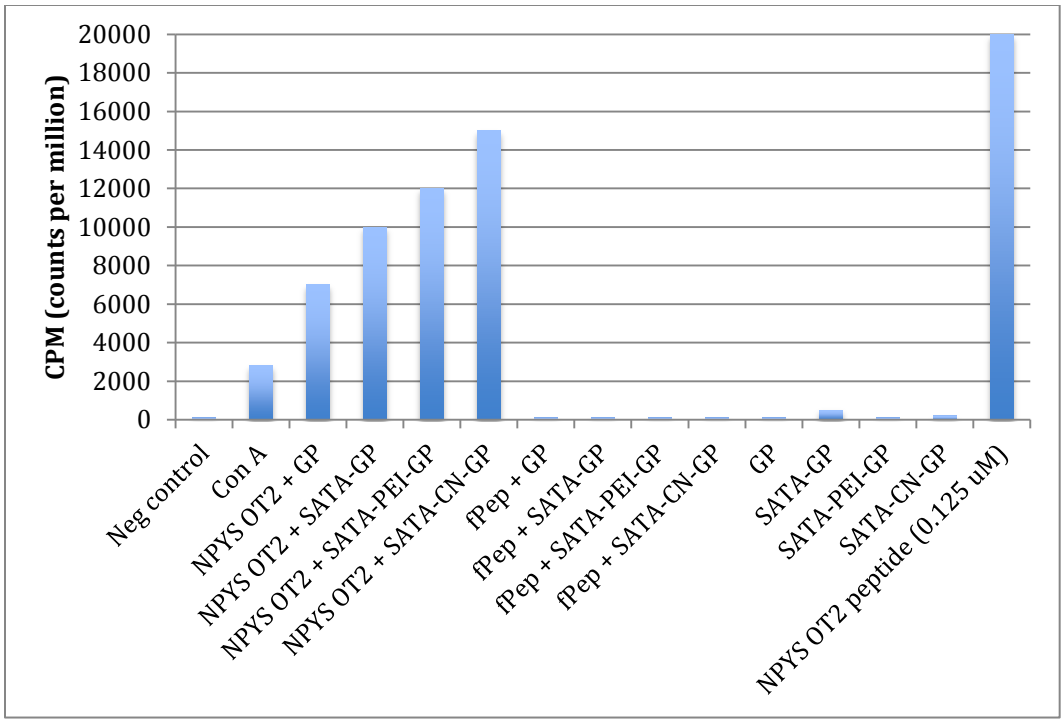

Figure 15: NPYS – OTII Peptide T Cell Proliferation.

DISCUSSION

In this project, several novel strategies for the delivery of peptides using beta glucan particles were evaluated. Glucan particles have been used for macrophage-targeted delivery of different payload macromolecules including DNA, siRNA, insoluble drugs, and nanoparticles (Soto et al., 2011) and are an effective targeted vaccine antigen/adjuvant delivery system (Levitz et al., 2010). There is a growing interest for extending the applicability of the glucan particles to deliver low molecular weight drug molecules. This project has focused on the delivery of peptides to phagocytic/macrophage cell lines as a potential vaccine formulation and use *in vivo.* Different loading strategies of peptide in GPs were tested, included synthesizing protein/GP/peptide/tRNA cores (a layer by layer approach), SATA-CN-GP cores (SATA derivatization inside the GP-CN cores), and surface-derivatized GPs (the GP surface is modified with biodegradable polymers).

Glucan particles have been successfully used to trap protein molecules such as Ovalbumin (OVA) and Bovine Serum Albumin (BSA) by forming a protein/tRNA nanoparticulate complex. The first approach tested in this project involved the formation of peptide/tRNA cores inside the GPs and its lipid enrobement to enhance the peptide retention. A fluorescent OTII peptide and albumin were loaded together into the glucan particles and then tRNA was added to form an insoluble polyplex. Efficient adsorption of payloads into glucan particles was achieved by soaking dry particles in the hydrodynamic volume of the particles. The wet samples were then lyophilized before tRNA addition to ensure maximum adsorption of the peptide.

Trapping polymer (tRNA) was then added to form the stable complex within the particles. The final compound added to this formulation is octanoic acid to enrobe or coat the payload core within the GPs to enhance the peptide retention capacity. The amount of OTII peptide bound to the particles was indirectly measured by measuring the amount of unbound fl-peptide. Three different loading strategies were evaluated for % OTII peptide binding: tRNA complexation and a lipid enrobement slightly increased the % peptide binding. The results in Figure 3 demonstrate efficient binding of the peptide. Formulations of both tRNA and octanoic acid contain 65% of peptide, while samples missing one of these components bound less peptide. In addition to binding assay the samples were evaluated under fluorescent microscope to confirm % peptide binding. These results confirmed the binding of the peptide and its retention inside the cores.

As another example of GP peptide core loading, a fluorescent Alexa HA peptide/Ova/tRNA complex was synthesized inside the GPs. In this method, lyophilization steps were carried out to maximize the adsorption of each core component and the core inside GP was enrobed with octanoic acid to increase retention capacity. The control sample prepared without tRNA and octanoic acid showed 6% binding, while trapping as a protein complex yielded 48% binding (Figure 4). Moreover, the samples were observed under fluorescent microscope at 100x to confirm peptide presence inside the GPs. Figure 5 clearly shows the fluorescence of the Alexa HA peptide/Ova sample, while no fluorescence traces were observed in the control sample. The combination of trapping polymers tRNA and octanoic acid ensured efficient retention of the payload inside the core.

However, there is a limit to the amount of peptide that can be encapsulated inside GPs as a polyplexed core. To overcome this limitation, an alternative method of encapsulating payload was devised. Formulation of chitosan (CN)/SATA/GP cores and its functionalization with f-Cys peptide via sulfhydryl linkages was tested. Due to the similarity between biologically active NYPS peptide and f-Cys peptide, the latter was used to optimize conditions for GP loading. The results obtained from CN/SATA/GP core functionalization did not show any evidence of SATA binding to GP-CN core. One of the possible reasons why SATA did not bind GP-CN core might be due to the small volume of the GP, which resulted in no reaction or that insoluble chitosan was not available for reaction with SATA.

We next designed a new peptide GP surface binding strategy. Surface derivatization methods, such as reductive amination and click chemistry, allow for many different molecules and polymers to be added to the GP surface. Due to inefficient binding of peptide to cores inside the hollow cavity of GPs the surface of GP were derivatized with cationic polymers and SATA to introduce sulfhydryl groups to the surface of GPs for further peptide conjugation reactions. First, cationic polymers such as PEI were added to the surface of GP, creating a payload binding mechanism. Once the functional groups were added to the surface, the GP samples were derivatized with SATA to form free sulfhydryl functional groups. The available primary amine on the peptide reacted with the introduced NHS ester to form a stable, covalent amide by nucleophilic attack. The free sulfhydryls (SH) were formed following the hydroxylamine-mediated deprotection step and quantified using Ellman's Assay. The results presented in Table 1 show the concentration of

quantified free SH groups in the synthesized GP samples. The SATA-PEI-GP yielded the highest number of free SH groups, while the remaining samples showed less free thiols available for reaction with peptide. After the amount of free SH groups in the samples was quantified, the samples were reacted with f-cys peptide, and the binding capacity of the GPs were measured indirectly. The measurements shown in Figure 6 reveal that the SATA-PEI derivatized samples were effective in binding 90% of the peptide, while unmodified GP bound only 10%. The SATA-GP sample showed only 13% binding, revealing that a cationic polymer enhances peptide binding to the GPs. A fluorescence activated cell sorter (FACS) assay (flow cytometry) was also used to confirm binding of fluorescent peptide to the synthesized SATA-GPs and unmodified GP. The results showed that unmodified GPs do not bind the f-cys peptide but binds to SATA derivatized GPs (Figure 7). The particles that showed clear evidence of binding were transferred to slides and examined under fluorescent microscope at 100x. The obtained microscopy images confirmed the binding assay and FACS analysis results; Figure 8 shows efficient f-cys peptide binding to SATA-PEI-GP, SATA-GP, and SATA-CN-GP, while unmodified GP showed minimal or no binding. The synthesized GPs were evaluated for peptide-binding-release stability at pH 5 and pH 7. F-cys peptide release from the synthesized SATA-CN-GP and SATA-GP samples was mediated by both glutathione solution (GSH, pH 7) and glycine-HCl buffer (pH 3) at 37˚C, and the % released peptide was measured over 48 hour period (Figure 9). It was hypothesized that glutathione will facilitate the release of the peptide by cleaving the disulfide bonds between peptide and thiols, while glycine buffer of low pH will inhibit thiol-disulfide

interchange reactions (McKenzie et al., 1972). The observed results yielded ~50% peptide released from the samples after 48 h GSH incubation. Although the peptide is cleaved from SATA in the presence of GSH, it is possible that some of it remains non-covalently bound to the chitosan through charge interactions. A flow cytometry assay was used to confirm the release of peptide after GSH treatment. Figures 10 and 11 show a difference between SATA-GP and SATA-CN-GP samples before and after GSH, corroborating the results from the release assay.

At low pH, CN should not be able to bind peptide, and the percent released should be higher. However, the data from the experiment did not show any significant difference in % peptide release over time in low pH solution. This might have been due to at least 2x larger binding capacity of SATA-CN-GP than that of SATA-GP, which results in more peptide needed for cleavage from SATA-CN-GP.

Next, the synthesized samples were evaluated for cellular uptake of fluorescent peptide using the phagocytic NIH3T3-D1 and DC2.4 dendritic cell line expressing glucan receptors. The cells were incubated loaded with synthesized GPs for 1, 3, and 24 hours, and observed under fluorescent microscope at 20x. The GP surface-modified samples loaded in these cell lines showed clear fluorescence after 1h and 3h incubation periods. However, no evidence of fluorescent particles was observed after 24 hr incubation. A possible reason for this may be a complete cleavage of the f-cys peptide from the particles over 24 h time period followed by peptide degradation. Microscopic evaluation of the synthesized samples shows efficient peptide binding and uptake of SATA-PEI-GP and SATA-CN-GPs by both cell lines. The SATA-GP sample showed less % binding and thus less efficient uptake by

the cells, while the unmodified GP sample resulted in no cell uptake. These particle uptake experiments confirmed the stability results (Figure 12 and 13), the PEI surface modification of GPs or the binding of f-cys peptide to the SATA-PEI-GPs had no negative impact on cellular toxicity. The results from these studies of f-cys peptide binding allowed for optimization of conditions to test the NYPS-Ova peptide for binding to the GPs and its biological activity. Samples were reacted with varying concentrations of NPYS-Ova peptide to assess binding capacity. Lower concentrations of NYPS peptide (0.5 mM) showed 50% binding to the SATA-PEI-GP sample, while no binding to SATA-CN-GP and unmodified GP was observed. An increased concentration of the peptide (2.5 mM) significantly enhanced peptide binding to SATA-PEI-GP (73%) and SATA-CN-GP (16%). Finally, peptide of doubled concentration (5 mM) was reacted with samples, yielding 56% and 61% binding to SATA-PEI-GP and SATA-CN-GP, respectively. The unmodified GP bound 18% peptide due to the higher concentration of the peptide reacted (Figure 14). The use of derivatized surface GPs allows efficient peptide binding compared to the unmodified GPs, demonstrating the visible effects or toxicity on the dendritic cells.

Since our final aim was to develop a vaccine formulation of Npys-OVA-peptide loaded GPs, we evaluated the effect of these formulations to induce presentation by dendritic cells to T-cells in an *in vitro* setting. The Npys-Ova-peptide was loaded in surface-modified GPs, as well as f-Cys peptide. The activity of these formulations was evaluated by a [^{3}H]-thymidine T-cell proliferation assay. Different Npys-Ova and f-Cys peptide-modified GP samples were cultured with CD4+ OT-II cells, and the proliferation of T cells was assessed by [^{3}H]-thymidine

incorporation. As seen on Figure 15, Npys-OVA-loaded surface-derivatized GPs led to improved CD4+ T cell proliferation, compared to Npys-OVA-GPs, while f-Cys peptide GPs did not generate any biological response, which was proved by lack of signal in these samples. SATA-GP was also tested to confirm that the response resulted from the NPYS-OVA peptide. The results presented show that the Npys-OVA-peptide encapusulated in surface modified GPs significantly enhances OVA-specific T cell proliferation *in vitro*.

CONCLUSIONS AND FUTURE WORK

In this project, three strategies for a targeted delivery of peptides with glucan particles (GPs) were tested. Synthesizing cores inside GPs using a layer-by-layer approach, SATA derivatization inside GP-CN-cores, and SATA and polymer derivatization of the GP surface were evaluated revealing that surface-derivatized GPs enhances the delivery of the OVA-peptide to the cells and is biologically active. The Npys peptide bound to the surface-derivatized GPs with a binding efficiency greater than 60%, and stimulated a strong T cell proliferation *in vitro*. In the future, the next step will be to test the Npys-OVA-GP formulation *in vivo* in mice, as well as finding an alternative biodegradable polymer with minimal toxicity effects on cells. Additionally, new formulations that may provide higher drug binding capacity and controlled release need to be studied.

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