EFFECT OF PROTEIN COATINGS ON THE DELIVERY PERFORMANCE OF LIPOSOMES

by

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ABSTRACT

Modifications of the surface properties of liposomal drug carriers, such as coating with therapeutic or targeting proteins, greatly impact their delivery performance. Many of the currently-used methods to associate proteins with the surface of the liposomes can be laborious, inefficient, and significantly change the delivery characteristics. In this dissertation, two relatively simple methods are explored for their ability to associate proteins with the liposome membrane and their impact on the liposome's delivery properties.

The first method that is characterized is the non-covalent conjugation of a polyhistidine (His)-tagged protein to the surface of liposomes containing nickel-chelating lipid. It is shown that the His-tagged model protein, yellow fluorescent protein (YFP-His), associates with the liposome membrane without impacting the stability or uptake properties of liposomes. Next, the effect of the liposome surface-associated protein antigen on the performance of a liposomal vaccine carrier that has previously shown to be efficient in inducing both cellular and humoral immune responses was tested. Not only was the cellular immune response uncompromised by this new liposome formulation, but also the humoral immune response to encapsulated antigen was enhanced. Thus, this study shows that additional antigens can be non-covalently coupled to the liposome membrane in this manner without impacting the delivery properties of the

encapsulated antigen and while enhancing the overall immunogenicity of the vaccine.

The second method investigated is the association of recombinant amphiphilic protein with the liposomal membrane via hydrophobic interaction. A truncated form of the amphiphilic protein apolipoprotein B (apoB), apoB23, was fused to YFP and used to associate YFP with the liposome membrane. The apoB23-YFP fusion protein did not affect the liposome's properties *in vitro*. An apoB23 fusion protein was then prepared with a HER2-specific antibody fragment and used to coat the liposome membranes. The density at which apoB23 fusion proteins coated the liposome membrane, however, was low and no significant selectivity for HER2-overexpressing cells was observed with apoB23-αHER2-coated liposomes. Despite this, it was demonstrated that the apoB fusion protein could effectively associate with the liposome membrane without affecting the liposome's characteristics and is suggestive of its utility in other targeting applications.

CHAPTER 1

LIPOSOME SURFACE MODIFICATION FOR ENHANCED DELIVERY OF THERAPEUTICS

INTRODUCTION

Therapeutic Delivery Strategies

The safe and effective administration of soluble therapeutics pose many challenges. The exposure to degradative enzymes can result in the delivery of a decreased percentage of therapeutic to the target site, thus requiring a relatively higher dose to be effective. Such large doses can be difficult to maintain from a cost and supply standpoint, while a greater concern is the increased potential for toxicity. The challenge of balancing effectiveness with safety is particularly difficult and risky for drugs with narrow therapeutic windows.

In an attempt to overcome some of these challenges, a lot of research has been focused on the use of particulate delivery systems. Many of these systems can protect associated therapeutics from degradation and deliver a larger payload to the target site than is possible with soluble therapeutics. Synthetic structures, including dendrimers and polymeric microspheres, have gained favor due to the ability to easily manipulate their structure [1, 2]. Dendrimers are highly branched polymeric structures in which therapeutics or targeting moieties are coupled to the surface [1]. Biodegradable delivery systems such as poly(lactide-

co-glycolide) (PLGA) and poly(l-lactide) (PLA) microspheres provide controlled release of therapeutics and have shown promise in the delivery of recombinant bone morphogenetic protein for bone regeneration [2-5]. However, the use of synthetic, exogenous materials in some of these structures has raised concerns about their biocompatibility [6]. In addition, the relatively harsh procedures involved in the production of synthetic delivery systems can affect the activity of encapsulated or surface-bound macromolecule therapeutics [7]. An alternative delivery system based on endogenous carriers can potentially avoid the above challenges faced by polymeric structures. Vehicles such as lipoproteins and red blood cells have shown the ability to transport therapeutic agents that are associated with their core or surface [6, 8]. A major drawback to carriers derived from endogenous vehicles is the inefficiency in obtaining a sufficient supply, as these vehicles must be continuously isolated from the blood [6]. In addition, modifications to the vehicle surface during drug loading or the addition of a targeting component can result in increased immunogenicity of the carrier [6].

Lipid-based carriers including lipid emulsions, micelles, and liposomes are composed of biologically compatible lipids and can be relatively easily prepared in a laboratory setting [9-11]. While hydrophilic drugs can be encapsulated in the aqueous core of liposomes, more lipophilic drugs can be encapsulated in the hydrophobic core of micelles or lipid emulsions as well as incorporated in the lipid bilayers [12-14]. Their relatively high loading capacity and versatility in use have made the lipid-based carriers attractive as delivery systems [11, 15]. A more

detailed description of the benefits and challenges of liposomal delivery systems are discussed in the following review.

LIPOSOMES FOR THE DELIVERY OF THERAPEUTICS

Liposomes are biocompatible spherical structures consisting of one or several concentric lipid bilayers and an aqueous core. Due to their relatively high carrying capacity and ability to protect encapsulated agents from degradative enzymes and neutralizing antibodies, liposomes are efficient delivery systems [15]. In addition, pharmaceuticals that are associated with liposomes exhibit lower volumes of distribution and decreased cytotoxicity in normal cells [12, 15]. Modifications to the liposome formulation, size, charge, and surface properties can be implemented in a relatively easy fashion and can influence its cell uptake, protein interaction, and overall pharmacokinetics [16-20]. In addition, the association of various molecules to the liposome surface can have a profound impact on the liposomes's circulation time, immunogenicity, and selective delivery [21-24]. Although liposomes can vary in size and in the number of bilayers, decreased clearance rates and enhanced accumulation in tumors have been reported with smaller unilamellar liposomes (~100 nm) [25-27]. Due to the leaky vasculature and impaired lymphatic drainage at many tumor sites, small liposomes can passively target tumors via what is known as the Enhanced Permeability and Retention (EPR) effect [28-30].

Despite the likeness of liposomes to cell membranes, *in vivo* pharmacokinetic studies discovered that the majority of injected liposomes are internalized by the liver and spleen within a half hour [26]. This characteristic has

been exploited for therapeutics that target cells of the reticuloendothelial system (RES) such as in the case of liposomal vaccines, leishmaniasis therapy, or immunomodulators [31-33]. Liposomes were found to not only effectively deliver antigen to the antigen-presenting cells (APCs), but also to provide an adjuvant effect whereby they stimulated an immune response that was higher than that with soluble antigen [33, 34]. This finding is particularly important for subunit vaccines, which are weakly immunogenic on their own [35, 36].

An additional exciting discovery for liposomal vaccines was in their ability to stimulate both a cell-mediated and humoral immune response [33, 35, 37]. Exogenous antigen is typically processed by APCs through the endosomal pathway, in which the antigen is degraded into peptides that are bound by major histocompatibility complex (MHC) class II molecules for subsequent surface presentation (Figure 1.1) [38, 39]. Once presented, the MHC class II-bound peptides are recognized and bound by CD4+ T helper cells, which can lead to the production of antibodies through activation of a humoral immune response [40]. Alternatively, agents that reach the cytosol of APCs (i.e. viral proteins), are predominantly degraded by the ubiquitin/ proteasome pathway and the resultant peptides bound and presented by MHC class I molecules [41]. This type of surface presentation is recognized and bound by cytotoxic T lymphocytes (CTLs) bearing the co-receptor CD8, leading to their activation and subsequent target cell lysis as part of a cell-mediated immune response [40]. The presence of cytokines (e.g. IL-2, IFN-γ) help drive the immune response and are necessary for proper T cell activation [40]. Although soluble protein antigens are usually

only presented by MHC Class II molecules, the association of that antigen with liposomes can result in MHC Class I presentation as well and thus activate a CTL response [33, 42, 43]. The discovery that liposomes can activate CTLs through a cell-mediated immune response is significant since this type of response is essential for immunity against certain viruses [44, 45]. Furthermore, it has been shown that the magnitude of the cell-mediated immune response can be increased with the use of components that enhance cytosolic delivery [46].

Subcellular Delivery of Liposomal Contents

The internalization of liposomes within cells has been shown to be mediated by endocytosis [47]. Once internalized, the liposomes and their contents are susceptible to degradation within the endosomal pathway. Thus it is not surprising that the extent of cytosolic delivery of liposomal contents is generally suboptimal. For therapeutics whose site of action is in the cytosol or nucleus, the ability to escape degradation and be delivered to the cytosol is critical. Enhanced cytosolic delivery of liposomal contents has been demonstrated through several approaches. The ability of certain viruses to gain efficient access to the cytosol of host cells has provided insight into the development of virosomes, in which viral envelope proteins are inserted in the carrier's membrane [48]. Similarly, pH-dependent fusogenic peptides that mimic viral envelope proteins can be incorporated into the liposome core for endosomal escape [49]. The properties of certain phagosome-escaping bacteria have been studied and have led to the use of bacterially secreted hemolysins in liposome formulations for enhanced cytosolic delivery [50].

Listeriolysin O (LLO)

Listeriolysin O (LLO) is a pore-forming endosomolytic protein secreted from the facultative intracellular bacterial pathogen *Listeria monocytogenes* [51]. It plays a crucial role in the pathogenicity of the bacterium through its formation of pores in the endosomal membrane, such that *L.monocytogenes* can avoid degradation and travel to the cytosol where it subsequently replicates before infecting neighboring cells [52, 53]. While LLO is most active at the acidic pH of the endosome, its effects are attenuated upon introduction into the cytosol, a neutral pH compartment [54, 55]. The cytosol delivery capabilities of LLO have been applied to various delivery systems including the delivery of toxins or antigens via pH-sensitive liposomes as well as the delivery of plasmid DNA using liposomes or polymers [56-60].

pH-sensitive liposomes

pH-sensitive liposomes can be prepared with particular lipid compositions such that the liposomes are stable at physiological pH, but become unstable upon exposure to the acidic endosomal pH. The use of phosphatidylethanolamine (PE) and the cholesterol derivative cholesteryl hemisuccinate (CHEMS) is one such liposome formulation that displays pH-sensitivity. At pH 7.5, negatively charged CHEMS stabilizes PE and promotes the formation of bilayer membrane, and thus an intact liposomal vesicle. When the pH drops below 5.5, CHEMS becomes protonated and the lipids transition from the lamellar to the hexagonal phase, leading to membrane destabilization [61, 62]. Studies utilizing pH-sensitive liposomes have demonstrated the

cytosolic delivery of nucleic acid, proteins, and antigens [46, 63, 64]. Furthermore, the intracellular delivery of liposomal contents has been shown with pH-sensitive liposomes in which poly(ethylene)glycol (PEG) or antibodies are coupled to the membrane suggesting that the addition of various macromolecules to the liposome surface does not abolish its pH-sensitivity [65, 66]. However, the superior delivery of the contents encapsulated in pH-sensitive liposomes over pH-insensitive liposomes is more distinctive *in vitro* as opposed to *in vivo,* presumably due to the decreased pH-sensitivity seen in the presence of serum [33].

Future of Liposomal Delivery Systems

Liposomal delivery systems that target non-RES cells continue to be developed despite the initial setbacks that resulted from their relatively short *in vivo* half-lives. Modification of the liposome surface with molecules such as PEG has greatly improved the *in vivo* residence time of liposomes and has resulted in the development of several successful liposome formulations. As of 2005, there have been 14 liposomal drugs either commercially available or undergoing clinical trials, most of which are primarily for cancer therapy [25]. In addition, the use of liposomes for a variety of applications is constantly being pursued. Diagnostic imaging, sustained release of insulin, and the treatment of infection are just a few additional liposome applications from an ever-expanding list [67-70].

IMPROVING THE LIPOSOMES' CHARACTERISTICS THROUGH SURFACE MODIFICATION

Targeted Delivery of Therapeutics

The selective delivery of therapeutics associated with liposomes has been pursued through the attachment of various targeting ligands to the liposome surface. Such strategies have resulted in the enhanced delivery of agents to the target site and also provide a potential means of decreasing side effects that result from the delivery of drugs to normal cells [71-73]. In addition, the elevated expression of tumor-associated antigens has prompted research into the use of a wide range of targeting molecules with affinity for specific cell markers. Although a fairly wide variety of targeting moieties have been used in liposome delivery, only a few will be discussed below.

Vitamins

The unregulated growth and metastasis of many cancers can result in the upregulation of receptors for various vitamins and nutrients that are necessary for the increased synthesis of cellular components [12, 74, 75]. The expression of vitamin receptors at the cell surface combined with their potential ability to internalize bound ligands makes them a promising marker for targeted drug delivery [12]. Even so, reports of vitamin receptor targeting with liposomes has almost been exclusively limited to targeting of the folate receptor with folic acid-conjugated liposomes [72, 76].

Peptides

The diversity in overexpressed receptors in cells has led to the development of a variety of peptides for use in targeted liposome delivery. Asai et al. developed a peptide that could selectively target drug-loaded liposomes to the angiogenic tumor vasculature [13]. Similarly, Schiffelers et al. targeted the $\alpha_v\beta_3$ integrins in the tumor endothelium by coupling the RGD peptide to the liposome surface [77].

Antibodies

The coupling of antibodies or their fragments to liposome membranes (immunoliposomes) represents the most commonly-used targeted liposomal delivery strategy. Although predominantly researched for their use in cancer therapy due to the elevated levels of tumor-associated antigens at the tumor site, antibodies have also been used in other areas such as in enhancing the delivery of liposomal vaccines to dendritic cells [78]. Antibody fragments (i.e. Fab or single-chain Fv) have gained popularity in use over whole antibodies due to their ease of production and lack of an Fc segment that has shown to enhance uptake via the Fc receptor on macrophages [31, 79-81]. An additional consideration in the choice of antibody is its ability to be internalized upon binding the targeted receptor (or antigen): Sapra and Allen showed significantly enhanced efficacy with immunoliposomes that targeted internalizing over non-internalizing epitopes [82].

One of the most extensively utilized tumor-associated antigens in the targeted delivery of chemotherapeutics is HER2, the appeal of which is primarily

due to the FDA approval of the HER2-specific monoclonal antibody, Herceptin. HER2 is a member of the human epidermal growth factor receptor (HER) family of transmembrane receptor tyrosine kinases along with HER1 (EGFR), HER3, and HER4 which are involved in the regulation of cell growth and differentiation [83, 84]. Although a direct physiological ligand for HER2 has not been identified, it has been shown to be the preferred partner in the formation of heterodimers with other HER receptors [85]. HER2 expression is low in normal cells, but has been found to be overexpressed in a variety of breast, ovarian, prostate, and gastrointestinal cancers [86-89]. The overexpression of HER2 promotes the formation of heterodimers, resulting in very potent intracellular signaling that can lead to aggressive tumor growth and a poor prognosis for the patient [86, 90].

The essential role that HER2 plays in tumor growth and metastasis, along with increasing knowledge regarding its mechanism and pathway and its differential expression level in tumor and normal tissues have prompted research into the use of HER2 as a target for cancer therapy [91, 92]. In addition, HER2 is an attractive target due to its homogeneous expression within local and metastatic tumor sites as well as its ability to rapidly endocytose bound antibodies or anti-HER2 immunoliposomes [93-95]. The recombinant human anti-HER2 monoclonal antibody (trastuzumab, Herceptin) has been shown to inhibit tumor growth and has been especially effective when combined with chemotherapy [96, 97]. In 1998, Herceptin became the first monoclonal antibody approved by the Food and Drug Administration for the treatment of solid tumors [91]. Although the exact mechanism of action is unknown, it has been

hypothesized that Herceptin blocks HER2's signaling pathway either through inhibition of its dimerization or through HER2 downregulation [90, 98].

Despite the low HER2 expression levels in the myocardium, cardiotoxicity remains a significant side-effect of Herceptin, presumably due to the disruption of HER2 signaling which is involved in myocardial development [99-101]. Immunoliposomes that selectively deliver chemotherapeutics to $HER2^+$ cells have been developed in an attempt to increase efficacy and decrease non-specific cytotoxicity. In contrast to Herceptin, anti-HER2 immunoliposomes do not require high steady-state levels of anti-HER2 antibodies [94]. In addition, HER2-specific antibody fragments that do not impact HER2 signaling can be developed and incorporated into anti-HER2 immunoliposomes [102]. The above two reasons combined with the delivery capabilities of liposomes highlight some of the reasons why anti-HER2 immunoliposomes may be a successful treatment option for $HER2^+$ cancers.

In addition to the use of targeting ligands for enhanced delivery of therapeutics, they can also be used for inhibition of specific responses; one such targeting ligand used in this manner is CTLA4. CTLA4, and its homolog CD28, are membrane receptors on cytotoxic T cells with affinity for the APC surface ligands B7-1 and B7-2 [103]. Whereas the interaction of CD28 with the B7 ligands provides the necessary costimulatory signal for T cell activation, CTLA4 ligation displays an inhibitory effect and thus negatively regulates T cell activation [104, 105]. A soluble form of CTLA4 was created by fusing the receptor's ectodomain with the Fc portion of IgG (CTLA4Ig) and has shown promise as an

immunosuppressant for use in organ transplantation and autoimmune disorders [106, 107]. Furthermore, CTLA4Ig-conjugated liposomes have been shown to enhance the *in vitro* inhibition of the B7-1 binding site over soluble CTLA4Ig, and thus is a potential improvement in the pharmaceutical application of CTLA4Ig [31].

Enhanced Stability

Despite the enhanced selectivity displayed by targeted liposomal delivery systems, the relatively short *in vivo* half-lives of liposomes remain a major hurdle to achieving optimal efficacy with liposome-based pharmaceuticals [108]. The incorporation of a small molar percentage of negatively charged glycolipids such as phosphatidylinositol and monosialoganglioside (GM_1) or the polymer poly(ethylene)glycol (PEG) into the lipid membrane can increase the liposome stability in serum and prolong circulation times [9, 17, 26, 109]. The long circulation times seen with these liposome formulations have led to their being referred to as Stealth® liposomes [110]. PEG is a biocompatible molecule with a large hydrodynamic radius that is thought to provide steric hindrance against opsonin binding and subsequent clearance by APCs [111]. Liposomes in which PEG is incorporated in the lipid membrane (PEGylated liposomes) have demonstrated *in vivo* half-lives of up to 45 h in humans and a concomitant decrease in their uptake by the liver and spleen [112, 113]. In addition, the enhanced stability of Stealth® liposomes has been shown to result in their increased accumulation in tumor tissues while further enhancement in delivery has been demonstrated through the conjugation of targeting ligands to the distal

end of the PEG molecule [17, 21, 112, 114]. The ubiquitous use of PEG for its stabilizing effects attests to its many advantages. However, there are some limitations that must be addressed before the use of PEG can become more widely implemented. Different chain lengths of PEG can greatly affect the properties of PEGylated liposomes; therefore a low degree of polydispersity, though technically challenging, is essential for consistent and predictable results [111, 115, 116]. In addition, studies with PEGylated liposomes have indicated their ability to activate the complement system as well as to stimulate PEG-specific antibodies, both of which can negatively impact the efficacy of subsequent doses [117, 118].

Enhanced Immunogenicity

The discovery that upon injection, liposomes are rapidly internalized by the mononuclear phagocytic cells in RES spurred research into the use of liposomes as vaccine carriers. Many of the inherent qualities of liposomes (i.e. protection of encapsulated agents, large capacity for one or multiple agents) have been exploited for antigen delivery. In addition, the utility of associating various antigens with the liposome surface has been investigated. The conjugation of weakly immunogenic small molecules or peptides to the liposome surface can greatly enhance their immunogenicity, more so than through conjugation to alternative carrier molecules such as proteins or polysaccharides [119, 120]. Several studies have compared the immunogenicity of encapsulated versus surface-associated antigen with varying, and at times conflicting, results. Latif and Bachhawt reported an enhanced antibody response to membrane-

associated versus encapsulated antigen, while Shek and Sabiston concluded that encapsulated antigen was more immunogenic [23, 121]. Meanwhile, Gregoriadis et al. reported no difference between surface-linked and encapsulated antigen [122]. The discrepancy between these results has been attributed to various factors including differences in the level of non-specific adsorption of the antigen to the liposome surface as well as the amount of entrapped versus surface-linked antigen [122, 123]. A more detailed analysis of the type of response generated by the different methods of antigen association revealed that encapsulated antigen generated a high IgG:IgM ratio, while surface-linked antigen stimulated rapid and prolonged IgG and IgM responses [124]. These results have led many to conclude that the optimal method of incorporating an antigen into a liposome depends on the antigen, liposome formulation, dose administered, and desired response [124].

CONJUGATION STRATEGIES FOR THE ASSOCIATION OF MOLECULES WITH THE LIPOSOME SURFACE

Covalent Conjugation

One of the most commonly used techniques for coupling molecules to the liposome surface is chemical conjugation, predominantly via amide, disulfide, or thioether bonds [25, 31, 81, 125]. Successful covalent bond formation usually requires prior chemical modification of the ligand or liposome surface. The development of an immunoliposome via the formation of a thioether bond, for example, is often achieved by first thiolating the antibody followed by conjugation to a liposome with grafted maleimide groups. In order to render them selectively

reactive toward the maleimides, the antibodies can be reduced in order to expose internal sulfhydryls; alternatively, sulfhydryl groups can be introduced by reagents such as *N*-Succinimidyl S-Acetylthioacetate *(*SATA) or *N*-Succinimidyl S-Acetylthiopropionate (SATP) which react with the antibody's free amines [31, 81, 126]. A potential drawback of this approach is that due to the potential exposure of sulfhydryls at multiple locations within the antibody, the orientation of the conjugated antibody is undefined. This can result in decreased binding affinity or exposure of the Fc segment of the antibody which can enhance the liposome's internalization by macrophages [31, 80]. In addition, covalent conjugation often requires multiple steps with buffers of various composition and pH, thus raising concerns about the potential impact on the activity of the protein [23, 127]. However, for systems in which the characteristics of the protein are not negatively affected, high coating densities can be achieved through covalent conjugation [127].

Non-covalent Conjugation

One example of a method of non-covalent conjugation exploits the high-affinity interaction between avidin and biotin [128]. A biotin reagent is first used to label the primary amines of antibodies or other proteins, which can then be coupled via the molecule avidin or streptavidin to liposomes containing biotinylated lipids. The antibody coating density achieved with this procedure is reportedly less than that with covalent conjugation [127]. However, the utilization of the avidin-biotin conjugation technique in such applications as targeted

liposomal delivery has proven successful due to the ability of liposomes with low antibody coating density to selectively bind cells [127, 129].

Alternatively, proteins with a polyhistidine (His) tag can be associated with liposomes containing metal ion-chelating lipids [127, 128, 130]. The relatively high affinity between histidine residues and metal ions is commonly utilized for the purification of His-tagged proteins with Ni-NTA agarose [131]. This interaction can also be exploited to link that same protein with a liposome membrane, thereby eliminating the need for further modification of the protein, which can be time consuming and potentially damaging to the protein [23, 31]. In the case of the chelator lipid 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DGS-NTA (Ni)), nitrilotriacetic acid (NTA) chelates nickel (Ni) through four of its coordination sites, leaving two free for interaction with His tags. Although the affinity of DGS-NTA (Ni) for His-tagged proteins is relatively high, it has been reported that a proportion of the associated His-tagged proteins can dissociate in the presence of serum [132]. However, the successful utilization of this coating technique in numerous *in vitro* and *in vivo* studies suggests that some His-tagged protein remains bound to liposomes. Nielsen et al. demonstrated the ability to target $ErbB2^{+}$ cells with immunoliposomes coated with His-tagged ErbB2 antibody fragments via the Ni-His interaction in an *in vitro* study [133]. Additionally, His-tagged antigen associated with lipid-based nanoparticles containing DGS-NTA (Ni) reportedly elicited enhanced immune responses in comparison to soluble antigen administered with the adjuvant alum in multiple *in vivo* studies [134, 135].

Since prior reports have demonstrated the need for a physical association between antigen and liposomes in order to see an adjuvant effect, the results from these *in vivo* studies suggest that His-tagged antigen remains associated with nickel-liposomes after injection [136]. The stability of the association of His-tagged proteins with liposomes containing nickel-chelating lipid was further improved through the development of tris-NTA lipid in which each lipid head group contains three NTA chelating groups [130, 132]. Tris-NTA-containing liposomes coated with the His-tagged form of the T cell costimulatory molecule, B7.1, were shown to target T cells both *in vitro* and *in vivo* [130].

Hydrophobic Interaction

Hydrophilic proteins can be modified with a hydrophobic reagent in order to associate them with the surface of liposomes. Phospholipid-containing reagents that react with free sulfhydryls or amines on proteins such as antibodies have been used for subsequent immobilization on liposome membranes. Sinha et al. demonstrated the ability to link hydrophobically modified IgG and antibody fragments with liposomes without destabilizing the liposome membrane, while Torchillin et al. showed the ability to associate ~250 molecules of hydrophobically modified α-chymotrypsin on the surface of ~100 nm liposomes [137-139].

Membrane coupling through hydrophobic interaction is also possible for those molecules with inherent hydrophobic domains. Iovannisci et al. used an endogenous amphiphilic protein, apolipoprotein A-I (apoA-I) to couple an antibody fragment to the surface of the lipid membranes of nanodisk carriers through the creation of a fusion protein consisting of apoA-I and the antibody

fragment [140]. The use of the amphiphilic protein apolipoprotein B has been explored in this thesis work for its utility in linking proteins to the liposome surface and is discussed in more detail below.

Apolipoprotein B-100

Apolipoprotein B-100 (apoB-100) is an amphipathic glycoprotein which serves as the sole protein component of low density lipoproteins (LDLs) [141]. Unlike the exchangeable apolipoproteins (i.e. apoA, apoC, apoE) which have the ability to transfer between lipoprotein particles, apoB-100 is a non-exchangeable apolipoprotein, and thus remains associated with LDL throughout its circulation lifetime [142]. LDLs are spheroidal particles ~22 nm in diameter and contain a core of predominantly cholesteryl esters and triacylglycerols, while the outer layer contains phospholipids, unesterified cholesterol and a single molecule of apoB-100 [143]. Due to the large size (550 kDa, 4536 amino acids) and highly insoluble nature of apoB-100, characterization of its structure has been difficult [144]. Furthermore, the conformational flexibility of apoB-100 has complicated high resolution structure analysis by X-ray crystallography and electron microscopy [145]. In spite of these obstacles, a combination of physico-chemical techniques has been employed in attempt to characterize the structure of apoB. Although the detailed configuration of apoB-100 is still unknown, it is generally agreed upon that apoB-100 associates with LDL through multiple amphipathic α-helix and β-sheet motifs and contains regions that penetrate the phospholipid membrane as well as regions that are exposed to the aqueous environment (Figure 1.2) [146-150]. Through these interactions, it is believed that apoB-100

wraps around LDL and maintains its structural integrity as well as targets the LDL for the LDL receptor (LDLr) via its C-terminal LDLr-binding domain [141, 143, 151].

The discovery that the LDLr is upregulated in various tumor cell lines has led to the exploration into the use of apoB-100 in drug targeting [152-154]. Furthermore, through chemical modification of apoB-100, researchers have shown the ability to target receptors other than LDLr [6, 153]. The long residence time of LDLs (2-3 days) makes it seem plausible that apoB-100 may also enhance the circulation time of delivery vehicles *in vivo* [155]. Due to the need for separating and delipidating apoB-100 from *in vivo*-derived LDL samples, in addition to resolving issues related to the insolubility of apoB-100, the use of apoB-100 with liposome delivery systems can be quite tedious [153]. Attempts at elucidating the role and structural characterization of apoB-100 has led to the development of various N-terminal fragments [156-160]. It has been shown that apoB-100 can be reduced in size down to the amino-terminal 17% (amino acids 1-782, apoB-17) and still possess some globular structure as well as lipid membrane binding affinity [156, 158, 161, 162]. Lacking the C-terminal LDLr binding pocket, these N-terminal fragments may provide a potential means of associating targeting moieties that are either fused or chemically conjugated to the apoB fragment with the liposome surface.

CONCLUSION

Surface modification has led to the development of liposomes with longer residence times *in vivo*, the ability to selectively deliver associated agents to

target cells, and liposomal vaccines with enhanced immunogenicity. The methods utilized to associate molecules with the liposome surface vary not only in their degree of complexity, but also in their potential for impairing the activity and integrity of the molecule and liposome membrane, respectively. In this dissertation, we examine the impact of protein coating on the properties of liposomes while taking into account the coating procedure utilized.

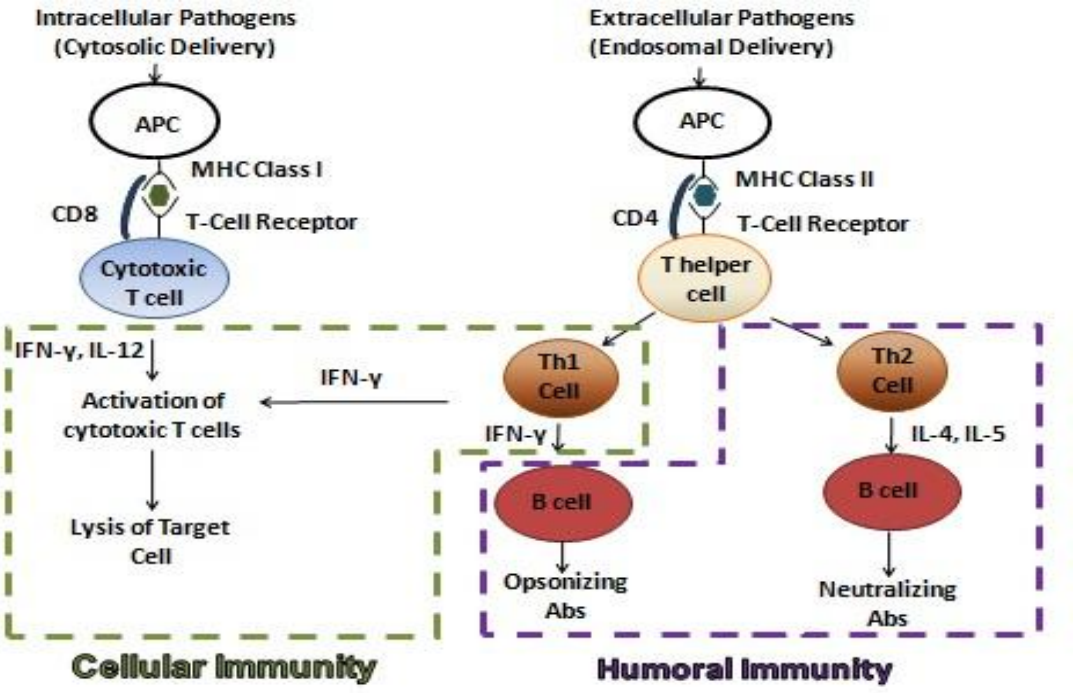

Figure 1.1: Overview of Cell-Mediated and Humoral Immunity

Agents that reach the cytosol of APCs (i.e. intracellular pathogens) are typically displayed on the surface by MHC class I molecules for recognition and binding by $CD8^+$ cytotoxic T-cells. This can result in the activation of cytotoxic T cells responsible for the lysis of the antigen-displaying cell. Agents that only reach the endosomes of APCs are usually displayed at the surface by MHC class II molecules for recognition and binding of $CD4^+$ T helper cells. This can result in the activation of Th1 cells which can help activate cytotoxic T cells or stimulate the production of opsonizing antibodies. CTL and Th1 cell activation are part of the cell-mediated immune response. Alternatively, MHC class II presentation can lead to Th2 cell activation and resultant production of neutralizing antibodies. The production of opsonizing and neutralizing antibodies is part of the humoral immune response. The secretion of various cytokines (e.g. IFN-γ, IL-4, IL-5, IL-12) helps drive specific types of responses.

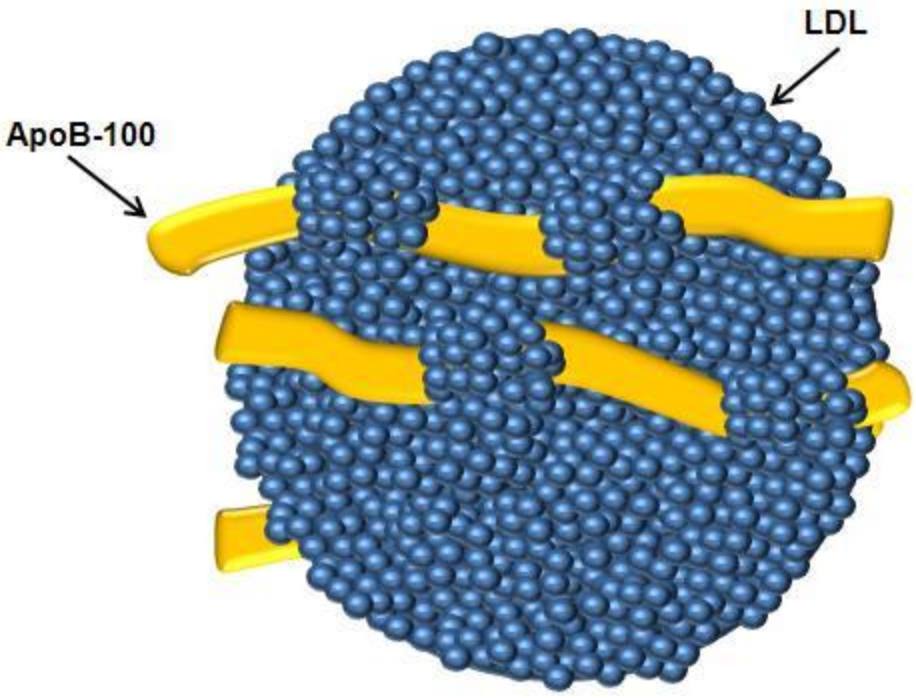

Figure 1.2: Proposed Model of ApoB-100 with LDL

Low-density lipoproteins (LDL) contain a core of predominantly cholesteryl esters and triacylglycerols. The surface of LDL is composed of unesterified cholesterol, phospholipids, and a single molecule of apolipoprotein B-100 (apoB-100). ApoB-100 is believed to associate with LDL through hydrophobic interaction with portions of the protein buried in the lipid membrane and portions exposed to the aqueous environment.

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CHAPTER 2

CHARACTERIZATION STUDIES ON THE USE OF NI-HIS AND TRUNCATED APOB FOR THE ASSOCIATION OF PROTEIN TO THE LIPOSOME SURFACE

SUMMARY

Coating the surface of liposomal delivery vehicles can be employed for modifying and improving various properties of the drug carriers including the liposome stability, selectivity, and overall efficacy of the delivery system. The method utilized to associate the molecule with the liposome is of great importance as it can affect the overall stability of the liposomes, activity of the bound protein, coating efficiency, stability of the protein-lipid interaction, and general simplicity of the process. In this study, two relatively simple methods to associate a model protein, yellow fluorescent protein (YFP), with the liposome membrane were investigated and compared. The first method was to attach histidine (His)-tagged YFP (YFP-His) to liposomes containing lipids with a nickel-chelating group. The second method involved the use of truncated apolipoprotein B-100 fused to yellow fluorescent protein (YFP) (apoB23-YFP), which binds to liposomes via the hydrophobic regions of apoB. We compared these coating methods for their effects on liposome stability as measured through membrane leakage and cell internalization assays. YFP-His and apoB23-YFP associated with the liposomes with no detectable loss of encapsulated

fluorophore. The membrane integrity was also preserved when the coated liposomes were incubated in the presence of serum over a 4 h time period. In addition, apoB23-YFP-coated liposomes and YFP-coated liposomes containing a low percentage of nickel-chelating lipid exhibited levels of uptake by bone marrow-derived macrophages (BMMs) similar to that of uncoated liposomes. This study demonstrates the potential use of these coating methods for associating protein with the liposome surface without impacting the stability of the liposome.

INTRODUCTION

Surface modification of liposomes can change the liposomes' characteristics and improve their overall efficacy as drug delivery vehicles. The association of targeting moieties with the liposome surface can enable the carrier to selectively bind cells that over-express the respective receptors or antigens, leading to more effective treatments with decreased toxicity in non-targeted cells. This relative selectivity has been demonstrated through the incorporation of various antibodies, vitamins, peptides, or mannose residues for targeted delivery to different cell types including tumor cells and antigen-presenting cells [1-5]. In addition, enhanced immunogenicity for liposomal vaccines has been achieved through the association of protein or peptide antigens or other costimulatory molecules to the surface of liposomes [6-10]. A further application of liposome surface modification is to increase circulation time *in vivo.* Liposomes grafted with monosialoganglioside (G_{M1}) or polyethyleneglycol (PEG) have demonstrated

as much as a 15-fold increase in their *in vivo* half-life over that of conventional liposomes [11-15].

The traditional method of associating coating molecules with the liposome surface is through covalent conjugation, which typically requires modification of the coating molecule for subsequent conjugation to the reactive groups incorporated in the liposome. Although commonly used, covalent conjugation techniques can be inefficient, tedious, and affect the liposome stability or protein activity [8, 16, 17]. Alternatively, noncovalent conjugation can be versatile, simple, and is capable of linking proteins to liposomes in a defined orientation without impacting their activity [16, 18]. One appealing example is the incorporation of polyhistidine (His) tags in proteins for specifically binding liposomes containing metal ion-chelating lipids [19-21]. The affinity between histidine residues and metal ions is commonly utilized for the purification of His-tagged proteins with Ni-NTA agarose [22]. This interaction can also be exploited to link that same protein with a liposome membrane, thereby eliminating the need for further modification of the protein, which can be time-consuming and potentially damaging to the protein. Chelator lipids such as DGS-NTA (Ni) can be easily incorporated into the liposome membrane and have demonstrated their utility in associating antibodies, proteins, and peptides with the liposome membrane [4, 18, 23].

Hydrophobic interactions can also be utilized for associating molecules with liposome membranes [24]. Unlike the conjugation methods, chemical modification of the protein or liposome is not required. An example of a protein

capable of associating with a lipid surface through hydrophobic interactions is the ~550 kDa amphiphilic protein apolipoprotein B-100 (apoB-100). ApoB-100 wraps around the surface of the cholesterol-carrying low-density lipoproteins (LDLs); it associates with the LDL membrane and lipid core through multiple lipid-binding domains distributed throughout apoB-100, while leaving hydrophilic regions exposed to the aqueous environment. In so doing, apoB-100 stabilizes the structure of LDLs [25, 26]. As an endogenous protein with affinity for LDL receptors, apoB-100 offers the potential of conferring stability and selectivity to drug delivery vehicles [27]. While full-length apoB has been successfully employed as a coating molecule for liposomes, the extent of its use has been limited due to difficulties with its isolation and association with the liposome surface [28, 29]. The production of recombinant truncated forms of the apoB-100 protein that have been demonstrated to retain the lipid binding property of the parent molecule, offer a potential solution to the aforementioned problems associated with the full-length apoB-100, thus maintaining its potential for use as a liposome-coating molecule [30, 31]. Furthermore, the use of a recombinant N-terminal apoB fragment eliminates LDL receptor interactions by the removal of the C-terminal LDL binding site, while offering a potential use as a fusion protein as a way to associate other proteins with the liposome surface [32, 33].

In this chapter, two relatively simple methods of associating a variant of yellow fluorescent protein (YFP) with the liposome membrane are examined: through non-covalent conjugation of His-tagged YFP with liposomes containing nickel-chelating lipid, and through hydrophobic interaction of a fusion protein

composed of an N-terminal apoB fragment (23% of the total amino acids) fused to YFP (apoB23-YFP) (Figure 2.1, A and B). The above-mentioned coating proteins were assessed for their versatility in coating density as well as for their impact on the integrity of the liposome membrane. The effect of the coating proteins on the internalization of liposomes in bone marrow-derived macrophages (BMMs) was also examined.

MATERIALS AND METHODS

Materials

All chemicals and reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE), 8-hydroxypyrene-1,3,6-trisulfonate (HPTS), p-xylene-bispyridinium bromide (DPX), and all tissue culture media were purchased from Invitrogen (Carlsbad, CA). 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DOGS-NTA (Ni)) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Calbiochem (La Jolla, CA).

Mice

C3H/HeJ ($Tlr4^{Lps\text{-}d}$, female, 7-8 week old, Jackson Laboratory) mice were used and handled according to the Institutional Guidelines.

Cell Lines

Bone marrow was harvested from the femurs and tibia of female 7-8 week old C3H/HeJ mice as described by Stier et al. [34]. Bone marrow cells were differentiated into bone marrow-derived macrophages (BMMs) in DMEM containing 20% heat-inactivated fetal bovine serum (HI-FBS), 30% L-cell conditioned media, 100 μg/mL streptomycin, 100 U/mL penicillin, and 55 μM 2-mercaptoethanol and harvested on day 6 and frozen in liquid N_2 until day of use. BMMs were cultured in complete DMEM containing 10% HI-FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, and 2 mM L-glutamine.

Preparation of YFP-His

The construct for the YFP variant (Citrine, [35]) used in these studies was a gift of Dr. Joel Swanson (University of Michigan, Ann Arbor, MI). YFP with a C-terminal hexahistidine tag (YFP-His) was expressed in the BL21 RIPL strain of *E.coli* transformed with the pET29b plasmid containing the gene for YFP-His. Bacterial cultures were induced for 4-6 h with 1mM IPTG for overexpression of YFP-His, and pelleted (9,000 x g, 10 min., at 4°C). Cell pellets were resuspended in wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) with 2 mM phenylmethylsulfonylfluoride (PMSF) and lysed with a French press (Thermo Spectronic, Rochester, NY). Lysate was centrifuged at 12,000 xg for 40 min. at 4°C and the supernatant incubated with Ni-NTA agarose. Resin was washed extensively and protein eluted with wash buffer containing 400 mM imidazole. Eluate was dialyzed into HBSE 8.4 (10 mM HEPES, pH 8.4, with 140 mM NaCl and 0.1 mM EDTA) or HBS 7.4 (10 mM

HEPES, pH 7.4, with 140 mM NaCl) overnight. All protein-containing solutions were dialyzed at 1:500 (v/v) two times in the specified buffer. Protein concentration was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) per the manufacturer's instructions. Purity was evaluated by resolving the samples in a SDS-PAGE gel and the proteins were detected using Krypton protein stain (Thermo Scientific) visualized with a Typhoon 9200 Imager (GE Healthcare).

Preparation of ApoB23-YFP

The construct for apoB23-YFP was created using the apoB23-HER2 gene in the pFastBac plasmid (a donation of Dr. Gregory Shelness, Wake Forest University, Winston-Salem, NC) along with the YFP-His gene in the pET29b plasmid. The YFP-His construct was mutated by PCR to add the *Aat* II (5') and *Kpn* I (3') restriction sites and ligated to *Aat* II and *Kpn* I-digested pFastBac vector containing the gene for apoB23. The resultant sequence contained a His-tag at the N-terminus of apoB23 and YFP at its C-terminus (Figure 2.2A). The new construct was transformed into One Shot Mach 1 T1 *E.coli* cells (Invitrogen) by the heat-shock method and the plasmid DNA purified using the Miniprep kit (Qiagen) per the manufacturer's instructions. After the sequence was confirmed (University of Michigan DNA Sequencing Core), the purified DNA for apoB23-YFP was transformed into DH10Bac competent cells (Invitrogen) by the heat-shock method and used to inoculate LB Agar plates containing 50 μg/ mL kanamycin, 7 μg/ mL gentamycin, 10 μg/ mL tetracycline, 300 μg/ mL X-gal, and 40 μg/ mL isopropyl β-D-thiogalactopyranoside (IPTG) (37°C, 48 h, 5% CO_2).

Colonies with a white phenotype were selected and the baculovirus shuttle vector (bacmid) was extracted as instructed in the Bac-to-Bac Baculovirus Expression System manual (Invitrogen). The recombinant bacmid DNA was transfected into 9 x 10^5 SF9 insect cells/ well of a 6-well plate per the manufacturer's instructions and viral supernatant was harvested 72 h later. The optimal multiplicity of infection (MOI) and time of harvest was determined empirically and the supernatant from the culture with the best apoB23-YFP expression amplified by infecting a 2 L culture using the determined optimal MOI and time of harvest. Cells were pelleted by spinning at 12,000 x g for 20 min. at 4°C and to the supernatant were added the following at these final concentrations: 0.05% NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ mL pepstatin A, and 1 µg/ mL leupeptin. The viral supernatant was filter-sterilized, concentrated, and diafiltered into wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) using a Millipore Pellicon XL tangential flow filtration device per the manufacturer's instructions. The purification procedure was later amended to improve yield by replacing the filtration, concentration and diafiltration steps with precipitation of the apoB23-YFP protein with 40% ammonium sulfate, followed by centrifugation at 10,000 x g for 20 min. at 4°C, and resuspension of the pellet with Ni-NTA wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). The protein solution was adjusted to 0.4 M NaCl and 0.5% Triton X-100 and incubated with Ni-NTA agarose. After extensive washing, the apoB23-YFP protein was eluted with wash buffer containing 250 mM imidazole, concentrated in an Amicon Ultra centrifugal filter device (molecular weight cutoff

= 100 kDa, Millipore), and dialyzed into HBSE 8.4 containing 1 mM benzamidine, 1 mM PMSF, and 1.5 mM sodium azide. Protein concentration and purity were determined as described above for YFP-His. The excitation and emission spectra for apoB23-YFP was determined by performing a fluorescence scan of a sample of apoB23-YFP in a Fluoromax-2 fluorometer (Instruments S.A. Inc., Edison, NJ).

Liposome Preparation

All lipid films were prepared from a 1:9:5 molar ratio of DOPA:DOPC:Chol as described by Provoda et al. [36]. For nickel-containing liposomes (Ni-liposomes), DGS-NTA (Ni) was incorporated at the indicated molar amounts. For the cell uptake assay, 0.5 mol % Texas Red-DHPE was additionally included in the lipid formulation. Lipid films were hydrated in HBSE 8.4 for protein-coating density studies and cell uptake studies or in HPTS/DPX (35 mM HPTS, 50 mM DPX in HEPES, pH 7.4 for YFP-His studies and pH 8.4 for apoB23-YFP studies) for membrane leakage and serum stability studies. Liposomes were subjected to four freeze/ thaw cycles, followed by extrusion through 0.2 μm and 0.1 μm filters for a total of 4 cycles through each. For the YFP-coating density study and the cell uptake study, liposomes were incubated with YFP-His at (DGS-NTA (Ni) lipid):(YFP-His) molar ratios ranging from 20:1 to 1:1 for 2 h at 4°C. For the stability studies, YFP-coated liposomes were formed by incubating the liposomes with YFP-His at a 2:1 (DGS-NTA (Ni) lipid):(YFP-His) molar ratio. For apoB23-YFP-coated liposomes, apoB23-YFP was incubated with 0.7 M guanidine hydrochloride (GdnHCl) for 25 min. before adding to liposomes at a 3000:1 total

phosphate (phospholipid):apoB-YFP molar ratio for a final concentration of 50 mM GdnHCl and 2.4 mM lipid and incubated for 20 h at 4°C. The uncoated liposomes for the apoB23-YFP studies were also incubated in the presence of 50 mM GdnHCl for consistency. Unassociated protein was removed by Sepharose CL-4B (GE Healthcare) gel filtration. Lipid concentration was determined by the method of Bartlett [37]. Standard curves of YFP-His or apoB23-YFP fluorescence were formed and used to determine the coating density of CL-4B-purified liposomes of known lipid concentration via fluorescence measurements at 485 nm excitation, 528 nm emission. Liposome diameter was determined by dynamic light scattering with a NiComp 380 ZLS instrument (Particle Sizing Systems, Santa Barbara, CA).

Membrane Leakage Assay

PA:PC:Chol liposomes were formed as described above with HPTS/ DPX encapsulated. For nickel-containing liposomes, 2 mole percent (of total phospholipid) DGS-NTA (Ni) was also incorporated. Liposomes were separated from unencapsulated HPTS/ DPX in a 1 x 25 cm Sephadex G-50 gel filtration column before coating with YFP-His or apoB23-YFP as described above. The unencapsulated HPTS fluorescence associated with 25 nmol of uncoated or protein-coated liposomes in buffer (HBS 7.4 for YFP-His studies or HBSE 8.4 + 50 mM GdnHCl for apoB-YFP studies) was measured immediately (time 0 h) and after the incubation period in a Fluoromax-2 fluorometer (Instruments S.A. Inc., Edison, NJ). Triton X-100 was added at a final concentration of 0.2 % at the end of the reading to lyse the liposomes and determine total fluorescence.

Measurements were normalized for total fluorescence; the contribution of YFP or apoB23-YFP to HPTS fluorescence measurements was found to be negligible. The % HPTS Released was calculated as: [((Fluorescence of unencapsulated HPTS at X h) - (Fluorescence of unencapsulated HPTS at 0 h)) / (Fluorescence of encapsulated HPTS at 0 h)] x 100 where X = 2 h for YFP-His binding studies and X = 20 h for apoB23-YFP binding studies.

Serum Stability Study

CL-4B-purified YFP-coated, apoB23-YFP-coated, or uncoated liposomes with HPTS/ DPX encapsulated were added to 75%, 10% or 0% calf serum (buffer: HBS 7.4 for YFP-His studies or HBSE 8.4 for apoB23-YFP studies) immediately before measuring the HPTS fluorescence of 1 nmol of lipid from each sample. Samples were diluted 1:200 in buffer to avoid high background readings from serum. Measurements were performed at 37°C and all liposome samples were stored under argon in a 37°C water bath in the dark. Fluorescence readings were taken at the time points indicated. Total fluorescence was determined by the addition of Triton X-100 as described above. Measurements were normalized for total fluorescence; the contribution of YFP-His or apoB23-YFP to HPTS fluorescence measurements was found to be negligible. The background contribution of serum was subtracted from 10 % and 75 % serum sample measurements. The % HPTS Released was calculated as above only X = designated time point (1.5, 2.5, or 4 h).

Cell Uptake Assay

BMMs from C3H/HeJ mice were plated at 1.25 x 10^6 cells/ well in a 24-well plate in complete DMEM on the day prior to the assay. Cells were washed with serum-free DMEM and pulsed with 200 µM of the different liposome formulations in serum-free DMEM for 4 h at 37°C. The cell-associated fluorescence was determined as previously described [38, 39]. Briefly, the cells were washed three times with cold PBS and dislodged by treatment with PBS buffer containing 5 mM EDTA (10 min. at 20°C). They were then washed once with PBS and cell density adjusted to 1 x 10^6 cells/ mL. The Texas Red-DHPE fluorescence (Ex 590/Em 645) was measured and used to calculate the nmoles of liposomes internalized per million cells based on standard curves.

Statistical Analyses

Significant differences in cell uptake for YFP-coated and uncoated Ni-liposomes and for apoB23-YFP-coated liposomes were analyzed by ANOVA and the Dunnett's Multiple Comparison test and the two-tailed Student's *t*-test, respectively. Data were concluded to be statistically significant if $p<0.05$.

RESULTS

Production of Protein-Coated Liposomes

The YFP-His and apoB23-YFP proteins were expressed and affinity-purified for their use as liposomal coating proteins. YFP-His migrated at ~ 33 kDa in an SDS-PAGE gel (Figure 2.2B). ApoB23-YFP (with an N-terminal His-tag), migrated at ~140 kDa in an SDS-PAGE gel (Figure 2.2C), and showed

excitation and emission peaks at 513 nm and 525 nm, similar to those of YFP-His (516 nm and 529 nm, respectively [35]).

To examine the coating efficiency of YFP-His, PA:PC:Chol liposomes were formed with 1, 2, 5, or 10 mol% DGS-NTA (Ni) incorporated into the lipid bilayer and incubated with YFP-His to initiate coating. Measurement of the particle size of purified YFP-coated and uncoated liposomes revealed liposomes of similar size (106.3 ± 1.1 nm for YFP-coated liposomes and 101.0 ± 1.6 nm for uncoated liposomes, respectively). Analysis of purified YFP-coated liposomes via fluorescence measurements revealed that the YFP-coating density increased with increased incorporation of nickel-chelating lipid (Figure 2.3). Additional control over the coating density was demonstrated through modification of the nickel-chelating lipid:YFP-His ratio. For a set mol % incorporation of nickel-chelating lipid, nickel-chelating lipid:YFP-His ratios ranged from 20:1 to 1:1, resulting in coating densities of 49-131 YFP-His/ liposome for 1 mol % nickel-chelating lipid to 291-926 YFP-His/ liposome for 10 mol % nickel-chelating lipid. All coating densities were calculated based on an approximated average liposome diameter of 100 nm. At higher ratios of nickel-chelating lipid:YFP-His, higher recoveries of YFP-His in the bound form were achieved: at a ratio of 20:1, 100% of the maximal recoverable amount of YFP-His was achieved, whereas at a ratio of 1:1, only 12% of YFP-His was recovered. These results demonstrate the versatility of coating density achievable through the Ni-His coating method.

Coating of the liposomes with the amphiphilic protein apoB23-YFP was achieved with a 3000:1 molar ratio of lipid (liposomes):apoB23-YFP for 20 h, and

resulted in typically 1-2 molecules of apoB23-YFP/ liposome. Purified apoB23-YFP-coated liposomes were similar in diameter to uncoated liposomes (94.7 ± 3.3 nm and 97.1 ± 0.4 nm, respectively). Due to the requirement for high (0.7 M) guanidine hydrochloride (GdnHCl) concentration in the apoB23-YFP protein solution followed by a high dilution (final = 50 mM) of GdnHCl when added to the liposome preparation, coating densities much higher than the coating density stated above were not achievable. These results demonstrate that the apoB fusion protein can be used to associate another protein with the liposome surface.

Liposome Membrane Integrity is Preserved Upon Addition of YFP-His or ApoB23-YFP

To investigate the effect of the coating protein on the integrity of the liposome membrane, liposomes were formed with the membrane-impermeant, small molecule dye, HPTS, and its quencher, DPX, co-encapsulated. When confined to the internal space of a liposome, HPTS fluorescence is quenched by the high concentration of DPX, and is dequenched upon its release and dilution of the HPTS and DPX after membrane destabilization [40]. HPTS fluorescence (λ_{Ex} 413 nm, λ_{Em} 520nm) associated with 25 nmol of lipid from each liposome formulation was measured in buffer before and after coating the liposomes with YFP-His or apoB23-YFP. The addition of Triton X-100 to lyse the liposomes at the end of each reading provided a measurement of the total fluorescence and, along with the initial measurement, was used to calculate the '% HPTS Released'. The HPTS that was released from uncoated liposomes was measured in parallel for comparison.

PA:PC:Chol liposomes with 2 mole % nickel-chelating lipid were coated with YFP-His using a 2:1 nickel-chelating lipid:YFP-His molar ratio while apoB23-YFP-coated liposomes were coated as described above. The amount of HPTS that was released from the liposomes during the coating step with either protein was minimal (0.1 ± 0.06 % for YFP-coated Ni-Lip, 1.3 ± 0.6 % for apoB23-YFP-coated Lip), demonstrating that the addition of the coating molecules to the liposome membrane did not induce leakage.

YFP-Coated and ApoB23-YFP-Coated Liposomes Maintain Membrane Integrity in Serum Solution

The presence of serum has been shown to increase the leakage of liposomal contents with prolonged incubations [41]. To evaluate the effect of serum on our protein-coated liposomes, coated and uncoated liposomes were prepared as described for the membrane leakage assay and incubated in 0 % (buffer), 10 %, and 75% serum. Keeping temperature constant at 37°C, the amount of HPTS fluorescence released in each liposome sample was measured at 0 h, 1.5 h, 2.5 h, and 4 h (Figure 2.4). For both sets of liposomes, there were no significant differences between the coated and uncoated formulations in each serum solution tested: little to no leakage of HPTS was detected for all liposomes incubated in 0 % serum (buffer), whereas the leakage of HPTS gradually increased over time for all liposomes incubated in 10 % serum. The magnitude of the leakage of HPTS was further increased in 75 % serum: ~ 2-fold for YFP-coated or uncoated Ni-Lip and ~ 6-fold for apoB23-YFP or uncoated liposomes.

The Addition of YFP-His or ApoB23-YFP to the Liposome Surface Does Not Enhance Uptake by BMMs

The liposome formulation used in this study included the negatively charged phosphatidic acid (PA) at 10 mol % of total phospholipids; the binding and internalization of liposomes by cells can be greatly affected by the liposome's membrane charge density, with negatively charged liposomes generally taken up by cells to a greater extent than neutral liposomes [38]. To detect the internalization of liposomes, the fluorescent dye Texas Red-DHPE was incorporated in the lipid bilayer of all liposome formulations. For the YFP-coated liposome study, liposomes were formed with 0, 1, 5, or 10 mole % of nickel-chelating lipid incorporated and coated with YFP-His at different coating densities by varying the nickel-chelating lipid:protein molar ratio as discussed above. The addition of different concentrations of YFP-His to liposomes did not appear to affect uptake properties as similar levels of uptake were detected for YFP-coated liposomes containing 1 mol % or 5 mol % nickel-chelating lipid as for the uncoated liposomes with the same incorporation of nickel-chelating lipid (Figure 2.5). The level of cell uptake for uncoated liposomes with 1 mole % or 5 mole % nickel-chelating lipid incorporated into the liposome membrane was similar to that of liposomes with 0 mole % nickel-chelating lipid. In contrast, the incorporation of 10 mole % nickel-chelating lipid into liposomes resulted in a 3-fold increase in cell uptake.

For the apoB23-YFP-coated liposome study, liposomes containing Texas Red-DHPE were coated with apoB23-YFP as discussed above; analysis of the coating density revealed that the liposomes were coated with approximately 2

apoB23-YFP molecules per liposome. The addition of the apoB23-YFP proteins to the liposome surface did not affect uptake by BMMs, as internalization was similar for both formulations.

DISCUSSION

The effect of the association of the protein YFP to the surface of liposomes was explored through two simple methods: an affinity interaction between the His-tag of YFP-His and the nickel-chelating lipid incorporated into the lipid membrane, and the hydrophobic interaction of a truncated form of the amphiphilic protein apolipoprotein B expressed as a fusion protein with YFP (apoB23-YFP). Both methods work effectively to associate YFP onto the liposome membrane without affecting the liposome membrane integrity or liposome-cell interaction properties by BMMs.

The use of YFP as a model protein allowed for the simplified quantification of its coating density through fluorescence measurements. YFP was produced as a His-tagged protein (YFP-His) and as an apoB fusion protein (apoB23-YFP), both of which were of high purity. In YFP binding studies, considerable flexibility in the YFP coating density was demonstrated by varying the mole % of nickel-chelating lipid incorporated in the Ni-Liposomes or of the molar ratio of nickel-chelating lipid:YFP-His during the incubation step. The adjustability of the YFP-His coating density suggests its versatility in many applications. The different possibilities of the types of coating molecules that can be attached to the liposomes with this method range from targeting moieties to therapeutics to antigens—all systems with potentially different coating density requirements. In

addition, association of YFP-His to the Ni-Liposomes was an efficient process with recoveries dependent on the nickel-chelating lipid:YFP-His ratio used. At higher ratios, such as 20:1, 100% of the maximal recoverable amount of YFP-His was bound to liposomes. These results demonstrate the potential utility of this process to associate proteins or peptides that are costly or in low supply [42].

The second alternative approach that was explored to associate proteins with the liposome membrane utilized a truncated form of the amphiphilic protein apoB which associates with lipid membranes via its hydrophobic domains. Although the exact manner in which the apoB fragment associates with liposomes is unknown, a hypothetical model could be proposed based on the full-length apoB-100's interaction with LDL in which it both penetrates the LDL membrane and fully wraps around the lipoprotein's surface [30, 33]. Due to this characteristic binding pattern of apoB, a high coating density was not expected. ApoB23-YFP typically coated liposomes of approximately 100 nm in diameter at densities of 1-2 molecules per liposome. This coating density was achieved by using a fixed lipid:protein molar ratio and lipid concentration during the incubation step. Lower lipid:protein ratios were found to result in the formation of precipitates, and therefore were not pursued. In addition, higher lipid concentrations were limited based on the final dilution of GdnHCl. In a study published by Ledford et al., truncated apoB protein was found to form multimers that lacked affinity for lipids unless the apoB multimers were first disrupted by GdnHCl [30]. Thus, to form apoB23-YFP-coated liposomes, apoB23-YFP was first incubated with 0.7 M GdnHCl (a concentration shown to result in the binding

of 90% of free truncated apoB to lipid vesicles [30]), before adding to liposomes for a final concentration of 50 mM GdnHCl. The low final concentration of GdnHCl was necessary to maintain the integrity of the liposome membrane as shown by the results from a leakage assay (Chapter 4). Thus, it is likely that using the current protocol the coating density of 1-2 molecules of apoB23-YFP/ liposome is the highest achievable density.

Any modification of the liposome membrane, such as via conjugation of a macromolecule or via non-covalent coating of its surface with protein, introduces the possibility of compromising the liposome stability or membrane integrity. The addition of YFP-His or apoB23-YFP to the liposome surface did not induce any detectable leakage of encapsulated fluorophore. This study examining liposome membrane integrity before and after the addition of a His-tagged protein to a Ni-liposome is to our knowledge the first study of its kind. However, others performed similar stability studies with liposomes in which full-length apolipoprotein A, E, or B were associated and reported results similar to those obtained in our studies with apoB23-YFP [28, 43].

To more accurately predict how the coated liposomes would act *in vivo*, it was important to examine the effect of the coating proteins on the integrity of the liposome membrane in the presence of serum as well as their influence on the uptake by mononuclear phagocytic cells of the reticuloendothelial system (RES) (e.g., antigen-presenting cells (APCs)) since both components can have a profound effect on the liposome circulation half-life *in vivo*. *In vitro* leakage studies conducted by Allen et al. showed that the presence of as little as 10 %

serum increased the rate of leakage of an encapsulated fluorophore by 20-fold in comparison to liposomes in buffer alone. The leakage rate was further increased another 2-fold in the presence of 100% serum [41]. The enhanced membrane destabilization in the presence of serum may at least be partly attributed to the binding of serum proteins to the liposome surface [44]. Surface coating molecules such as PEG or G_{M1} have been shown to decrease protein binding and enhance the overall stability of liposomes [44-46]. It was hypothesized that the addition of our coating molecules may have stabilizing effects similar to those of PEG or G_{M1}. YFP-His theoretically extends away from the liposome surface to a similar extent as PEG (compare 42 Å vs. 50 Å in length, respectively [47, 48]), and at a sufficiently high density has the potential to display stabilizing effects similar to those seen with PEG. Alternatively, apoB23-YFP, with its unique method of covering the liposome surface through multiple points of contact, was also predicted to stabilize liposomes in the presence of serum. Our results, however, did not indicate any stabilizing effect of YFP-His or apoB23-YFP; the extent of HPTS leakage from coated liposomes was similar to that of uncoated liposomes for all time points and serum conditions. Although YFP-His and apoB23-YFP did not improve the stability of the liposomes, it is encouraging to note that neither coating method resulted in destabilization of the liposome surface, and therefore may offer the potential to be used to associate proteins with the liposome surface for purposes other than imparting enhanced stability.

The above conclusions from the serum stability assay results were formed based on the assumption that both apoB23-YFP and YFP-His remained

associated with the liposome membrane during the course of the assay. As a non-exchangeable apolipoprotein, apoB-100 remains associated with LDL throughout the course of LDL's lifetime [33]. Although it is possible that the apoB fragment also remains associated with the liposome membrane in the presence of serum, this property should be evaluated in order to more accurately interpret the results of the serum-induced leakage assay. For the non-covalent Ni-His conjugation technique, it was previously reported that for a particular His-tagged protein analyzed, ~40 % remained associated with the membrane of liposomes containing 1 mol % DOGS-NTA (Ni) after incubating in serum for 4 h [49]. Even though the amount of YFP-His that remains associated with Ni-Lip in serum may differ due to differences in affinity or liposome composition, the above-mentioned results suggest that some YFP-His remain associated with the liposome membrane during the course of the assay.

A measure of the *in vivo* performance of the coated liposomes was also approximated through a cell internalization study. Liposomes have been found to be rapidly internalized by the cells of the RES [50-52]. This property has been exploited for situations in which APCs are targeted such as in vaccine delivery systems [53, 54]. However, in the case of drug delivery systems that target cell types other than RES cells, it is generally preferable to avoid uptake and internalization by the RES. The fact that YFP-coated liposomes with a relatively low molar amount of nickel-chelating lipid showed similar levels of uptake in BMMs as uncoated non-nickel liposomes indicates that the addition of YFP-His

to Ni-liposomes does not enhance interaction with APCs which could lead to a decrease in circulation time *in vivo*.

An alternative approach to decreasing recognition and uptake of drug delivery vehicles by cells of the RES has been through the use of carriers mimicking endogenous particles such as red blood cells or low-density lipoproteins [55, 56]. Similarly, we hypothesized that the addition of truncated apoB in the form of the apoB23-YFP fusion protein may be able to disguise the liposomes as an endogenous vesicle and thus decrease its recognition as a foreign particle, leading to reduced internalization by BMMs. Instead, it was found that the addition of apoB23-YFP as a coating molecule did not alter the uptake properties from that of the uncoated formulation. Although this result was not as we hypothesized, it suggests that the apoB fusion protein can be used as a method of coating liposomes with a protein while maintaining liposome stability.

Conclusion

Two methods of associating the model soluble protein, YFP, with the surface of liposomes were demonstrated: through affinity interaction between the His-tag of YFP and a nickel-chelating lipid in the liposome membrane, and through hydrophobic interaction between the membrane and an amphiphilic protein fused to YFP, apoB23-YFP. Both processes demonstrated successful coating of the liposome surface without destabilizing the membrane as shown in the leakage and serum stability assays. Additionally, apoB23-YFP-coated liposomes and YFP-coated liposomes of low molar percentage of nickel-chelating lipid did not either enhance or retard the uptake of the liposomes by

BMMs. These results suggest that either method could be utilized as a protein-coating technique in liposomal drug delivery systems. The potential of these methods extends to a wide array of pharmaceutical applications including targeted delivery, vaccines, and diagnostic imaging.

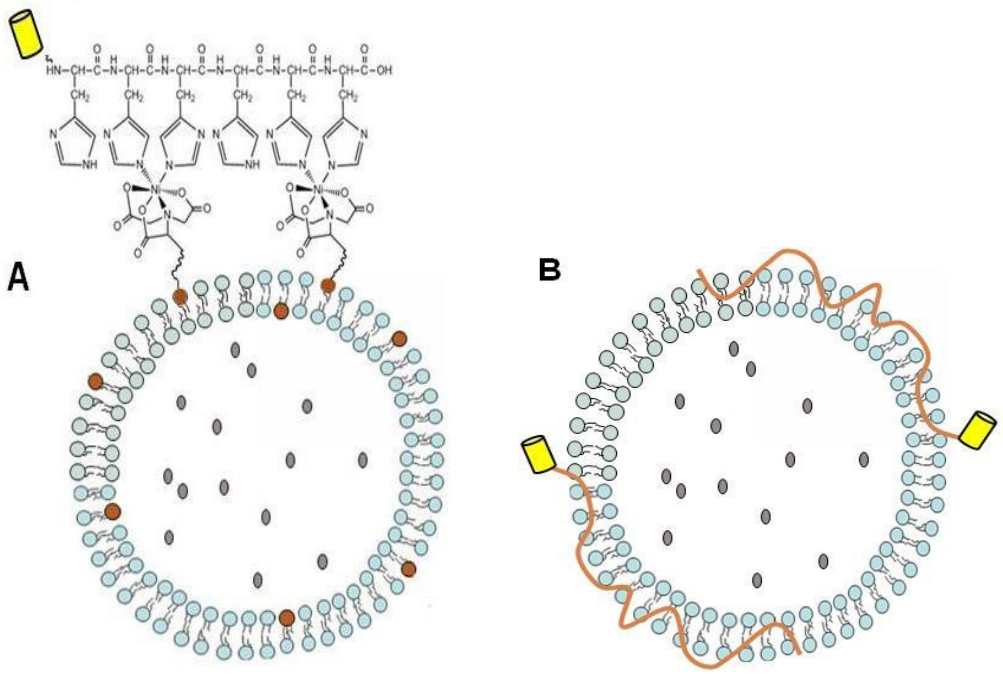

Figure 2.1: YFP-His and ApoB23-YFP as Liposome Coating Molecules

(A) Depiction of a YFP-coated liposome with YFP molecules in yellow and nickel-chelating lipid shown as red lipid head groups incorporated in the lipid membrane. Each nickel group has two coordination sites with which it can interact with the histidine residues on the YFP molecule as shown. (B) Proposed model of the interaction of apoB23-YFP with a liposome with the YFP molecules in yellow and apoB23 fragments in orange. The hypothetical drawing is based on the proposed interaction of the full-length apoB-100 with the LDL in which the apoB protein wraps around the lipoprotein and interacts with the lipid membrane through its multiple hydrophobic regions.

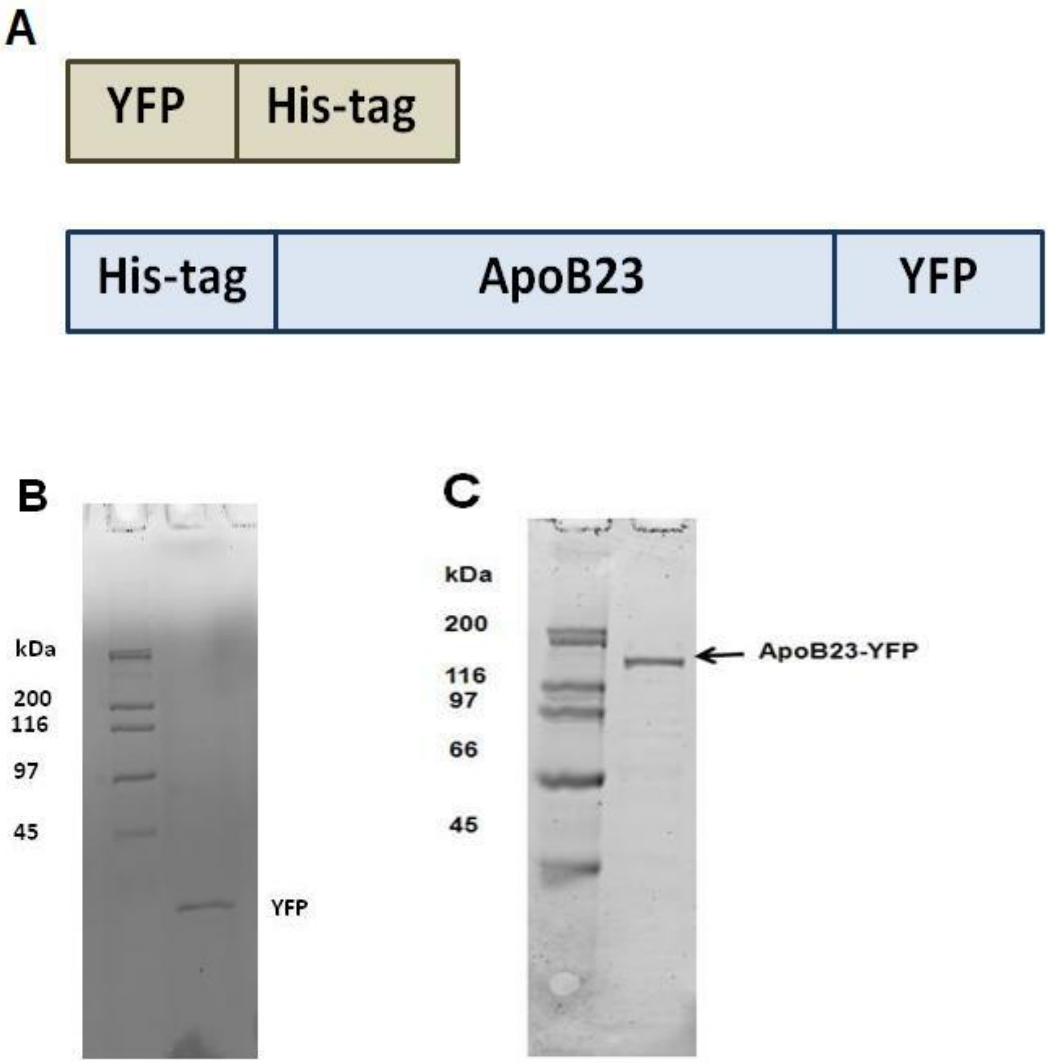

Figure 2.2: Expression of YFP and ApoB23-YFP Proteins

(A) Constructs of YFP-His and apoB23-YFP showing the presence of a hexahistidine (His)-tag at the C-terminus of YFP and N-terminus of apoB23-YFP. YFP lies at the C-terminus of apoB23-YFP. (B) YFP with a C-terminal hexahistidine tag (YFP-His) was expressed in *E.coli* and purified using nickel affinity chromatography. (C) ApoB23-YFP with an N-terminal His-tag was expressed in SF9 cells and purified with nickel chromatography. Analysis of the purity on an SDS-PAGE stained gel revealed single bands for YFP-His and apoB23-YFP around 33 kDa and 140 kDa, respectively.

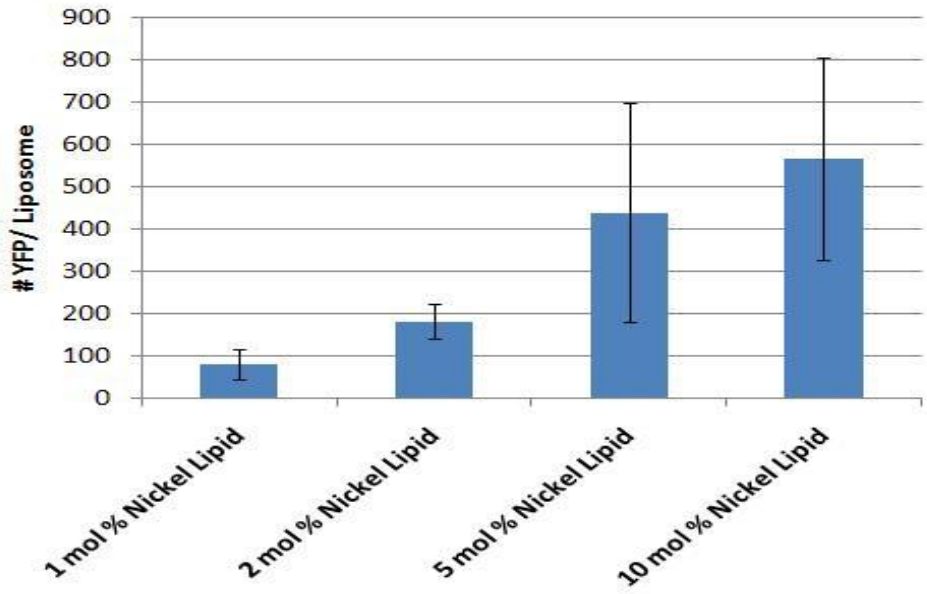

Figure 2.3: Versatility of YFP Coating Density

PA:PC:Ni:Chol liposomes were formed with the above amounts of nickel lipid incorporated. For each set of liposomes, the nickel-chelating lipid:YFP-His molar ratio used during the incubation period was varied between 20:1 to 1:1. The above results were obtained from fluorescence measurements of purified coated liposomes.

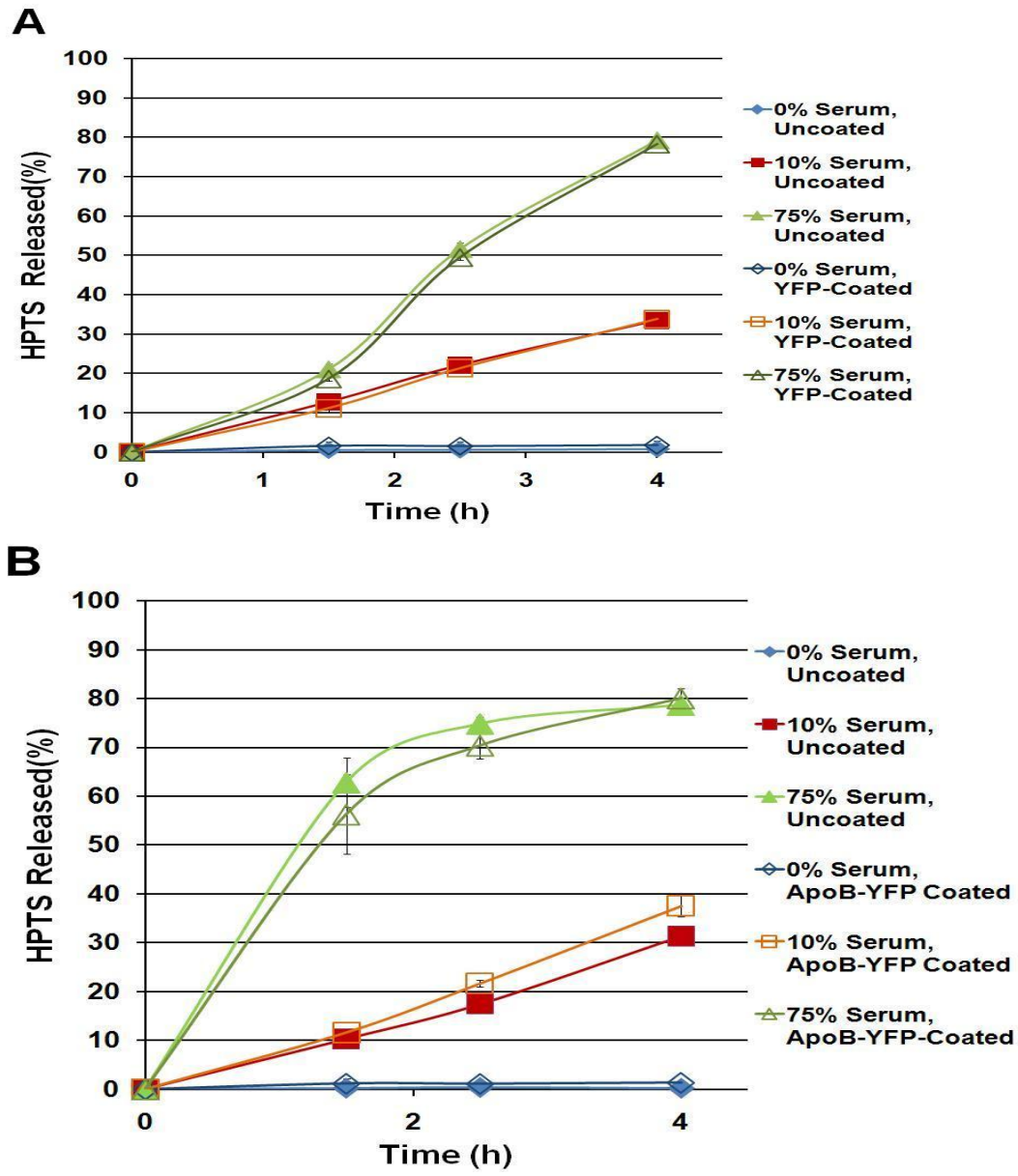

Figure 2.4: Stability of Liposomes in Serum-Containing Buffer

Coated or uncoated PA:PC:Chol liposomes with encapsulated HPTS/ DPX were incubated in 0 % (buffer), 10 %, or 75 % serum at 37°C. At the designated time points, the leakage of HPTS was detected by the increase in fluorescence resulting from the dilution of the collisional quencher DPX upon leaking out of liposomes. Measurements were normalized for total fluorescence and % HPTS released calculated. (A) YFP-coated or uncoated PA:PC:Chol liposomes with 2 mole % nickel lipid incorporated. (B) ApoB23-YFP-coated or uncoated PA:PC:Chol liposomes. The mean ± SD is shown.

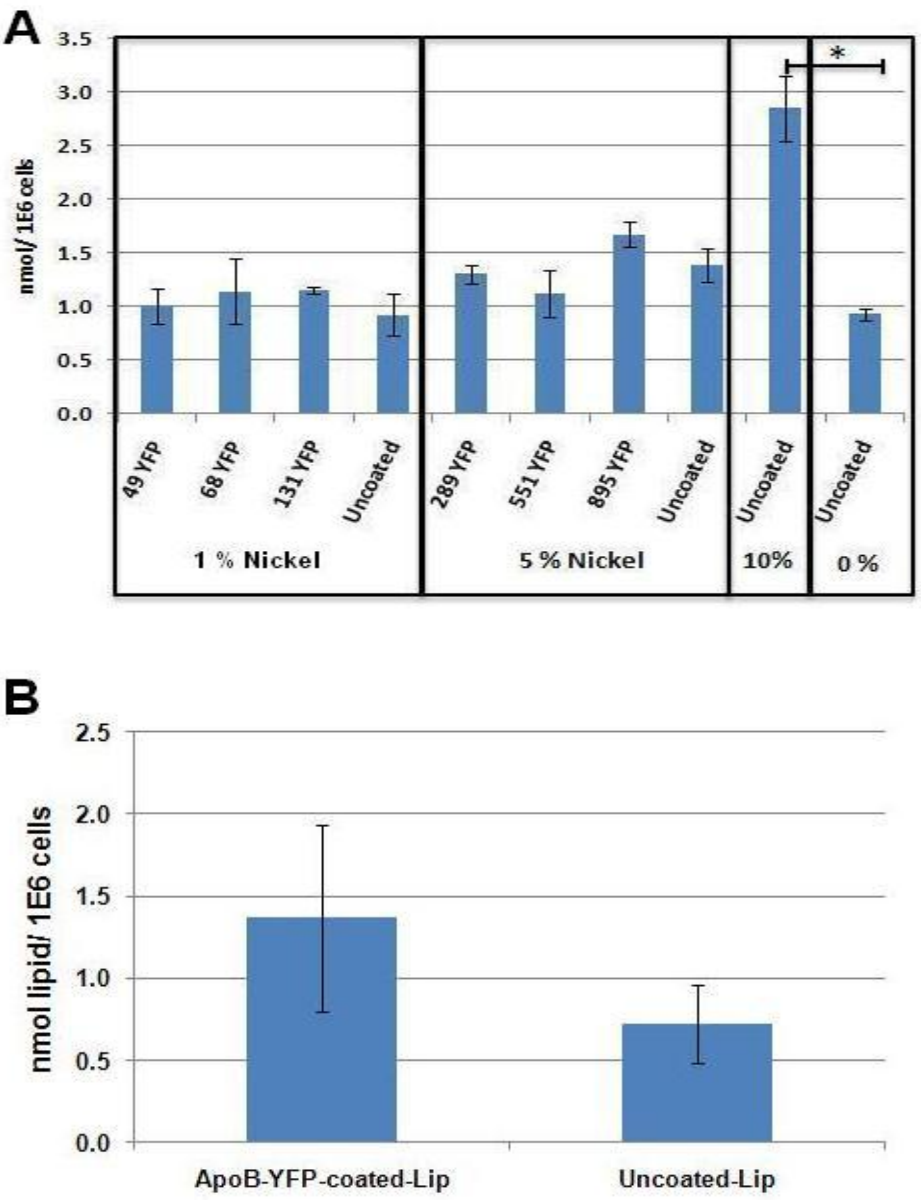

Figure 2.5: Effect of Coating Density on BMM Uptake

PA:PC:Chol liposomes with 0.5 mol % Texas Red-DHPE were formed. (A) For the YFP studies, 0-10 mol % nickel-chelating lipid was also incorporated; liposomes with 1 mol % or 5 mol % nickel were incubated with YFP at different nickel chelating-lipid:YFP molar ratios as described in Figure 2.3 to achieve the above-noted coating densities. (B) For the apoB23-YFP studies, liposomes were coated with apoB23-YFP or left uncoated. BMMs were pulsed with 200 μM purified liposomes for 4 h at 37°C, washed, resuspended, and the fluorescence associated per million cells determined. Data are displayed as the mean ± SD. * $p<0.01$

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CHAPTER 3

INFLUENCE OF A PROTEIN COATING ON THE DELIVERY PERFORMANCE OF A LIPOSOMAL VACCINE CARRIER

SUMMARY

Vaccine formulations containing multiple antigens from the same or different pathogens have been pursued due to their potential to increase efficacy and to decrease the cost of administration. Liposomes as vaccine carriers offer a distinct advantage of being able to carry multiple antigens that are either membrane-associated or encapsulated. We have previously demonstrated that the co-encapsulation of listeriolysin O (LLO) with a protein antigen in liposomes engenders induction of both arms of antigen-specific immunity. Here, LLO-containing pH-sensitive liposomes were prepared with two model antigens: ovalbumin (OVA) co-encapsulated and histidine (His)-tagged yellow fluorescent protein (YFP-His) membrane-associated. Nickel-chelating lipid (2 mole percent) was incorporated in the lipid bilayer (Ni-liposomes) for interaction with YFP-His. The effect of coating the Ni-liposomes with YFP-His on the delivery performance of LLO-liposomes and resulting OVA-specific immune responses was evaluated. The YFP-coated liposomes delivered OVA to the cytosol of antigen-presenting cells, as shown *in vitro* in bone marrow macrophages and *in vivo* by the stimulation of cytotoxic T lymphocyte (CTL) responses in mice. The level of CTL

activation *in vivo* by coated liposomes was similar to that of uncoated liposomes. Moreover, mice immunized with YFP-coated liposomes had significantly higher levels ($p<0.01$) of OVA-specific IgG antibodies over mice immunized with the uncoated liposome formulation. Additionally, YFP-coated liposomes stimulated the production of YFP-specific IgGs. This study shows that a His-tagged antigen can be associated with the membrane of Ni-liposomes without impairing the immunogenicity of the encapsulated antigen, and also be used as an effective antigen itself.

INTRODUCTION

Concerns over the safety of traditional vaccines as well as the need for more effective and economical vaccines have led to the development of many promising new delivery systems specifically geared toward the delivery of recombinant protein antigens. One such vehicle that has gained a lot of attention in vaccine delivery due to its inherent characteristics is the liposome. Having a propensity to interact with antigen-presenting cells (APCs), liposomes are an appropriate choice for antigen delivery [1, 2]. Liposomes have also exhibited adjuvant properties through their ability to enhance the immune response to associated antigens and are capable of stimulating cell-mediated as well as humoral immunity [1-3]. This property is particularly useful for subunit vaccines such as peptide epitopes and proteins which are weakly immunogenic on their own [4, 5]. Additionally, liposomes have the ability to carry multiple agents either through encapsulation or via membrane association. Due to the decreased cost and increased compliance associated with the use of multiple antigens against

different pathogens in a single vaccine, these combined vaccines are highly recommended by the World Health Organization [6]. Diphtheria, tetanus and pertussis (DTP) vaccine, measles, mumps and rubella (MMR) vaccine, and the influenza vaccine containing both hemagglutinin (HA) and neuraminidase (NA) are all examples of combined vaccines that have been used with much success [6, 7]. Furthermore, delivery of multiple antigens has been shown to have synergistic effects through the production of antigen-specific antibodies at a level much greater than that elicited from single antigen delivery, leading to an overall enhanced immune response [8, 9].

The association of antigen with a liposome can be through encapsulation or interaction with the membrane. Encapsulation of vaccine components offers the advantages of some degree of protection from enzymatic degradation *in vivo* and reduction of any potential toxicities associated with the encapsulated agent. On the other hand, membrane attachment of antigens has its own unique advantages [10, 11]. Although dependent on the formulation, coating density, and route of administration, several studies have found that antigen linked to the liposomal membrane stimulated a more pronounced and prolonged antibody response than when the antigen was encapsulated [12-15]. Thus, one may attach antigen to the liposomal surface in order to achieve a desired response. Liposome association through surface linkage may be preferred for small molecules or peptides that stimulate a very weak immune response on their own. Through conjugation to the liposome membrane, a more enhanced immune response may be possible [16, 17]. Additionally, liposomes coated with tumor-

associated antigens are being investigated for their potential in inhibiting tumor growth [18]. The financial and efficacy benefits of combined vaccines in conjunction with the advantages of both surface-linked and encapsulated antigens suggest that a vaccine delivery system with antigens associated with the liposome in both manners could be advantageous and thus warrant further investigation into the impact of a membrane-associated protein on the delivery properties of an encapsulated antigen.

In this study, citrine—a variant of yellow-fluorescent protein (YFP) is used as a model antigen and is coupled to the surface of a liposomal vaccine delivery system containing encapsulated ovalbumin (OVA), another model antigen. The reasons for this study were two-fold: 1) to investigate the effect of a membrane-associated protein on the liposome's performance in delivering encapsulated antigen and 2) to examine the overall immunogenicity of this multiple antigen delivery system. The type of immune response generated by an antigen is dependent on the way in which it is delivered to antigen-presenting cells (APCs). Antigen that is internalized by APCs (i.e. macrophages, dendritic cells, etc.) is processed and its peptides typically presented at the cell surface by major histocompatibility complex (MHC) class I molecules (if delivered to the cytosol) or by MHC class II molecules (if delivered only to the endosome) (Figure 3.1). Once presented, T cells then bind to the presented peptide via the T cell receptor (with specificity for the peptide) and co-receptor CD8 or CD4 (with specificity for MHC class I or II molecules, respectively). From here, the process may lead to a humoral immune response which is involved in the production of antibodies, or it

may lead to a cell-mediated immune response that involves the activation of cytotoxic T lymphocytes (CTLs). The activation of either of these arms of the immune system can lead to the secretion of specific cytokines (e.g. interferon (IFN)-γ) that help amplify the response [19]. Activation of CTLs, which plays a central role in anti-viral and anti-tumor immune responses, requires that the antigen be processed in the cytosol and presented by the MHC class I molecules, which is the ubiquitous pathway for presenting cytosolic proteins [20, 21]. However, exogenous antigens typically do not have access to the cytosolic pathway for MHC class I processing and presentation; instead they are generally endocytosed and degraded within the endolysosomal pathway for MHC class II presentation [20]. Although liposomal delivery of antigens can elicit CTL activation, this response is greatly enhanced by a strategy to actively augment cytosolic delivery, such as that achieved via the incorporation of listeriolysin O (LLO) in the liposome formulation [22]. LLO is a pore-forming endosomolytic protein secreted by the intracellular pathogen *Listeria monocytogenes*: LLO is most active at the acidic pH of the endosome [23]; and the utility of LLO in the cytosolic delivery of toxins, plasmid DNA, and antigens has been demonstrated [24-28]. We have previously shown that co-encapsulation of LLO with the model antigen OVA in pH-sensitive liposomes resulted in a strong OVA-specific CTL and antibody response [24]. In this chapter, we expanded our work by investigating whether the addition of a coating antigen to our LLO-containing liposomal vaccine would alter its ability to generate OVA-specific CTLs and anti-OVA antibodies similar in level to our previous uncoated formulation. The ability

of YFP to elicit a humoral immune response through generation of YFP-specific antibodies was also examined.

Various chemical-crosslinking methods have been employed to attach protein to the liposome surface. However, such methods can be tedious and may affect the activity of the protein [14]. A more simplified and efficient coating method is through the incorporation of nickel-chelating lipid (Ni) into liposomes for binding to histidine (His)-tagged proteins. Polyistidine tags, consisting of a string of 4-10 repeating histidine residues, are commonly employed for labeling or purifying proteins while retaining the protein's biological activity [29, 30] . The Ni-His interaction is one of relatively high affinity and has been used to associate costimulatory molecules, antibody fragments and peptides with lipid-based vehicles [18, 30-32].

In this chapter, the development of a liposomal vaccine with LLO and OVA co-encapsulated and His-tagged YFP associated with the liposomal membrane is discussed. YFP-His was attached through interaction with the nickel-chelating lipid that was incorporated in the liposome bilayer. The effect of the coating protein on the delivery performance of a liposome-based vaccine was evaluated in terms of the ability of the encapsulated OVA antigen to activate both OVA-specific antibodies and CTLs. In addition, the ability of YFP-His in its liposomal membrane-associated form to stimulate the production of antibodies against itself was also investigated.

MATERIALS AND METHODS

Materials

All chemicals and reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. The fluorophore 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) and its quencher p-xylene-bispyridinium bromide (DPX), and all tissue culture media were purchased from Invitrogen (Carlsbad, CA). L-α-phosphatidylethanolamine, transphosphatidylated (egg, chicken) (ePE) and 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) (DGS-NTA (Ni)) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas Red-DHPE) was purchased from Invitrogen. Sheep red blood cells (RBCs) were procured from Lampire Biological Laboratories (Pipersville, PA).

Mice

C57BL/6 (female, 8-12 weeks old; Harlan Laboratories, Indianapolis, IN) and C3H/HeJ ($Tlr4^{Lps\text{-}d}$,female, 7-8 week old, Jackson Laboratory) mice were used and handled according to Institutional Guidelines.

Cell Lines

Bone marrow was harvested from the femurs and tibia of female 7-8 week old C3H/HeJ mice as described by Stier et al. [33]. Bone marrow cells were differentiated into bone marrow-derived macrophages (BMMs) in DMEM containing 20 % heat-inactivated fetal bovine serum (HI-FBS), 30 % L-cell

conditioned media, 100 µg/ mL streptomycin, 100 U/ mL penicillin, and 55 µM 2-mercaptoethanol and harvested on day 6. Cells were stored in liquid N_2 until day of use. BMMs were cultured in complete DMEM containing 10 % HI-FBS, 100 µg/ mL streptomycin, 100 U/ mL penicillin, and 2 mM L-glutamine. B3Z cells, a $CD8^+$ T-cell hybridoma line specific for the OVA SIINFEKL peptide, were maintained in RPMI 1640 media with 25 mM HEPES, 10 % HI-FBS, 2 mM glutamine, 100 µg/mL streptomycin, 100 U/ mL penicillin, and 50 µM 2-mercaptoethanol.

Preparation of Recombinant Listeriolysin O (LLO)

The construct for recombinant listeriolysin O (LLO) containing an N-terminal hexahistidine (His) tag and AcTEV affinity tag (His-AcTEV-LLO) was provided by Dr. Jiayan Liu (University of Michigan, Ann Arbor, MI). His-AcTEV-LLO were expressed in the BL21 RIPL strain of *E.coli* as described by Mandal et al. with some modifications [24]. Bacteria were induced with isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h and pelleted (9,000 x g, 10 min., at 4°C). Cell pellets were resuspended in wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) with 2 mM phenylmethylsulfonylfluoride (PMSF) and lysed with a French press (Thermo Spectronic, Rochester, NY). Lysate was centrifuged at 12,000 x g for 40 min. at 4°C and the supernatant incubated with Ni-NTA agarose. Resin was washed extensively and protein eluted with wash buffer containing 400 mM imidazole. Eluate was dialyzed into wash buffer overnight. All protein-containing solutions were dialyzed at 1:500 (v/v) two times in the specified buffer. AcTEV protease was expressed in the BL21 RIPL strain

of *E.coli* with a 20 h induction with 1 mM IPTG and then purified as described above. AcTEV was concentrated using an Amicon Ultra 15 Centrifugal Filter Unit (MWCO 3 kDa) and then dialyzed overnight in wash buffer. To remove the His-tag from purified His-AcTEV-LLO, AcTEV was added to His-AcTEV-LLO at a 1:1 mass ratio in wash buffer and incubated overnight at room temperature. Purified LLO was collected in the flow-through after running the reaction mixture in a Ni-NTA agarose column and LLO was dialyzed against HBS 8.4 (10 mM HEPES, pH 8.4, with 140 mM NaCl). Protein concentration was determined using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) per the manufacturer's instructions. Purity was evaluated by resolving the samples in a 4-12 % Bis-Tris SDS-PAGE gel and the proteins were detected using Krypton protein stain (Thermo Scientific) visualized with a Typhoon 9200 Imager (GE Healthcare). To confirm the absence of the His-tag, the proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane and probed with mouse anti-polyhistidine monoclonal antibody (1:3,000, Sigma), followed by an alkaline phosphate conjugated goat anti-mouse antibody (1:20,000, Sigma). Bands were visualized with a Typhoon 9200 Imager.

LLO activity was evaluated through its ability to lyse sheep red blood cells (RBCs). Serial 2-fold dilutions of LLO were made in MBSE 5.5 (10 mM MES, pH 5.5, with 140 mM NaCl and 1 mM EDTA) containing 1 mg/mL bovine serum albumin (BSA). RBCs were washed in PBS, then diluted in MBSE 5.5 and plated at 20 x 10^6 cells/ well in a round-bottom 96-well plate. The LLO dilutions

were added to the RBCs and the sealed plate was incubated for 15 min. at 37°C on a rotator. Cell debris was pelleted by centrifuging the plate at 1400 x g for 15 min. at 4°C. The supernatants were transferred to a 96-well flat-bottomed plate and the absorbance was measured at 450 nm. Values were normalized to the absorbance of a positive control consisting of RBCs and LLO-His, a recombinant C-terminally His-tagged LLO (without AcTEV affinity tag) that has known and well-characterized hemolytic activity. The Hemolytic Fraction, calculated as: (Absorbance at 450 nm)/ (Absorbance of positive control at 450 nm), was plotted against LLO mass and used to determine the mass of LLO that lyses half as many RBCs as the positive control (defined as the HD_{50}). An antigen presentation assay (described below) was performed to compare the cytosolic delivery capabilities of AcTEV-cleaved LLO and LLO-His.

Preparation of YFP-His

The construct for YFP with a C-terminal hexahistidine tag (YFP-His) was a gift of Dr. Joel Swanson (University of Michigan, Ann Arbor, MI). YFP-His was expressed in the BL21 RIPL strain of *E.coli*. Bacterial culture was induced for 4-6 h with1 mM IPTG for overexpression of YFP-His, centrifuged at 9,000 x g for 10 min. at 4°C, and the resuspended pellets were purified over Ni-NTA agarose as described for LLO. Eluate was dialyzed against HBS 8.4 after which protein concentration and relative purity were determined by BCA assay and SDS-PAGE, respectively.

Liposome Preparation

All lipid films were prepared with a 2:1 molar ratio (10 μmol: 5 μmol) of ePE:CHEMS as described by Provoda et al. [25]. For nickel-containing liposomes (Ni-Lip), 2 mole percent (of total phospholipid) DGS-NTA (Ni) was also incorporated. For *in vitro* cell uptake assay, 0.5 mol percent Texas Red-DHPE was included in lipid formulation. Lipid films were resuspended with 500 μL HBS 8.4 containing AcTEV-cleaved LLO at 0.3 mg/mL and OVA at 20 mg/mL. Liposomes were subjected to four freeze/ thaw cycles, followed by four one-minute bursts in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY). For YFP-coated liposomes, Ni-liposomes were incubated with YFP-His at a 5:1 lipid:protein molar ratio and 10 mM lipid concentration for 2 h at 4°C. Unencapsulated and unbound proteins were removed by Sepharose CL-4B (Sigma-Aldrich) gel filtration in a 1 x 25 cm column. Lipid concentration was determined by the method of Bartlett [34]. The concentration of encapsulated and membrane-associated protein was determined by running purified liposomes along with known quantities of LLO, OVA, and YFP to generate a standard curve in 4-20 % Tris-Glycine SDS-PAGE, followed by detection with Krypton stain and quantification using a Typhoon 9200 Imager. For YFP-coated liposomes pretreated with anti-YFP monoclonal antibody (MAb), YFP-coated liposomes were incubated with murine anti-YFP MAb (Applied Biological Materials Inc., Richmond, BC) at a 1:1 molar ratio of YFP-His:anti-YFP MAb for 1 h at room temperature and used immediately.

pH-Sensitivity Assay

Lipid films composed of DOPE:CHEMS or DOPE:CHEMS with 2 mol % nickel-chelating lipid were formed as described above and resuspended with 500 μL HPTS/DPX (35 mM HPTS, 50 mM DPX in HEPES, pH 8.4). Following the freeze/thaw cycles and sonication steps, the liposomes were separated from unencapsulated HPTS/DPX by gel filtration before coating with YFP-His as described above. The HPTS fluorescence associated with 10 nmol of uncoated or protein-coated liposomes in pH 7.4 buffer (PBS) or pH 4.5 buffer (50 mM sodium acetate) was measured in a Fluoromax-2 fluorometer (Instruments S.A. Inc., Edison, NJ). Triton X-100 was added at a final concentration of 0.2 % at the end of the reading to disrupt the liposomes and determine total fluorescence. The % HPTS Released was calculated as: [(Fluorescence of unencapsulated HPTS)/ (Fluorescence of total HPTS)] x 100.

Cell Uptake Assay

The cell uptake assay was performed as described in Chapter 2. Briefly, BMMs from C3H/HeJ mice were plated at 1.25 x 10^6 cells/ well of a 24-well plate in complete DMEM on the day prior to the assay. Cells were washed with serum-free DMEM and pulsed with 200 μM of the different liposome formulations in serum-free DMEM for 4 h at 37°C, 5% CO_2. The cells were washed three times with cold PBS and dislodged by treatment with PBS containing 5 mM EDTA (10 min. at room temperature), followed by washing once with PBS and adjusted to 1 x 10^6 cells/ mL. The Texas Red-DHPE fluorescence was measured

at 645 nm and used to calculate the nmoles of liposomes internalized per million cells based on standard curves.

Antigen Presentation Assay

The antigen presentation assay was performed as described by Lee et al. [26] with minor modifications. C57BL/6 BMMs were plated at 2 x 10^5 cells/ well in a 96-well plate in complete DMEM on the day prior to the assay. Cells were washed with serum-free DMEM and treated with liposomes serially diluted based on OVA concentrations in serum-free media for 2 h. The cells were washed, then chased in complete DMEM for 3 h, fixed with 1% paraformaldehyde in PBS, and treated with 0.2 M lysine (in DMEM). Cells were washed again and 2 x 10^5 B3Z cells/ well were added to 96-well plates and incubated for 15 h at 37°C, 5% CO_2. Chlorophenol red-β-D-galactopyranoside (CPRG; Calbiochem, San Diego, CA) substrate (prepared in PBS containing 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, and 0.125 % NP40) was incubated with washed cells for 4 h. Relative β-galactosidase levels were detected through the conversion of its colorimetric substrate, CPRG, to chlorophenol red and reported as absorbance at 570 nm using an Emax plate reader (Molecular Devices).

Immunization Protocol

C57BL/6 mice were primed with liposomal formulations containing 15 μg OVA in 100 μL HBS 8.4 via subcutaneous injection. Mice were euthanized on day 10; blood was collected through cardiac puncture and the spleens were harvested for assays.

Cytotoxic T-Lymphocyte (CTL) Assay

Naïve mice were euthanized and spleens removed and forced through a 70 µm cell strainer to isolate spleen cells. Cells were washed extensively with mouse media (RPMI 1640 containing 2 mM L-glutamine, 10 % HI-FBS, 55 µM 2-mercaptoethanol, 100 U/ mL penicillin, 100 µg/ mL streptomycin) and RBCs lysed with ACK lysing buffer (Invitrogen). Cells were then washed with mouse media and pulsed with either OVA SIINFEKL peptide or Influenza nucleoprotein (NP) peptide$_{147\text{-}155}$ (Anaspec, Freemont, CA) (1 µg/ mL, 2 x 10^7 cells/ mL for 1 h at 37°C, 5% CO_2). Pulsed cells were washed with mouse media and adjusted to 1 x 10^8 cells/ mL with PBS + 0.1 % HI-FBS. OVA SIINFEKL peptide-pulsed cells were labeled with 4 µM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Invitrogen) while NP peptide-pulsed cells were labeled with 0.4 µM CFSE by slowly adding 50 µM or 5 µM CFSE, respectively, to cells while vortexing. Reaction proceeded for 10 min. at room temperature in the dark and was stopped by adding an equal volume of cold HI-FBS and incubating 5 min. on ice. Cells were washed three times with PBS + 5 % HI-FBS and adjusted to 2 x 10^7 cells/ mL before combining in a 1:1 (v/v) ratio of the two cell populations. 200 µL (2 x 10^6 cells/ population) were injected into naïve and immunized mice via tail vein on day 9 after immunization. Mice were euthanized 18 h later and spleens removed. Splenocytes were isolated by forcing spleens through a 70 µm cell strainer, washed extensively with mouse media, and resuspended in L-15 media. An equal volume of Ficoll-Paque PREMIUM (GE Healthcare) was carefully layered on the bottom of the tube and cells were centrifuged for 20 min. at 1,100 x g at room temperature with the brake off. The lymphocyte layer was recovered

and viable cells were analyzed for CFSE fluorescence in a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA). The percent specific lysis was calculated as: [1-((ratio of OVA peptide-pulsed cells lysed in immunized mouse)/(ratio of OVA peptide-pulsed cells lysed in naïve mouse))] x 100. This equation accounts for slight differences in the relative amounts of the two cell populations injected by normalizing to the naïve mouse.

Interferon (IFN)-γ Secretion Assay

Splenocytes were harvested on day 10 from the Ficoll density gradient as described above and 50,000 cells/ well stimulated with $NP_{147-155}$ peptide (5 μM final) or OVA SIINFEKL peptide (5 μM final), OVA (50 μg/mL final), or concanavalin A (5 μg/mL final; Sigma-Aldrich) for 72 h at 37°C, 5 % CO_2 in a 96-well plate, after which supernatants from duplicate wells were combined and assayed for IFN-γ secretion via ELISA. Collected supernatants were added in duplicate to a 96-well MaxiSorp Immuno plates (Nunc) previously coated with rat anti-mouse IFN-γ antibody (BD Biosciences) for 5 days at 4°C, and blocked with PBS + 1% BSA for 1 h at room temperature. The presence of IFN-γ in cell supernatant was detected with biotinylated rat anti-mouse IFN-γ antibody (BD Biosciences) followed by avidin-HRP (BD Biosciences), and finally TMB substrate. Plates were washed six times with PBST (PBS containing 0.05% Tween 20) using an ELX405 plate washer (BioTek) between each step. Development of substrate was stopped by addition of 2 N H_2SO_4, and the absorbance at 450 nm was immediately measured.

Anti-Antigen Total IgG ELISA

Blood was harvested from euthanized mice via cardiac puncture and incubated for 1 h at 37°C, 5% CO_2 followed by overnight at 4°C to allow clotting. Sera were separated from RBCs by spinning samples for 10 min. at 250 x g and removing supernatant. MaxiSorp Immuno plates were coated with 0.5 µg OVA or 0.5 µg YFP-His overnight at 4°C, washed six times with PBST, then blocked overnight at 4°C with PBS containing 1% BSA. Plates were washed again and serial dilutions (in PBST with 1% BSA) of sera added and placed at 4°C for 2-3 days. OVA-specific or YFP-specific IgGs were detected with goat anti-mouse total IgG-biotin (Sigma), followed by avidin-HRP, and finally TMB substrate. Plates were washed six times with PBST between each step. Conversion of substrate to colorimetric product was halted by addition of 2N H_2SO_4, and absorbance at 450 nm was immediately measured. Data were fit to a four-parameter curve (Softmax Pro 5.2) to calculate titer (the dilution factor giving an absorbance of 0.5). For samples below the limit of detection, the lowest dilution measured was reported.

Statistical Analyses

Grubb's test was performed on all data sets to identify outliers. Data with homogeneous variance were analyzed via ANOVA and Tukey's post-test. Data with non-homogeneous variance were analyzed by the Kruskall-Wallis test and Dunn's post test. Data were concluded to be statistically significant if $p<0.05$.

RESULTS

Expression and Activity of LLO

To produce recombinant LLO that can be encapsulated in Ni-liposomes without interfering with the Ni-His interaction between YFP-His and Ni-Lip, we expressed LLO with an N-terminal AcTEV-cleavable His-tag. Incubation of His-AcTEV-LLO with the AcTEV protease followed by collection of the flow-through (FT) from Ni-NTA affinity purification yielded LLO of high purity and lacking the His-tag as confirmed by SDS-PAGE and an anti-His Western blot, respectively (Figure 3.2). The affinity of Ni-NTA for histidine allowed for retention of the cleaved His-tag along with the His-tagged AcTEV protease both of which were collected in the elution fraction after displacement with imidazole. The presence of the LLO band in the eluate fractions in the stained gel, but not the anti-His blotted membrane, signifies that some of the LLO with cleaved His-tag likely bound non-specifically to the column. The lack of detectable His-AcTEV-LLO in the elution fraction also shows that the efficiency of the reaction was close to 100 %.

The pore-forming activity of LLO can be examined through its ability to lyse red blood cells (RBCs) [35, 36]. The release of hemoglobin from lysed RBCs can be detected by absorbance of 450 nm light. Serial dilutions of AcTEV cleaved recombinant LLO were incubated with RBCs and analyzed for lytic activity in comparison to an active LLO-His standard (C-terminally His-tagged LLO without an AcTEV cleavage site). A plot of the total LLO mass versus the Hemolytic Fraction revealed an HD_{50} of ~0.5 ng (data not shown), which was

comparable to the previously determined value of 1 ng for LLO-His, thus confirming the hemolytic activity of the AcTEV cleaved LLO. Further analysis of LLO activity as to its ability to deliver liposome-encapsulated OVA antigen to the cytosol of BMMs was assessed through an antigen presentation assay (Figure 3.3). Liposomes containing the same concentration of LLO-His along with OVA were run in parallel for comparison. LLO-His has been used in previous studies in our lab with successful cytosolic delivery of liposome cargo [25, 36-38]. The level of antigen presentation from cells treated with liposomes containing LLO was lower than with liposomes containing LLO-His. Based on this result, the concentration at which LLO was encapsulated in liposomes for the following studies were set at 0.3 mg/mL while the concentration of LLO-His previously used in successful liposomal vaccine formulations was typically 0.2 mg/mL [37]. Ni-liposomes containing LLO and OVA were incubated with YFP-His (~33 kDa) and unbound protein was removed by gel filtration, resulting in an average final nickel-chelating lipid:YFP molar ratio of 5.8:1. For liposomes of an average diameter of 100 nm, this equates to ~215 YFP molecules/ liposome.

pH-Sensitivity of YFP-Coated Liposomes

The effect of nickel-chelating lipid and the YFP-coating on the properties of the liposome membrane was analyzed by a pH-sensitivity assay. Three liposome formulations were prepared with the fluorescent dye HPTS and its quencher DPX co-encapsulated: uncoated liposomes (Uncoated Lip), uncoated liposomes containing nickel-chelating lipid (Uncoated Ni-Lip), and YFP-coated liposomes containing nickel-chelating lipid (YFP-coated Ni-Lip). The release of

HPTS in acidic buffer (pH 4.5) was greater than that in buffer at physiological pH (pH 7.4) for all liposome formulations tested (data not shown). However, the magnitude of HPTS released from Uncoated Lip in acidic buffer was ~2-3-fold greater than from YFP-coated Ni-Lip or Uncoated Ni-Lip. These results suggest that the pH-sensitivity of YFP-coated Ni-Lip is retained, though slightly compromised, in comparison to the Uncoated Lip formulation.

Internalization of YFP-Coated Liposomes in BMMs

The uptake in bone marrow-derived macrophages (BMMs) of three different liposome formulations was compared: Uncoated Lip, Uncoated Ni-Lip, and YFP-coated Ni-Lip. All liposomes tested were prepared with encapsulated LLO and OVA along with Texas Red-DHPE included in the lipid bilayer for monitoring the uptake by fluorescence intensity associated with cells. Results shown in Figure 3.4 are from studies in which cells were pulsed with 200 μM liposomes; a similar trend was seen with 100 μM liposomes (data not shown). The addition of YFP-His as a coating protein did not impede internalization: YFP-coated Ni-Lip were internalized at a level similar to that of Uncoated Ni-Lip. Liposomes that contained DGS-NTA (Ni) (Ni-Lip), however, were taken up significantly less than liposomes without the nickel-chelating lipid (Uncoated Lip). This decrease is evident in both the YFP-coated and Uncoated Ni-Lip suggesting that it is a property related to the DGS-NTA (Ni) lipid rather than to YFP-His coated onto the liposomes.

In the absence of LLO and OVA (i.e. empty liposomes), Ni-Lip were internalized to a similar extent as Uncoated Lip (Chapter 2). To investigate the

effect of LLO and OVA adsorption on the uptake of liposomes, empty liposomes were incubated with LLO and OVA in solution followed by purification via gel filtration. Analysis of the purified liposome fractions via SDS-PAGE revealed that both LLO and OVA adsorbed to the membranes of empty Ni-Lip to a greater extent than to empty Uncoated Lip (data not shown). Furthermore, the trend of the cell uptake of these empty liposomes with adsorbed LLO and OVA was similar to that seen in Figure 3.4 whereby Ni-Lip were taken up by BMMs less than Uncoated Lip (data not shown). Taken together, these results suggest that the decreased uptake for Ni-Lip and YFP-coated Ni-Lip seen in Figure 3.4 is a result of the adsorption of LLO and OVA to the liposome membrane during the encapsulation step.

YFP-Coated Liposomes Effectively Deliver OVA to the Cytosol of BMMs *in vitro*

LLO was added to the liposome formulation to enhance cytosolic delivery. To monitor the effect of the incorporation of nickel-chelating lipid into the liposome formulations on the LLO-mediated cytosolic delivery of the model antigen OVA, an *in vitro* antigen presentation assay was performed. BMMs were treated with serial dilutions of OVA-encapsulated liposomes with or without LLO. All formulations containing LLO (Uncoated Lip, Uncoated Ni-Lip, and YFP-coated Ni-Lip) showed enhanced antigen presentation that increased with OVA concentration, whereas liposomes without LLO (Uncoated Lip without LLO) exhibited levels of antigen presentation near the level of detection at all OVA concentration tested (Figure 3.5). The *in vitro* antigen presentation exhibited by YFP-coated Ni-Lip, though decreased relative to the Uncoated Lip (with both

OVA and LLO), showed enhanced antigen presentation compared to the Uncoated Lip without LLO. Therefore, these results indicate that nickel-liposomes encapsulating LLO and OVA may be coated with YFP-His and still exhibit an LLO-mediated cytosolic delivery of antigen *in vitro*.

YFP-Coated Liposomes Effectively Activate the Cellular Immune Response *in vivo*

Splenocytes from mice immunized with the liposomal vaccine formulations were stimulated *ex vivo* with OVA protein, and the resulting IFN-γ secretion was measured using an ELISA (Figure 3.6). Detection of IFN-γ in splenocyte samples from immunized mice is an indication that the immunization resulted in T cells specific for OVA peptide that upon binding the presented peptide in the *ex vivo* stimulation, led to a cell-mediated immune response. The cytokine IFN-γ was detected in mice immunized with YFP-coated Ni-Lip (2473 pg/ mL ±1282) at a level that was similar to that produced by mice immunized with the Uncoated Ni-Lip and Uncoated Lip vaccine formulations (1561 pg/ mL ± 616 and 3283 pg/ mL ± 1263, respectively). Splenocytes stimulated with irrelevant peptide (peptide sequence derived from Influenza NP) resulted in undetectable levels of IFN-γ; all cells stimulated with the positive control concanavalin A showed activation (data not shown). All splenocytes from mice immunized with the liposome formulations, regardless of the incorporation of nickel or YFP coating, stimulated similar levels of IFN-γ secretion. The activation of $CD8^+$ T cells was examined through the analysis of IFN-γ secretion after stimulation with the MHC Class I peptide SIINFEKL ($OVA_{257-264}$). However, levels of IFN-γ produced by SIINFEKL stimulation were at or below the level of detection.

YFP-Coated Liposomes Effectively Stimulate Cytotoxic T Lymphocytes *in vivo*

To examine OVA-specific CTL activity *in vivo*, immunized mice were injected via tail vein on day 9 with equal populations of CFSE-labeled naïve splenocytes pulsed with OVA peptide ($CFSE_{HIGH}$) and differentially CFSE-labeled naïve splenocytes pulsed with an irrelevant peptide ($CFSE_{LOW}$). On day 10, the immunized mice were euthanized and splenocytes isolated for evaluation in the flow cytometer. The production of OVA-specific CTLs results in the lysis of OVA peptide loaded $CFSE_{HIGH}$ cells, but not the cells presenting the NP peptide. This, in turn, results in a decrease in the peak height for the labeled cells pulsed with OVA peptide. As shown in Figure 3.7A, a decrease in the peak height for cells pulsed with OVA peptide ($CFSE_{HIGH}$, right peak), but not in the peak representing cells pulsed with the irrelevant peptide ($CFSE_{LOW}$, left peak) was seen in the immunized, but not in the naïve mice. The level of OVA-specific lysis was similar between uncoated liposomes (30.1% ± 1.3 for Uncoated Lip and 26.2% ± 5.8 for Uncoated Ni-Lip) regardless of whether nickel-chelating lipid was included in the formulation (Figure 3.7B). Coating the liposomes with YFP-His resulted in OVA-specific lysis (18.3% ± 5.4) similar to that of the uncoated formulations with no significant differences between the groups. These results show that coating liposomes with YFP-His does not inhibit the ability of LLO-containing liposomes to deliver the OVA antigen to the cytosol of APCs for activation of CTL.

YFP-Coated Liposomes Stimulate an anti-OVA IgG Response

The ability of YFP-coated Ni-Lip to stimulate the production of antibodies against the encapsulated OVA antigens was evaluated by monitoring the OVA-

specific total IgG in serum by ELISA. The two uncoated liposome formulations (Uncoated Lip and Uncoated Ni-Lip) (168 ± 91 and 83 ±34, respectively) stimulated anti-OVA IgG titers slightly above background (naïve = 35), whereas the YFP-coated Ni-Lip produced anti-OVA titer that was significantly higher (6559 ± 2383) ($p<0.01$) than that of the uncoated liposomes (Figure 3.8).

YFP-Coated Liposomes Stimulate an anti-YFP IgG Response

The immunogenicity of YFP-His was monitored by the detection of anti-YFP IgG in the sera of immunized mice using a total IgG ELISA. Both uncoated formulations resulted in anti-YFP IgG titers (100 ± 14 for Uncoated Lip, 69 ± 33 for Uncoated Ni-Lip) that were near the level of detection (as established by the naïve mouse: naïve anti-YFP IgG titer = 128); however, the anti-YFP titer was about 25-fold higher in mice immunized with YFP-coated Ni-Lip (3119 ± 1522) than that measured in mice immunized with the uncoated formulations (Figure 3.9).

YFP-Coated Liposomes Pretreated with anti-YFP MAb Deliver OVA to the Cytosol of BMMs *in vitro*

The impact of pre-existing YFP-specific antibodies on the delivery properties of the YFP-coated nickel liposomes was investigated *in vitro* through a cell uptake and antigen presentation assay. Pretreating the YFP-coated Ni-Lip with anti-YFP MAb had little effect on the uptake level of the liposomes as both liposome formulations were taken up to a similar extent (Figure 3.10A).

In an *in vitro* antigen presentation assay, OVA was delivered to the cytosol by YFP-coated Ni-Lip pretreated with anti-YFP MAb significantly less than the

non-pretreated formulation; however, the level of antigen presentation from the liposomes with the anti-YFP MAb pretreatment was significantly enhanced over the Uncoated Lip without LLO (Figure 3.10B). These results suggest that in the presence of YFP-specific monoclonal antibodies, YFP-coated Ni-Lip retain their LLO-mediated ability to deliver OVA to the cytosol of BMMs *in vitro.*

The presence of YFP-Specific MAb Does Not Prevent YFP-Coated Liposomes from Eliciting a Cellular and Humoral Immune Response *in vivo*

To investigate the effect of pre-existing YFP-specific antibodies on the delivery properties of the liposomal vaccine *in vivo*, a liposome formulation was prepared at the time of immunization consisting of YFP-coated Ni-Lip that had been pretreated with anti-YFP MAb. The assays for IFN-γ, CTL lysis, and anti-OVA IgG were carried out as described above in parallel with samples from mice immunized with the other liposome formulations. The results from each of these assays demonstrated that YFP-coated Ni-Lip pretreated with anti-YFP MAb performed similar to YFP-coated Ni-Lip *in vivo:* The level of IFN-γ production, CTL lysis, and anti-OVA IgG production from mice immunized with YFP-coated Ni-Lip pretreated with anti-YFP MAb was similar to that from mice immunized with YFP-coated Ni-Lip.

DISCUSSION

The demand for more effective vaccines has prompted research into the optimization of liposomal vaccine delivery systems through the incorporation of multiple antigens, adjuvants, and targeting moieties. However, it is essential that these agents do not impair the delivery of antigen to the APCs. We have

previously shown that co-encapsulation of LLO and antigen in pH-sensitive liposomes leads to a robust immune response in mice in which antigen-specific antibodies and CTLs are generated [24, 37, 39]. Here a His-tagged protein antigen associated with the membrane of liposomes containing nickel-chelating lipid, DGS-NTA (Ni), was tested for its ability to induce an immune response to itself without compromising the immune response to the encapsulated antigen. The liposomal vaccine formulation with membrane-associated His-tagged YFP maintained its ability to deliver encapsulated OVA to the cytosol of APCs for efficient CTL activation. In addition, the YFP-coated liposomal vaccine stimulated the humoral immune response as shown by the production of antibodies specific for OVA and YFP-His.

The ability of a protein to trigger an immune response is dependent on its ability to be internalized, processed, and presented by APCs such as macrophages [11]. Therefore, examination of the *in vitro* effect of a coating protein on a liposomal vaccine began by examining its effect on the liposome uptake by BMMs. In Chapter 2, it was shown that the incorporation of DGS-NTA (Ni) at a low mole percentage of total phospholipids in negatively-charged empty liposomes did not affect cell uptake. In the present chapter, however, Ni-Lip in which LLO and OVA were co-encapsulated were taken up by BMMs significantly less than liposomes without the nickel-chelating lipid. The main difference between the two studies was the use of empty liposomes in Chapter 2 and the co-encapsulation of LLO and OVA in the present chapter. The above-mentioned results in combination with the decreased uptake of empty Ni-Lip with adsorbed

LLO and OVA suggest that this difference in uptake is a result of the adsorption of LLO and OVA on the lipid membrane. This observation is not surprising given that non-specific adsorption is relatively common for encapsulated proteins and can be difficult to avoid [15, 40, 41]. It is possible that the heightened adsorption of proteins to the nickel-liposomes shielded the negatively charged liposomes, as it has been shown that anionic liposomes can be internalized 10-fold more than neutral liposomes [42]. Although the ability of the adsorbed protein to impact the liposome's immunogenicity cannot be ruled out, a study performed by Shek and Sabiston comparing liposomes with encapsulated bovine serum albumin (BSA) in addition to either membrane adsorbed BSA or trypsinized liposomes (removing adsorbed protein) did not find a difference in antibody response to BSA, suggesting that the adsorbed antigen has negligible influence on the immunogenicity of the encapsulated antigen [41].

Cytosolic delivery of antigen is a major pathway for CTL activation via MHC class I presentation. The evaluation of our delivery vehicle with respect to this property was performed using an *in vitro* antigen presentation assay. Co-encapsulation of LLO with OVA in uncoated pH-sensitive liposomes led to enhanced MHC class I-dependent OVA antigen presentation over liposomes without LLO (Figure 3.5), and as previously reported [24]. This enhancement over the non-LLO negative control is maintained with the addition of the YFP-coating. The slight decrease in presentation by YFP-coated Ni-Lip compared to Uncoated Lip may be explained by the decreased pH-sensitivity reported or the decreased internalization seen in Figure 3.4. These promising *in vitro* results

demonstrating the LLO-mediated cytosolic delivery of OVA encapsulated in YFP-coated Ni-Lip provided grounds for investigating their use *in vivo.*

The ability of OVA encapsulated in LLO-containing YFP-coated Ni-Lip to stimulate a cell-mediated immune response *in vivo* was first evaluated through the detection of the cytokine, IFN-γ. Detection of IFN-γ confirms that YFP-coated liposomes possess the capacity to stimulate a cell-mediated immune response to the encapsulated OVA antigen [43]. IFN-γ was detected at similar levels in the splenocytes of mice immunized with Uncoated Lip, Uncoated Ni-Lip, or YFP-coated Ni-Lip after *ex vivo* stimulation with the OVA protein In contrast to traditional protein vaccines, which primarily activate humoral immunity, LLO-liposomal vaccines have been shown to stimulate both humoral and cellular immunity, resulting in a more robust immune response [24, 37, 39]. The IFN-γ results demonstrate that the attachment of YFP-His to Ni-liposomes did not affect their ability to stimulate cellular immunity. Furthermore, immunization with YFP-coated Ni-Lip resulted in OVA-specific CTL activation at levels statistically indistinguishable from that of uncoated liposomes (Figure 3.7), suggesting that *in vivo* delivery of OVA to the cytosol of APCs was not adversely affected by YFP-His coating of liposome surface.

The ability of both liposomal membrane-associated and encapsulated antigen to stimulate the production of antibodies against itself is well documented [2, 13, 44, 45]. However, many studies have found that both a prime and boost injection are required to obtain a measurable response [22, 24]. Unsurprisingly, a prime-only immunization with Uncoated Lip or Uncoated Ni-Lip resulted in

weak anti-OVA IgG titers (Figure 3.8). Conversely, immunization with YFP-coated Ni-Lip effected a 100-fold enhancement in anti-OVA antibody titer. This apparent synergistic effect seen with OVA and YFP-His has been observed elsewhere in the co-delivery of multiple antigens or antigens with adjuvants [8, 46, 47]. For example, Alving et al. demonstrated an enhanced antibody response against lipid-encapsulated malaria peptide conjugate when lipid A was co-encapsulated in liposomes [47]. Similarly, Yan et al. showed a significant increase in the production of antigen-specific antibodies when the HIV antigens p24 and pNef were co-delivered with lipid nanoparticles versus the nanoparticle delivery of single antigens [8]. Although the exact mechanism in which multiple antigen delivery can increase the antibody production to an antigen is unclear, it is possible that the surface presentation and T cell binding of one antigen can stimulate the production of cytokines that enhance the antibody production to the other antigen. Thus, combined vaccines delivering multiple antigens have the benefit of stimulating enhanced antibody responses, therefore requiring a lower antigenic dose and possibly fewer injections. Taken together, the combined results from the CTL, IFN-γ secretion, and OVA total IgG assays demonstrate the feasibility of coating liposomal delivery systems with His-tagged protein via the Ni-His interaction without impairing the *in vivo* delivery efficiency of the encapsulated agent.

Antibody responses generated against the YFP-His antigen were also monitored and indeed anti-YFP antibodies were detected in the sera of mice immunized with YFP-coated liposomes at a titer about 25-fold higher than

background (Figure 3.9). Anti-YFP antibodies were generated in this study with 2 mole percent of DGS-NTA (Ni) and a 1:5 molar ratio of YFP-His:DGS-NTA (Ni) lipid (used during the binding step before liposome purification). An increase in either of these values can lead to an increase in coating density (as shown in Chapter 2) which should further enhance the antibody titer [44, 48]. These results suggest that the addition of protein to the external membrane of LLO-liposomes through the Ni-His interaction not only results in the successful delivery of the encapsulated antigen to the proper subcellular compartments, but also elicits an immune response to the coated antigen. Although the reported anti-YFP antibody titer is less than the reported anti-OVA titer, it is important to note that the antibody titers cannot be directly compared. The molar amount of OVA injected during the immunization was calculated to be about 3-fold more than YFP-His. In addition, discrepancies between the assays for detection of the different antibodies may occur due to differences in plate binding affinity of OVA versus YFP-His proteins or in substrate development.

The study conducted here involved a prime-only immunization; however, further optimization studies may necessitate subsequent booster injections in order to obtain a more desired response. Research has shown that multiple injections of therapeutic proteins as well as drug carriers can result in the development of pre-existing immunity to components of the delivery system in which subsequent injections lose their efficacy due to their accelerated blood clearance [49-51]. In a simplified approach, we investigated the effect of pre-existing YFP-specific IgG's on the delivery properties of encapsulated OVA by

examining the formulation of YFP-coated Ni-Lip that were pretreated with anti-YFP MAb. An *in vitro* antigen presentation assay revealed that although the anti-YFP MAb pretreatment compromised the level of MHC class I-dependent OVA antigen presentation, it did not inhibit the enhanced antigen presentation due to the effect of LLO (Figure 3.10). When the humoral and cell-mediated immune response elicited by the YFP-coated Ni-Lip pretreated with MAb was examined *in vivo*, the levels of IFN-γ, CTL, and OVA-specific IgG were found to be similar to that of YFP-coated Ni-Lip (Figure 3.11). These results suggest that pre-existing levels of YFP-specific IgG would not influence the immune response to encapsulated antigen *in vivo*. As acknowledged above, these results merely provide insight into the impact of the presence of antibodies against the surface component of vaccines on the delivery performance of our liposomal vaccine delivery system. As monoclonal antibodies are considered to be humanized antibodies, the impact of polyclonal antibodies and their isotypes (i.e. IgM, IgA) should be evaluated for a more thorough analysis.

The use of His-tagged YFP allowed for its simple purification as well as its facile association with liposomes containing nickel-chelating lipid. Concerns have been expressed regarding the deleterious effects of the nickel; however, the greatest threat of nickel toxicity is through chronic exposure to airborne nickel-containing compounds [52, 53]. Additionally, the lowest reported dose of nickel-containing compounds injected intramuscularly with toxic effects is around 10 mg/kg body weight; this toxic dose is ~1000 times higher than the amount that we intravenously injected in mice in our study [54, 55]. Although the health

concerns associated with nickel should not be ignored, it appears likely that it can be used in a safe manner in the drug delivery field.

CONCLUSIONS

We have shown that we can attach YFP-His to liposomes containing nickel-chelating lipid via the relatively straightforward Ni-His interaction without greatly affecting the delivery properties of the LLO-containing liposomal vaccine carrier. YFP-coated pH-sensitive liposomes containing OVA and LLO effectively delivered OVA antigen to the cytosol of APCs for presentation and elicitation of CTL activation. These results support the use of this strategy for attaching various proteins or peptides such as other antigens or antibodies to liposomes in a manner that does not require extensive manipulation. The co-delivery of both YFP-His and OVA antigens led to the enhanced production of anti-OVA and anti-YFP IgGs over that measured from delivery of OVA alone, thus furthering the case for the potential utility of this delivery formulation with biologically relevant antigens for a multiple antigen combination vaccine approach.

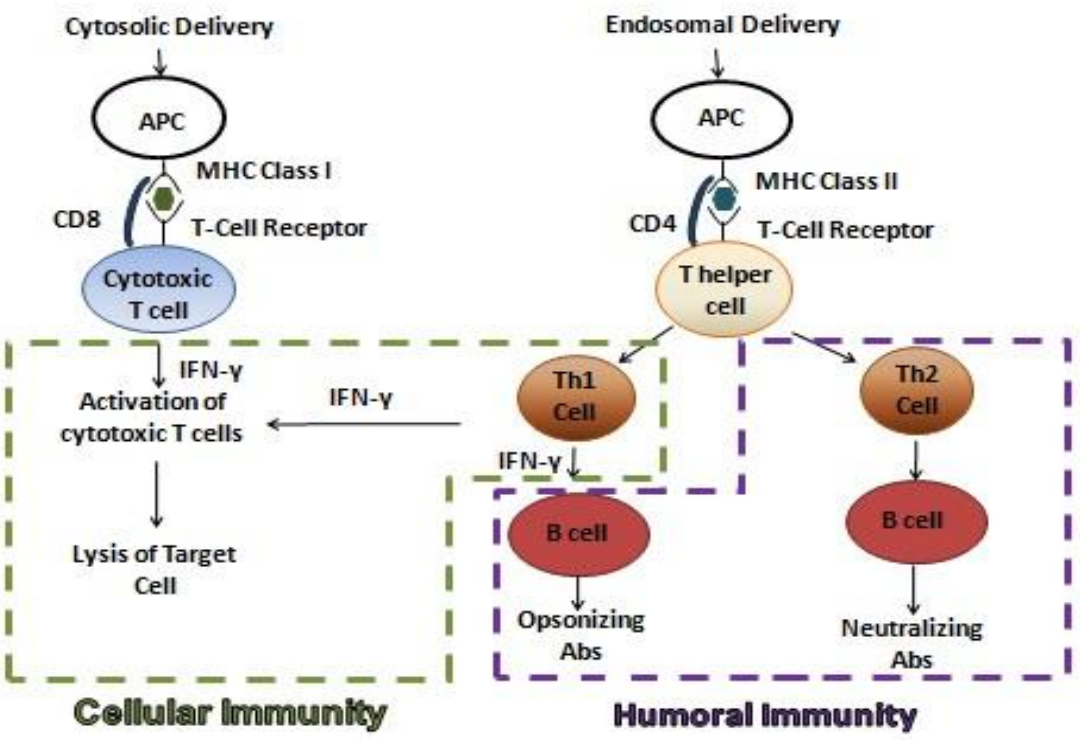

Figure 3.1: Overview of the Adaptive Immune Response

Agents that reach the cytosol of APCs (i.e. intracellular pathogens) are typically displayed on the surface by MHC class I molecules for recognition and binding by $CD8^+$ cytotoxic T cells. This can result in the activation of cytotoxic T cells responsible for the lysis of the antigen-displaying cell. Agents that only reach the endosomes of APCs are usually displayed at the surface by MHC class II molecules for recognition and binding of $CD4^+$ T helper cells. This can result in the activation of Th1 cells which secrete IFN-γ and can help activate cytotoxic T cells and lead to the production of opsonizing antibodies. CTL and Th1 cell activation are part of the cellular immune response. Alternatively, MHC class II presentation can lead to Th2 cell activation and resultant production of neutralizing antibodies. The production of opsonizing and neutralizing antibodies is part of the humoral immune response.

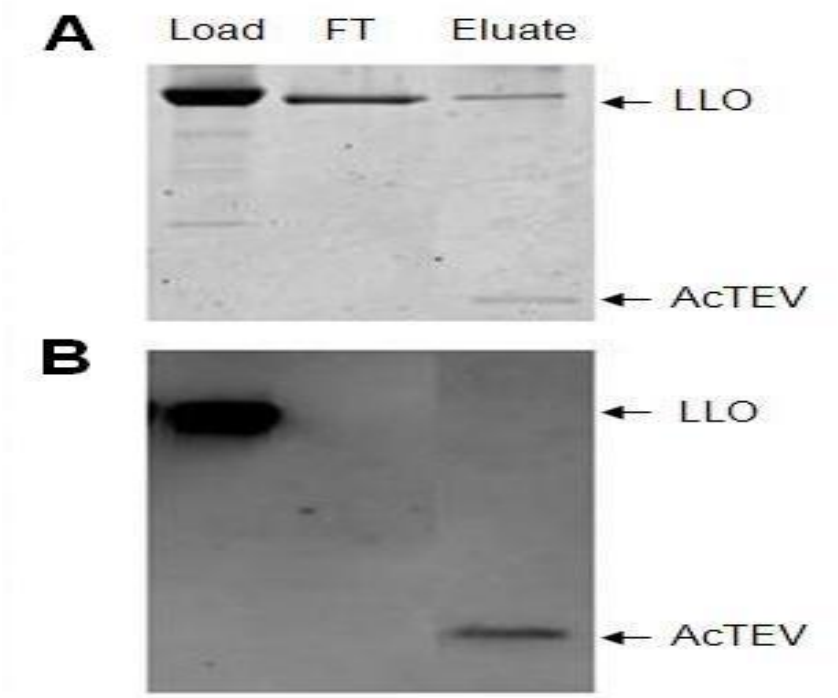

Figure 3.2: Production of LLO with AcTEV-Cleavable His-Tag

(A) Representative SDS-PAGE and (B) anti-His Western blot of His-AcTEV-LLO purification. *Load* indicates fraction of His-AcTEV-LLO before incubation with AcTEV protease. His-AcTEV-LLO and AcTEV were incubated overnight and the reaction was purified using Ni-NTA resin. Flow-through *(FT)* indicates fraction collected after incubation with Ni-NTA resin without affinity for resin. *Eluate* indicates fraction collected using buffer containing 400 mM imidazole to displace bound protein.

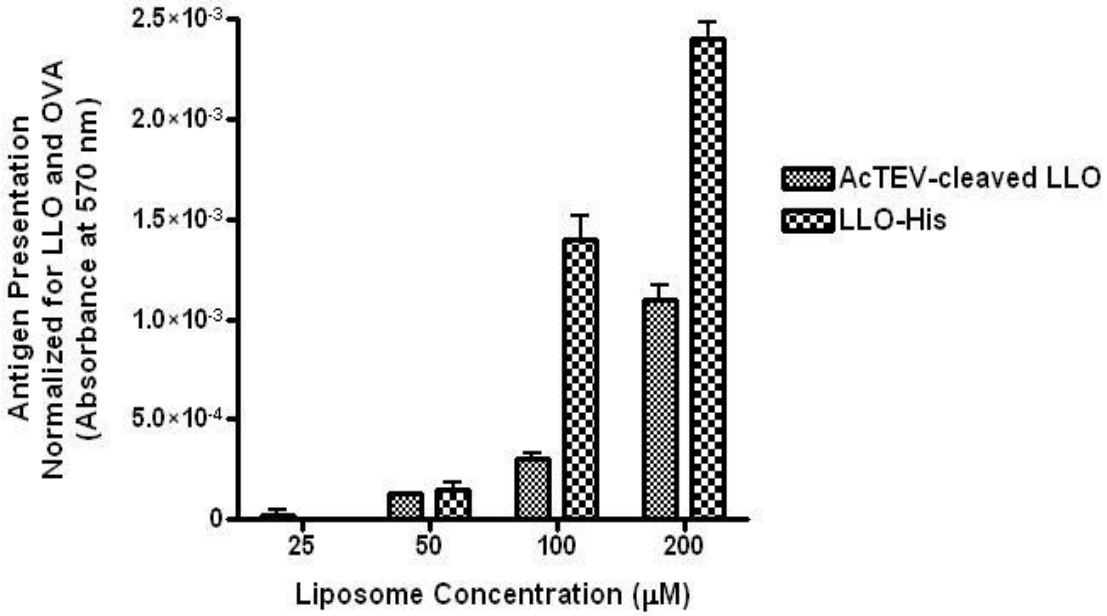

Figure 3.3: Confirmation of AcTEV-Cleaved LLO Activity

BMMs were pulsed with the above concentrations of pH-sensitive liposomes containing 20 mg/mL OVA and 0.2 mg/mL LLO (AcTEV-cleaved LLO or C-terminally His-tagged LLO (LLO-His)). The ability of OVA to be delivered to the cytosol was indirectly assessed by BMM MHC class I presentation of OVA SIINFEKL peptides to B3Z cells, which are in turn stimulated to produce β-galactosidase. The β-gal-mediated hydrolysis of CPRG to the chlorophenol red product was then measured by its absorbance of 570 nm light. Values are normalized for LLO and OVA content. Data for both experiments are the mean ± SD.

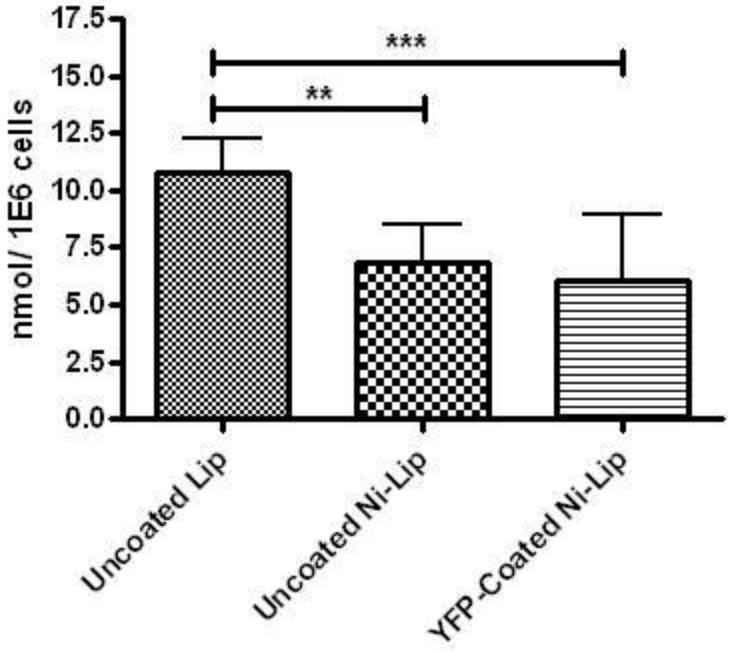

Figure 3.4: Internalization of LLO/OVA-Encapsulated Liposomes is Decreased with Nickel-Liposomes

BMMs were pulsed with uncoated liposomes (Uncoated Lip), uncoated nickel-containing liposomes (Uncoated Ni-Lip), or YFP-coated nickel-containing liposomes (YFP-Coated Ni-Lip) containing LLO and OVA for 4 h. Cells were washed, resuspended, and the amount of cell uptake per million cells monitored by Texas Red-DHPE fluorescence. Data are the average of three independent experiments and are displayed as the mean ± SD. **p<0.01, ***p<0.001

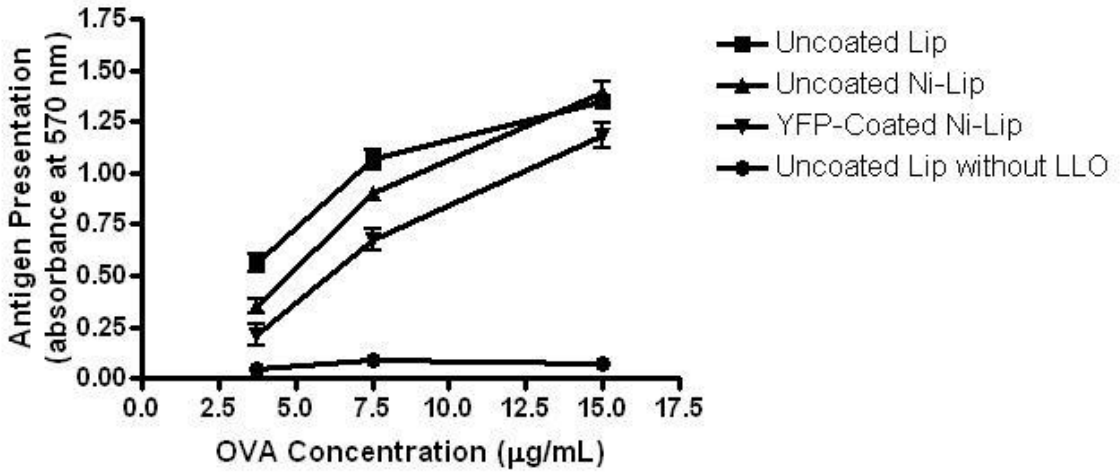

Figure 3.5: YFP-Coated Nickel-Liposomes Retain their Ability to Deliver OVA to the Cytosol of BMMs for Presentation by MHC Class I Molecules

BMMs were pulsed for 2 h with a 2-fold serial dilution of Uncoated Lip (■), Uncoated Ni-Lip (▲), or YFP-coated Ni-Lip (▼), all encapsulating LLO and OVA. Uncoated liposomes with OVA alone encapsulated (●) were also tested. Antigen presentation was measured as described in Figure 3.3. Each data point represents the mean of quadruplicates ± SD. $p<0.001$ for all samples and concentrations, which were compared against Uncoated Lip without LLO (●).

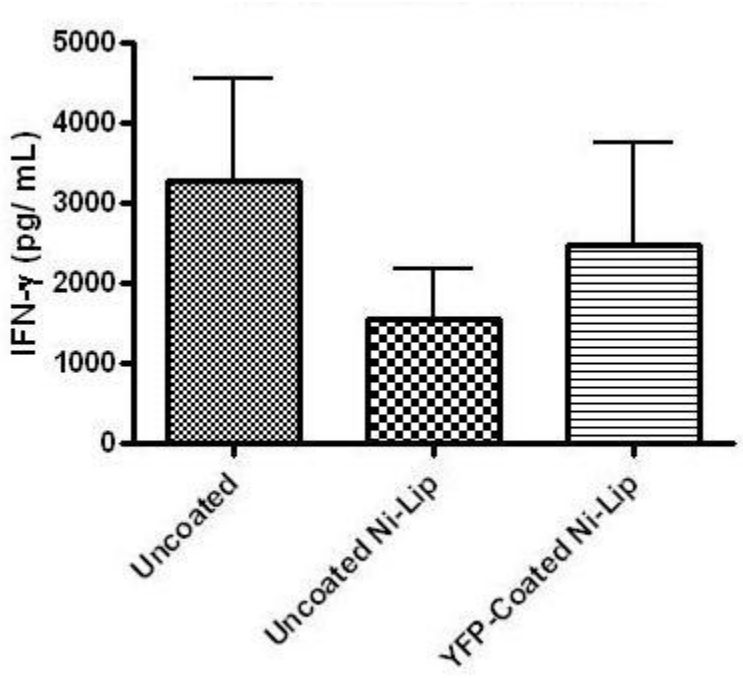

Figure 3.6: YFP-Coated Liposomes Stimulate a Cell-Mediated Immune Response

Mice were immunized subcutaneously with liposomes normalized to OVA content (15 µg) and euthanized on day 10. Splenocytes were isolated and stimulated *ex vivo* with OVA protein for 72 h. Production of IFN-γ was detected via ELISA. The mean ± SEM is shown (n=5-6 mice per group).

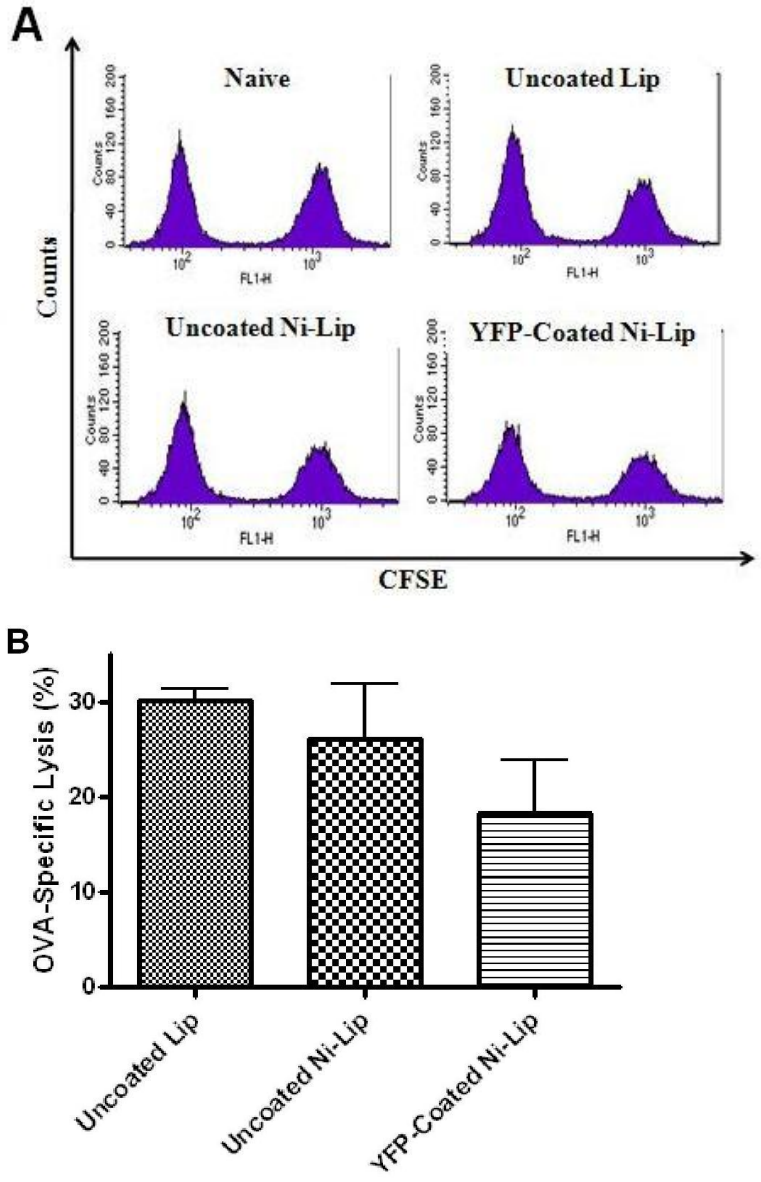

Figure 3.7: YFP-Coated Liposomes Produce CTL Activity *in vivo* Similar to that of the Uncoated Formulation

Mice were immunized subcutaneously with liposome formulations encapsulating LLO and OVA. On day 9, an equal ratio of OVA peptide-pulsed ($CFSE_{HIGH}$) and non-specific peptide-pulsed ($CFSE_{LOW}$) cells was prepared and intravenously injected into the immunized mice. Splenocytes were analyzed for the presence of peptide-specific CTLs. (A) Representative histograms from flow cytometric analysis. (B) Results from (A) were used to calculate OVA-specific lysis. Each data point represents the mean ± SEM of each group (n=6-7). Statistical analysis revealed no significant differences between groups.

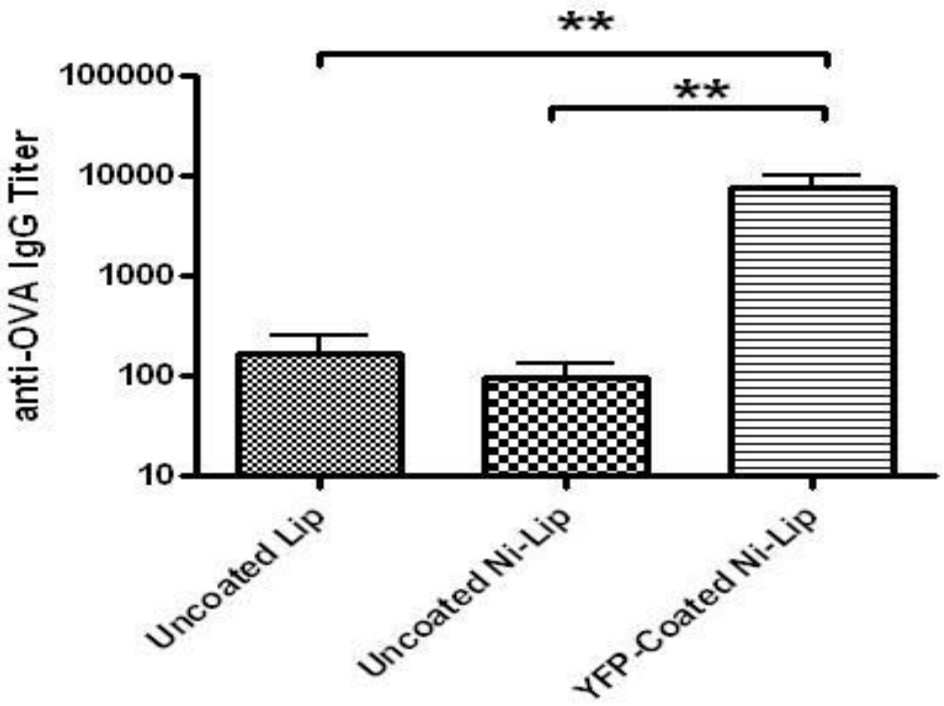

Figure 3.8: YFP-Coated Liposomes Stimulate an Enhanced anti-OVA IgG Response

Mice were immunized as described in Figure 3.7 and sera monitored on day 10 for the presence of anti-OVA IgG by ELISA. Titer was calculated as the dilution factor yielding an absorbance of 0.5 using non-linear regression analysis of the plotted ELISA values. Each data point represents the mean ± SEM of each group (n=5-6). **p<0.01

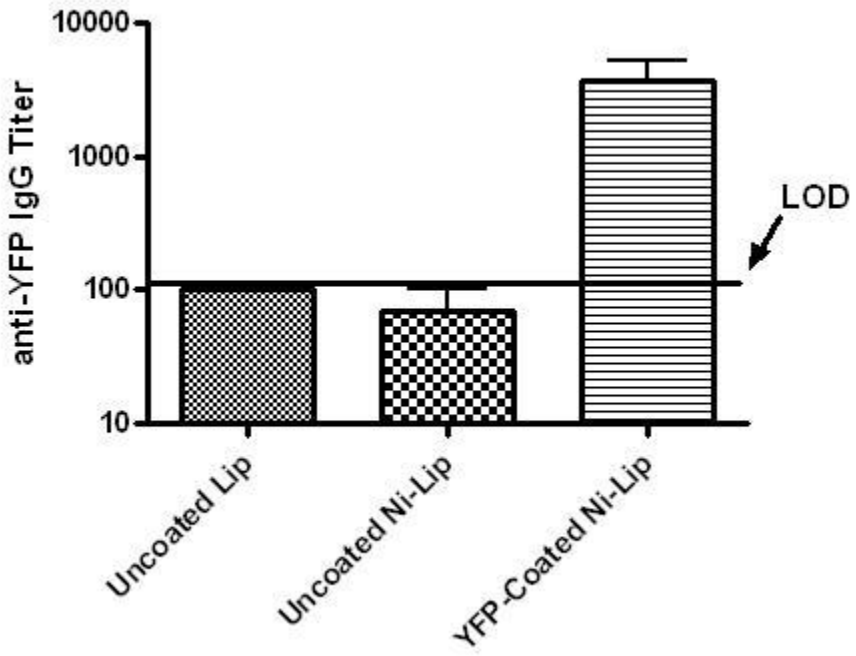

Figure 3.9: YFP-Coated Liposomes Can Elicit an anti-YFP Total IgG Response

Mice were immunized as described in Figure 3.7,Figure 3.7 sera was monitored on day 10 for the presence of anti-YFP IgG by ELISA, and the titer calculated as in Figure 3.8. Each data point represents the mean ± SEM of each group (n=4-6). LOD=limit of detection (as established by the naïve mouse)

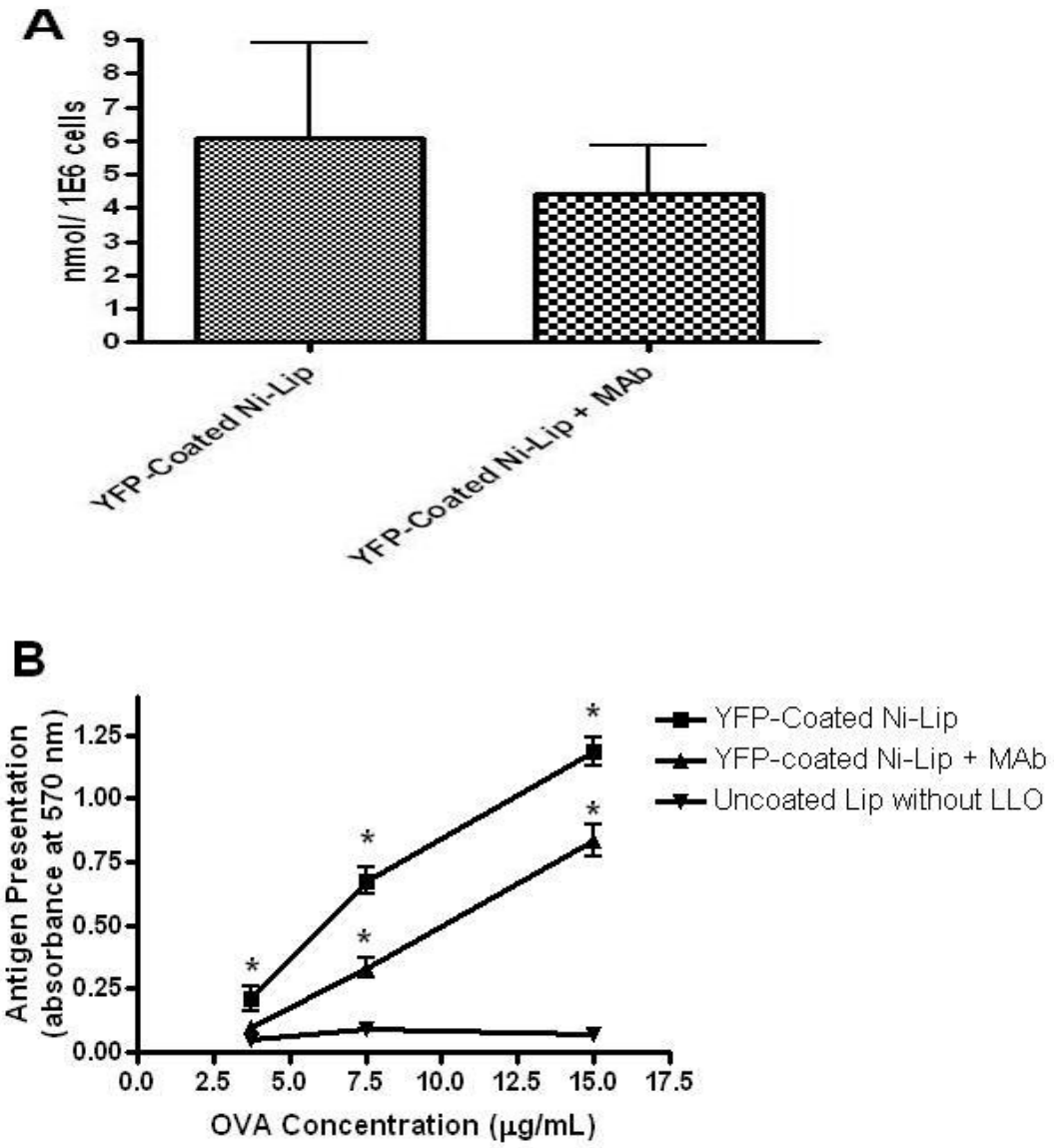

Figure 3.10: YFP-Coated Nickel-Liposomes Pretreated with anti-YFP MAb Retain their Ability to Deliver OVA to the Cytosol of BMMs for Presentation by MHC Class I Molecules

BMMs were pulsed for 1 h with YFP-coated nickel-liposomes with (YFP-Coated Ni-Lip + MAb) or without (YFP-Coated Ni-Lip) pretreatment with anti-YFP MAb. Both liposome formulations contained encapsulated LLO and OVA. The level of cell uptake (A) was determined as described in Figure 3.4 while the antigen presentation (B) was measured as described in Figure 3.5. For the antigen presentation assay, uncoated liposomes with OVA alone encapsulated (Uncoated Lip without LLO) were also tested. Data are displayed as the mean ± SD. * signifies significant difference ($p<0.01$) in comparison to Uncoated Lip without LLO.

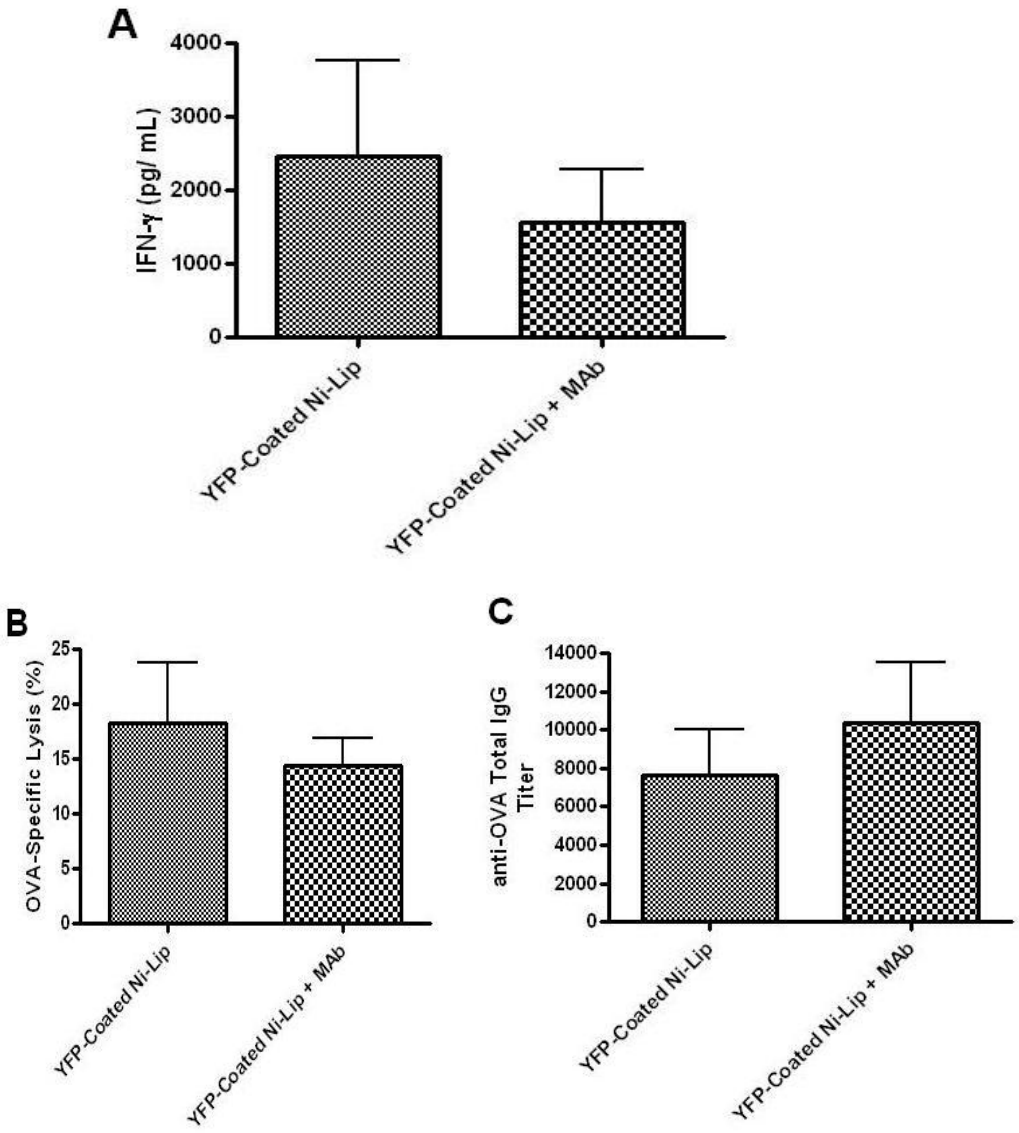

Figure 3.11: YFP-Coated Nickel-Liposomes Pretreated with anti-YFP MAb Effect an *in vivo* Response Seen with YFP-Coated Nickel Liposomes

Mice were immunized subcutaneously with liposome formulations encapsulating LLO and OVA. (A) IFN-γ, (B) OVA-specific lysis, and (C) anti-OVA Total IgG titer were determined as described in previous figures. Each data point represents the mean ± SEM of each group (n=4-7). Statistical analysis revealed no significant differences between groups.

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CHAPTER 4

TARGETING THE HER2 RECEPTOR WITH TRUNCATED APOLIPOPROTEIN B-ANTI-HER2 ANTIBODY FUSION PROTEIN

SUMMARY

Tumor-targeted therapy is an attractive method for delivering therapeutic agents to cancer cells with minimal cytotoxicity to normal tissues. Various targeting ligands have been conjugated to the surface of liposomes and have been demonstrated to selectively deliver encapsulated agents to the tumor site. In this study, an alternative method is examined in which protein fused to a truncated form of apolipoprotein B (apoB) hypothetically associates with liposome membranes through hydrophobic interactions between apoB and the lipid bilayer. To examine this approach, a single-chain Fv fragment of a HER2 antibody (αHER2) was fused to the amino-terminal 23% of apolipoprotein B (apoB23). Protein coating studies revealed that apoB23-αHER2 bound liposomes at a density of ~ 12 apoB23-αHER2 per 200 nm liposome. Measurement of the release of the encapsulated fluorophore HPTS revealed that the association of apoB23-αHER2 with the liposome membrane does not induce leakage of its contents nor does it affect the pH-sensitivity of the liposome formulation. Flow cytometry showed that FITC-labeled apoB23-αHER2 can bind HER2-overexpressing cell lines (BT-474 and SK-BR-3) 5-fold more than a low

HER2-expressing cell line (MCF7). However, the selectivity for HER2 was not maintained when apoB23-αHER2 was associated with liposomes. Potential explanations for the lack of selectivity seen with the apoB23-αHER2-coated liposomes are discussed.

INTRODUCTION

The administration of chemotherapeutics in a patient is often accompanied by various side effects due to the cytotoxic effect of the drug on healthy tissues. A promising method of alleviating this problem is through the selective delivery of therapeutics to cancer cells. By specifically targeting a drug to a particular cell population, the amount delivered to target cells is increased and systemic exposure decreased, thus improving the overall therapeutic index of the drug [1]. The overexpression of various receptors and antigens on tumor cells has prompted the development of targeted delivery systems with affinity to such tumor cell markers. The ability of delivery systems such as drug conjugates, lipid-based vesicles, and polymeric nanoparticles to target tumor cells has been demonstrated through the utilization of various targeting moieties [2-4]. Liposomes have become an attractive vehicle for cancer therapy due to their ability to passively accumulate at the tumor site by what is known as the 'Enhanced Permeability and Retention' (EPR) effect [5]. In addition, liposomes can deliver a relatively large amount of encapsulated drug that is not only protected from enzymatic degradation, but also restricted from interacting with healthy cells en route to the tumor site [6, 7]. Targeted delivery of liposome-

encapsulated agents has been demonstrated through the utilization of various targeting moieties including peptides, vitamins, and antibodies [8-10].

Several methods of linking targeting moieties to the liposome surfaces have been utilized [11-14]. Many commonly employed conjugation techniques, though highly efficient, can be tedious, requiring preliminary steps for the modification of the protein and lipid prior to their conjugation. Such procedures are not only time-consuming, but may also raise concerns about the final physicochemical state and/or biological activity of the protein or delivery vehicle [15-17]. A potential alternative approach is the fusion, via molecular biological techniques, of the targeting molecule to a protein capable of associating with the liposome membrane through hydrophobic interaction. As discussed in Chapter 2, apolipoprotein B-100 (apoB-100) is a large endogenous protein that covers the surface of the cholesterol-carrying low-density lipoproteins (LDL) and maintains its structural integrity. Due to the amphipathicity of apoB-100, its hydrophobic domains associate with the various lipid layers of the LDL particle while the hydrophilic domains remain exposed to the aqueous plasma [18, 19]. While the use of apoB-100-covered liposomes to target cells expressing LDL receptors or scavenger receptors has proven successful, the extraction from LDL and stability of this 550 kDa monomeric protein can be problematic, thus limiting its use [20, 21]. An attractive alternative to full-length apoB-100 is the use of a recombinant truncated form which lacks the C-terminal LDL receptor binding site but retains the ability to associate with lipid membranes [22-25]. Utilization of this apoB fragment as a fusion protein with targeting moieties offers a potential method for

associating the targeting ligand with the liposome surface. The apoB fusion protein offers the advantages of producing the ligand and linker as a single molecule and eliminating the protein and lipid modification steps that are required in many conjugation techniques, thus simplifying the coupling procedure as a whole.

To investigate this approach, we examined the use of a fusion protein, apoB23-αHER2, consisting of the N-terminal 23% of amino acids of apoB-100 fused to the single-chain Fv fragment of an anti-HER2 antibody for targeted liposome delivery. Single-chain Fv antibody fragments consist of the light and heavy variable domains of an antibody connected by a polypeptide linker [26]. The single polypeptide chain of the Fv fragment allows for its production as a single fusion protein with truncated apoB and eliminates the need for extra preparation steps such as fragmentation or conjugation as has been the case with other antibody fragments [3, 27]. HER2 is a member of the class I family of receptor tyrosine kinases involved in the regulation of cell growth, survival, and differentiation [28, 29]. Overexpression of HER2 occurs in many breast, ovarian, prostate and gastric cancers and is predictive of a poor prognosis [30, 31]. The development of HER2-specific monoclonal antibodies such as trastuzumab (Herceptin^{TM}) has demonstrated therapeutic efficacy through inhibition of the cell signaling pathway responsible for tumor cell growth [28, 29, 32, 33]. In addition, HER2 has been utilized as an attractive marker for the selective delivery of chemotherapeutic agents [2, 29, 34]. Furthermore, the conjugation of HER2-specific antibody fragments to the surface of liposomes has enabled the drug

delivery vehicles to be selectively bound and internalized by HER2-overexpressing cells, leading to cell-specific cytotoxicity [27, 35].

In this chapter, the development of a HER2-targeted liposome delivery system is discussed in which the single-chain HER2 antibody fragment is associated with the liposome membrane through hydrophobic interaction of the apoB23-αHER2 fusion protein. The ability of apoB23-αHER2 to associate with liposomes as well as the effect it had on the lipid membrane stability and pH-sensitivity are investigated. In addition, the ability of apoB23-αHER2-coated liposomes to target HER2-overexpressing cell lines was explored.

MATERIALS AND METHODS

Materials

All chemicals and reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. The fluorophore 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) and its quencher p-xylene-bispyridinium bromide (DPX), and all tissue culture media were purchased from Invitrogen (Carlsbad, CA). 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol was purchased from Calbiochem (La Jolla, CA).

Cell Lines

BT-474, SK-BR-3, and MCF7 cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). BT-474 cells were maintained

in ATCC Hybri-Care Medium supplemented with 1.5 g/ L sodium bicarbonate, MCF7 cells in Eagle's MEM supplemented with 0.01 mg/mL bovine insulin, and SK-BR-3 cells in McCoy's 5a. All media were additionally supplemented with 10% heat-inactivated fetal bovine serum, 100 µg/ mL streptomycin, and 100 U/ mL penicillin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. SF9 insect cells were grown in SF900-II serum-free media at 28°C in a shaker/ incubator.

Preparation of ApoB23-αHER2 and ApoB19.8

The pFastBac plasmids containing the gene for apoB23-αHER2 or for apoB19.8 were generous gifts from Dr. Gregory Shelness (Wake Forest University, Winston-Salem, NC). Both the apoB23-αHER2 and apoB19.8 constructs were transformed into One Shot Mach 1 T1 *E.coli* cells (Invitrogen) by the heat-shock method and the plasmid DNA purified using the Miniprep kit (Qiagen) per the manufacturer's instructions. After the DNA sequences were confirmed (University of Michigan DNA Sequencing Core), the purified DNA for apoB23-αHER2 or apoB19.8 were transformed into DH10Bac competent cells (Invitrogen) by the heat-shock method and used to inoculate LB Agar plates containing 50 µg/ mL kanamycin, 7 µg/ mL gentamycin, 10 µg/ mL tetracycline, 300 µg/ mL X-gal, and 40 µg/ mL isopropyl β-D-thiogalactopyranoside (IPTG) (37°C, 48 h, 5% CO_2). Colonies with a white phenotype were selected and the baculovirus shuttle vector (bacmid) was extracted as instructed in the Bac-to-Bac Baculovirus Expression System manual (Invitrogen). The recombinant bacmid DNA was transfected into 9 x 10^5 SF9 insect cells/ well of a 6-well plate per the

manufacturer's instructions and viral supernatant was harvested 72 h later. The optimal multiplicity of infection (MOI) and time of harvest was determined empirically and the supernatant from the culture with the best apoB23-αHER2 or apoB19.8 expression amplified by infecting a 2 L culture using the determined optimal MOI and time of harvest. Cells were pelleted by spinning at 12,000 x g for 20 min. at 4°C and to the supernatant were added: 0.05% NaN_3, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ mL pepstatin A, and 1 µg/ mL leupeptin (final concentrations). The viral supernatant was filter-sterilized, concentrated, and diafiltered into wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole) using a Millipore Pellicon XL tangential flow filtration device per the manufacturer's instructions. The purification procedure was later amended to improve yield by replacing the filtration, concentration and diafiltration steps with precipitation by 40% ammonium sulfate, followed by centrifugation at 10,000 x g for 20 min. at 4°C, and resuspension of the pellet with wash buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole). The protein solution was adjusted to 0.4 M NaCl and 0.5% Triton X-100 and incubated with Ni-NTA agarose. After extensive washing, the apoB protein was eluted with wash buffer containing 250 mM imidazole, concentrated in an Amicon Ultra centrifugal filter device (molecular weight cutoff = 100 kDa, Millipore), and dialyzed into HBSE 8.4 (10 mM Hepes, pH 8.4, with 140 mM NaCl and 1 mM EDTA) containing 1 mM benzamidine, 1 mM PMSF, and 1.5 mM sodium azide. ApoB19.8 and apoB23-αHER2 were stored at 2-8°C until time of use. Protein concentration was determined using the Pierce® BCA Protein Assay

Kit (Thermo Scientific, Rockford, IL) per the manufacturer's instructions. Total protein yield for apoB19.8 and apoB23-αHER2 averaged 0.4 mg/L. Purity was evaluated by resolving the samples in a 10% Tris-glycine SDS-PAGE and the proteins were identified using Sypro Red protein gel stain (Invitrogen) and visualized with a Typhoon 9200 Imager (GE Healthcare).

Liposome Preparation

All lipid films were prepared from a 2:1 molar ratio (10 μmol: 5 μmol) of ePE to cholesteryl hemisuccinate (CHEMS) as described by Provoda et al. [36]. Lipid films were resuspended with 500 μL HBSE 8.4 (10 mM Hepes, pH 8.4, with 140 mM NaCl and 1 mM EDTA) for targeting studies or in HPTS/DPX (35 mM HPTS, 50 mM DPX in HEPES, pH 8.4) for pH-sensitivity studies. Liposomes were subjected to four freeze/ thaw cycles, followed by four 30-second bursts in a bath sonicator (Laboratory Supplies Co., Inc., Hicksville, NY) and four cycles of extrusion through a 0.2 μm pore-size filter (GE Water and Process Technologies). Lipid concentration was determined by the method of Bartlett [37]. ApoB23-αHER2 or apoB19.8 in addition to guanidine hydrochloride (GdnHCl) were added to formed liposomes at a 20:1 mass ratio of phosphate:apoB for a final concentration of 50 mM GdnHCl and 270 μg/ mL lipid and incubated for 20 h at 4°C. The uncoated liposomes were also incubated in the presence of 50 mM GdnHCl for consistency. Unencapsulated and unassociated protein was removed by Sepharose CL-4B (Sigma Aldrich) gel filtration and the lipid concentration was again quantified by the method of Bartlett. To quantify the concentration of liposome-associated protein, apoB was

first precipitated with 10% trichloroacetic acid (TCA). The sample was spun for 10 min. at 4°C at 21,000 x g and the pellet washed with a 1:1 (v/v) solution of ether and ethanol. Pellets were dried briefly and resuspended with sample buffer before running in a 10% SDS-PAGE Tris-HCl gel along with known quantities of TCA-precipitated apoB23-αHER2 or apoB19.8 to generate a standard curve. Bands were detected with Sypro Red gel stain and quantified using a Typhoon 9200 Imager. Liposome diameter was determined by dynamic light scattering with a NiComp 380 ZLS instrument (Particle Sizing Systems, Santa Barbara, CA). To confirm the physical association of apoB with the liposomes, un-purified apoB23-αHER2-coated or apoB19.8-coated liposomes were run in a Ficoll density gradient as described by R.R.C. New [38]. Briefly, protein-coated liposomes were mixed in a 1:3 (v/v) ratio with 30 % Ficoll (in HBSE 8.4) and placed in the bottom of a centrifuge tube (Beckman, Palo Alto, CA), on top of which was carefully layered 10% Ficoll followed by 0% Ficoll (HBSE 8.4), and spun for 30 min. at 100,000 x g at 15°C. The interface between the 0% and 10% Ficoll layers containing the lipid-rich fraction was removed, and protein was precipitated with 10% TCA and quantified as described above. The lipid concentration of the sampled Ficoll fraction was determined by the method of Bartlett, with the following modifications: Ficoll-containing samples required twice the prescribed volume of H_2O_2 (added as a 30% solution rather than 9%) after the H_2SO_4 hydrolysis step; therefore, 200 µL 30% H_2O_2 was added to all samples and standards.

Membrane Leakage Assay

DOPE:CHEMS liposomes were formed as described above with HPTS/ DPX encapsulated. Liposomes were separated from unencapsulated HPTS/ DPX in a 1 x 25 cm Sephadex G-50 gel filtration column. To determine the effect of GdnHCl on liposome membrane integrity, 25 nmol of uncoated liposomes were added to HBSE 8.4 containing 0-2 M GdnHCl and the HPTS fluorescence (λ_{Ex} 413 nm, λ_{Em} 520nm) measured over a 5 min. time span in a Fluoromax-2 fluorometer (Instruments S.A. Inc., Edison, NJ). Triton X-100 was added at a final concentration of 0.2 % at the end of the reading to disrupt the liposomes and determine total fluorescence. The end-point fluorescence measurements were collected, background-corrected, and used to calculate the % HPTS Released: [(Fluorescence of unencapsulated HPTS)/(Fluorescence of total HPTS)] x 100.

The leakage of encapsulated HPTS due to apoB binding was measured as described in Chapter 2. Briefly, the HPTS fluorescence associated with 25 nmol of uncoated or protein-coated liposomes in HBSE 8.4 was measured immediately (time 0 h) and after the 20 h surface coating period. Triton X-100 was added at a final concentration of 0.2 % to completely lyse the liposomes and normalize the samples based on total fluorescence. The % HPTS Released was calculated as: [((Fluorescence of unencapsulated HPTS 20 h) - (Fluorescence of unencapsulated HPTS at 0 h)) / (Fluorescence of total HPTS)] x 100.

pH-Sensitivity Assay

DOPE:CHEMS liposomes were formed as described above with HPTS/ DPX encapsulated. Liposomes were separated from unencapsulated HPTS/

DPX by gel filtration before coating with apoB23-αHER2 or apoB19.8 as described above. The HPTS fluorescence associated with 25 nmol of uncoated or protein-coated liposomes in pH 7.4 buffer (PBS) or pH 5.5 buffer (10 mM MES, 140 mM NaCl, 1 mM EDTA) was measured in a Fluoromax-2 fluorometer (Instruments S.A. Inc., Edison, NJ). Triton X-100 was added at a final concentration of 0.2 % as described above. The % HPTS Released was calculated as: [(Fluorescence of unencapsulated HPTS)/ (Fluorescence of total HPTS)] x 100.

Evaluation of HER2 Receptor Expression in Cell Lines

BT-474, SK-BR-3, and MCF7 cells grown in 6-well plates were washed twice with cold PBS, and lysed with 100 µl lysis buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ mL aprotinin, 10 µg/ mL leupeptin, and 250 µg/mL sodium vanadate; 5 min. on ice). After cell debris was pelleted by centrifugation (21,000 x g for 10 min. at 4°C), protein concentrations in the supernatants were determined by BCA, and equal protein amounts were run in an 8% Tris-Glycine SDS-PAGE gel. The proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane and probed with HER2/ ErbB2 mouse monoclonal antibody (1:1,000, Cell Signaling Technology), followed by an alkaline phosphate conjugated goat anti-mouse antibody (1:30,000, Sigma). Bands were visualized with a Typhoon 9200 Imager.

Selectivity of Free ApoB23-αHER2 Protein for the HER2 Receptor

Conjugation of fluorescein isothiocyanate (FITC, Invitrogen) to ApoB23-αHER2 was performed by incubating a 500:1 molar ratio of FITC: ApoB23-αHER2 for 1 h at room temperature. FITC-conjugated ApoB23-αHER2 was purified using a Zeba Spin Column (Thermo Scientific) per the manufacturer's instructions and concentration was adjusted to 0.5 mg/mL with HBSE 8.4. MCF7, SK-BR-3, and BT-474 cells were counted by hemocytometer, density adjusted to 5 x 10^6 cells/ mL and 100 µL added per flow cytometry analysis tube. After washing the cells twice with PBS containing 0.5% bovine serum albumin (BSA) and 0.05% sodium azide, 5 µg of apoB23-αHER2-FITC conjugate was added and incubated in the dark for 45 min. on ice. The cells were then washed twice and analyzed for the presence of bound apoB23-αHER2-FITC conjugate using the flow cytometer.

Selectivity of ApoB23-αHER2-Coated Liposomes for HER2-Overexpressing Cell Lines

DOPE:CHEMS films were resuspended with 500 µL HPTS and liposomes prepared as described above. BT-474, SK-BR-3, and MCF7 cells were plated at 0.4 x 10^6 cells/ well of a 12-well plate on the day prior to the assay. Cells were washed with serum-free media and pulsed with 50 µM of the different liposome formulations in serum-free media for 2 h at 37°C, 5% CO_2. The cells were dislodged as previously described [39, 40]. Briefly, the cells were washed three times with cold PBS and dislodged by treatment with PBS containing 5 mM EDTA (10 min. at room temperature), followed by washing once with PBS before

measuring the HPTS fluorescence of viable cells on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA).

RESULTS

Preparation of ApoB23-αHER2-Coated Liposomes

Two truncated apoB proteins were produced for this study. The first protein, apoB23-αHER2, consisted of the amino-terminal 23% of amino acids of apoB-100 and was fused to a single-chain Fv fragment of an anti-HER2 antibody at its C-terminus. The second protein, apoB19.8, consisted of the amino-terminal 19.8 % of apoB-100 and served as a control that lacks HER2 affinity (**Error! eference source not found.**A). The expression and purification of apoB19.8 and apoB23-αHER2 led to the production of proteins ~100 kDa and 140 kDa in size, respectively (Figure 4.1B). For the experiments utilizing apoB-coated liposomes, free apoB was separated from liposome-bound apoB by gel filtration. Analysis of the liposome fraction revealed the presence of apoB at a coating density of ~25 molecules apoB19.8/ 200 nm liposomes and ~12 molecules apoB23-αHER2/ 200 nm liposome. To confirm that the apoB proteins detected in the liposome fraction were, in fact, bound to liposomes rather than as apoB multimers that co-purified with the liposomes, apoB-coated liposomes were run in a Ficoll density gradient and the distribution of protein analyzed by SDS-PAGE. Analysis of the upper, lipid-rich fraction of the density gradient revealed the presence of lipid-bound apoB19.8 and apoB23-αHER2 proteins (Figure 4.2). In the absence of liposomes, apoB remained in the bottom, lipid-poor fraction. Quantification of the apoB coating density using the lipid-rich Ficoll fraction

revealed coating densities similar to those obtained after CL-4B chromatography. Although this study utilized the pH-sensitive, negatively-charged liposome formulation DOPE:CHEMS, other liposome formulations were also analyzed for their ability to interact with apoB23-αHER2 and apoB19.8. Both proteins demonstrated the ability to also bind PC:CHEMS (negatively charged) liposomes as well as PC:Chol (neutral) liposomes at similar coating densities (data not shown).

The Association of ApoB23-αHER2 or ApoB19.8 with Liposomes Does Not Affect Membrane Stability or pH-Sensitivity

As an amphiphilic protein, apoB has a tendency to oligomerize in lipid-free buffer that lacks detergent or denaturants [22]. Thus, the apoB proteins were first incubated with GdnHCl before diluting into the liposome-containing buffer in order to dissociate any oligomers that may have formed. The incorporation of GdnHCl in the apoB binding protocol brought into question the effect of the GdnHCl on the integrity of the liposome membrane. To investigate the effect of GdnHCl, the fluorescent dye HPTS and its quencher DPX were co-encapsulated in DOPE:CHEMS liposomes. When in close proximity to DPX, as in the internal space of the liposome where DPX is at relatively high concentrations, the fluorescence of HPTS is quenched. Upon release of HPTS and dilution of DPX (i.e. leakage out of the liposome), the increase in HPTS fluorescence can be detected [41]. Thus, membrane leakage due to different GdnHCl concentrations could be examined through HPTS fluorescence measurements. A previous study has shown that 0.7 M - 2 M GdnHCl resulted in a relatively high recovery of apoB bound to liposomes [22]. However, our analysis of the effect of GdnHCl on

membrane integrity showed that these high salt concentrations resulted in the immediate release of ~25 % of HPTS (Figure 4.3A). In contrast, dilution of GdnHCl to 50 mM resulted in the release of 6.1 % HPTS which was similar to the level detected in liposomes measured in the absence of GdnHCl. Based on these results, apoB binding experiments were performed by adding a high concentration of GdnHCl (0.7 M - 1 M) to apoB to dissociate the protein into monomers before diluting to a final GdnHCl concentration of 50 mM upon liposome addition.

To analyze the effect of protein binding on liposome membrane integrity, the HPTS fluorescence associated with 25 nmol of lipid from each liposome formulation was measured in buffer before and after coating the liposomes with apoB23-αHER2 or apoB19.8. Triton X-100 was added at the end of each reading to lyse the liposomes so that the total fluorescence could be detected. The '% HPTS remaining' (encapsulated) was calculated based on the fluorescence measurements before and after coating the liposomes with apoB as well as the total fluorescence. Similar to the results shown in Chapter 2 for apoB23-YFP-coated PA:PC:Chol liposomes, the amount of HPTS that remained encapsulated in the liposomes after coating them with apoB23-αHER2 or apoB19.8 was virtually unchanged (93.1 ± 3.4 % for apoB23-αHER2-coated liposomes, 94.1 ± 0.1 % for apoB19.8-coated liposomes) (Figure 4.3B), demonstrating that the addition of the coating molecules to the liposome membrane did not induce leakage.

The DOPE:CHEMS liposome formulation is pH-sensitive; the liposomes are stable at physiological pH (pH 7.4), but are destabilized by the low pH of the endosomes (pH 4.5-5.5) [42]. This property has lead to an increase in the cytosolic delivery of liposomal contents *in vitro* [43, 44]. The effect of the apoB proteins on the pH-sensitivity of the DOPE:CHEMS liposome formulation was evaluated through the detection of HPTS released upon addition of the liposomes into buffers of acidic versus neutral pH. For comparison, a pH-insensitive liposome formulation, DOPC:CHEMS, was tested in parallel. When the liposomes were added to buffer at physiological pH (pH 7.4), all liposome formulations retained the majority of their encapsulated HPTS, releasing only 2-5% (Figure 4.4). At the low pH that is representative of the pH in the endocytic vesicles (pH 5.5), apoB23-αHER2-coated and apoB19.8-coated liposomes released 97.6 ± 4.9 % and 92.5 ± 8.2 %, respectively, of their encapsulated HPTS which was similar to the amount released by uncoated pH-sensitive liposomes (90.2 ± 6.9 %). As expected, the pH-insensitive PC:CHEMS liposomes were unaffected by the low pH and released only ~2-3% of its encapsulated HPTS at pH 7.4 and pH 5.5.

Selectivity of ApoB23-αHER2 for HER2-Overexpressing Cell Lines

Previous studies have reported the overexpression of HER2 in SK-BR-3 and BT-474 cell lines as well as its low level of expression in MCF7 cells [45, 46]. The differential in expression levels of HER2 in the above cell lines was verified by a Western blot of cell lysate using anti-HER2 monoclonal antibodies (Figure

4.5). As expected, the expression of HER2 was high for BT-474 and SK-BR-3 cells, but undetectable in MCF7 cells.

In the absence of liposomes, the apoB23-αHER2 protein was evaluated for its ability to selectively bind HER2-overexpressing cells. FITC-labeled apoB23-αHER2 was incubated with MCF7, SK-BR-3, and BT-474 cells and selective binding analyzed using flow cytometry. As shown in Figure 4.6, the incubation of FITC-labeled apoB23-αHER2 with the HER2-overexpressing cell lines, BT-474 and SK-BR-3, resulted in a ~6.7-fold shift in fluorescence compared to untreated cells, whereas the incubation with the low HER2-expressing MCF7 cells resulted in only ~ a 2-fold increase. These results demonstrate the ability of apoB23-αHER2 to selectively bind HER2-overexpressing cells.

Targeting ApoB23-αHER2-Coated Liposomes to HER2-Overexpressing Cells

To evaluate the ability of apoB23-αHER2-coated liposomes to selectively bind HER2-overexpressing cells, HPTS (without DPX) was encapsulated in DOPE:CHEMS liposomes. Selective binding was evaluated through the detection of HPTS fluorescence associated with each cell line by flow cytometry analysis. As shown in Figure 4.7, the treatment of MCF7, SK-BR-3, and BT-474 cells for 2 h at 37°C with 50 μM of uncoated, apoB19.8-coated, or apoB23-αHER2-coated liposomes resulted in similar levels of binding within a specific cell line.

Potential obstruction of cell-surface HER2 due to multilayered colony growth was investigated by treating 0.5 x 10^6 BT-474 and MCF7 cells in

suspension with 100 μM of apoB23-αHER2 and apoB19.8-coated liposomes. Liposomes displayed relatively high binding to both MCF7 and BT-474 cells (Figure 4.8). Within each cell line, apoB19.8-coated liposomes associated with the cells to the same extent as apoB23-αHER2-coated liposomes. Liposomes associate non-specifically with cells to different extents depending on the formulation [39]. Thus, it was hypothesized that a neutral liposome formulation might decrease the level of non-specific adsorption seen with the negatively charged DOPE:CHEMS. DOPC:Chol was used as a neutral liposome formulation and coated with apoB19.8 or apoB23-αHER2. A repeat of the binding assay revealed that the overall binding of DOPC:Chol to cells was lower than that with the negatively charged DOPE:CHEMS, but as with DOPE:CHEMS, binding to cells was roughly equivalent for apoB19.8 and apoB23-αHER2-coated DOPC:Chol liposomes (Figure 4.9).

The ability of apoB-coated liposomes to be internalized by cells was investigated by performing the cell-binding assay with apoB23-αHER2-coated liposomes at 4°C and 37°C. The cell-associated fluorescence for the 37°C pulse was about 3-fold greater than the same experiment performed at 4°C (Figure 4.10). These results suggest that the apoB23-αHER2-coated liposomes are internalized by cells. Finally, the cell binding assay was repeated with higher and lower liposome concentrations to ensure that the liposomes were at or near saturating concentrations; inadequate liposome concentrations could hide potential differences in selectivity, while elevated liposome concentrations could result in decreased accessibility of HER2 to unbound liposomes by those already

bound as proposed by Lee et al. [40]. The extent of cell binding was found to be similar between 100 μM apoB19.8-coated and apoB23-αHER2-coated liposomes with both SK-BR-3 and Bt-474 cells (Figure 4.11). Similarly, no discernible selectivity was observed in BT-474 cells treated with 5 μM apoB-19.8-coated or apoB23-αHER2-coated liposomes.

Discussion

The apoB23-αHER2 fusion protein, consisting of a truncated form of apolipoprotein B recombinantly fused to the single chain Fv segment of an anti-HER2 antibody, was explored as a means of attaching a targeting moiety to the surface of liposomes for use in targeted liposomal delivery. The apoB23-αHER2 fusion protein was coupled to pH-sensitive, negatively charged DOPE:CHEMS liposomes and evaluated for their ability to target HER2-overexpressing cells. A second truncated apoB protein, apoB19.8, which lacks HER2 specificity, was used as the negative control. The presence of apoB23-αHER2 or apoB19.8 in the top (buoyant liposome-containing) fraction of the Ficoll density gradient signified that the apoB fragments were indeed associated with the liposome membrane. Furthermore, the ability of both apoB proteins to associate with liposomes of different composition and charge was also verified. Previous studies that investigated the lipid-binding behavior of truncated apoB utilized lipid vesicles composed of neutral phosphatidylcholine, which is the predominant phospholipid in LDL [19, 22, 47]. This is the first known study that shows the affinity of truncated apoB for negatively charged liposome formulations, thus demonstrating its versatility for use with liposomal delivery systems.

In contrast to many protein-association methods in which the protein is linked to the liposome membrane through site-specific conjugation, the apoB protein interacts with the lipid surface through multiple hydrophobic domains [22]. In addition, the apoB fragments have the potential for penetrating the liposome bilayer, similar to how the full-length apoB-100 penetrates the LDL membrane [19, 25]. Given these characteristics of apoB, a legitimate concern with the use of this unique method of associating targeting molecules with the lipid membrane is its potential for affecting the liposome membrane integrity. However, analysis of the membrane stability over the 20 h period in which the apoB proteins interacted with the lipid membrane showed minimal leakage of the small molecule dye HPTS. Furthermore, the pH-sensitivity of the DOPE:CHEMS liposomes was maintained as demonstrated by the ability of apoB19.8- and apoB23-αHER2-coated liposomes to release elevated levels of HPTS at low pH. These results suggest that the interaction of the apoB fragments with liposomes has minimal impact on the liposome membrane.

The selectivity for HER2 was examined using the MCF7, SK-BR-3, and BT-474 cell lines. BT-474 and SK-BR-3 cells have reportedly high expression levels of HER2, while that in MCF7 is 10-30-fold lower [27, 46]. Furthermore, these cell lines have been used in previous studies that demonstrate the ability of immunoliposomes and immunotoxins to target HER2 [2, 27]. The differences in HER2 expression between the cell lines did not directly translate to differences in selective binding, as HER2-targeted immunoliposomes were shown to associate with the BT-474 and SK-BR-3 cells 450-700-fold greater than with MCF7 cells

[27]. In these studies, FITC-labeled apoB23-αHER2 protein on its own demonstrated the ability to preferentially bind SK-BR-3 and BT-474 cells over MCF7 cells. However, upon association with the liposome membrane, any selectivity for HER2 was abolished. ApoB23-αHER2-coated liposomes bound cells with high and low levels of HER2 expression to a similar extent. In addition, uncoated liposomes or those coated with apoB19.8 demonstrated levels of binding similar to those coated with apoB23-αHER2. To investigate whether the inherent differences in growth patterns and morphology between the MCF7, SK-BR-3, and BT-474 cell lines influenced the level of liposome association, a binding study was performed with the above cell lines in suspension. We hypothesized that incubating liposomes with the cells in suspension would provide a better comparison of the HER2 exposure on the cell surface. Treating the cells while in suspension led to a greater overall liposome association with the cells. However, apoB23-αHER2 and apoB19.8-coated liposomes associated with BT-474 as well as MCF7 cells to similar extents.

Studies have shown that the internalization of liposomes is not only dependent on cell type, but also on the net charge of the liposome membrane [39]. The cell association of apoB23-αHER2-coated neutral DOPC:Chol liposomes was lower overall in comparison to negatively charged DOPE:CHEMS liposomes (compare Figure 4.9 to Figure 4.7) and did not demonstrate enhanced binding over apoB19.8-coated liposomes.

Certain antibodies generated against the HER2 extracellular domain have shown the ability to be rapidly internalized upon binding the membrane protein

[48, 49]. Furthermore, liposomes with conjugated anti-HER2 antibody fragments have been demonstrated to undergo receptor-mediated endocytosis upon binding HER2 [27, 50]. The internalization of liposomes can be investigated by measuring the liposome fluorescence associated with cells after an incubation period at 37°C versus 4°C. Incubation at 4°C results in inhibition of endocytosis; therefore, a measurement of liposome association at this temperature can denote the amount of liposomes that are membrane-bound [40, 51, 52]. Liposomes have been found to associate with cells with similar affinity at both temperatures and while the number of liposomes found on the surface of cells at steady-state is similar at 37°C and 4°C, the total liposome association can be greater at 37°C due to endocytosis [40]. Alternatively, a lower fluorescence measured at 4°C may be due to the dissociation of cell surface-bound liposomes due to the presence of EDTA added to detach the cells from the plate. Although the use of EDTA precludes an accurate calculation of surface-bound liposomes at 4°C, a higher fluorescence measured at 37°C would suggest that liposomes were internalized, and thus not vulnerable to dissociation from cells due to EDTA. The incubation of apoB23-αHER2-coated liposomes with HER2-overexpressing cells at 37°C resulted in a greater total fluorescence than at 4°C. These results suggest that the apoB23-αHER2-coated liposomes are internalized.

The initial conditions utilized for the binding studies were a 2 h pulse with 50 μM liposomes at 37°C. The 2 h pulse in these studies was deemed sufficient based on previous binding studies that showed liposomes to be rapidly internalized upon binding, with the amount internalized plateauing within 2 h [27,

40, 53, 54]. Based on the established MCF7 doubling time of 29 h (ATCC) and an approximated liposome size of 200 nm, we calculated that the above conditions would lead to a maximum of 150,000 liposomes/ cell. This hypothetical liposome to cell ratio is well above reported internalized levels of ~10,000-20,000 neutral immunoliposomes / cell or ~30,000 negatively-charged uncoated liposomes/ cell at saturating liposome concentrations [10, 27, 39, 45]. However, to account for differences in liposome internalization rates due to the liposome formulation or affinity of the antibody fragment for HER2, cells were also pulsed at higher (100 μM) and lower (5 μM) concentrations of liposomes. However, these liposome concentrations did not result in detectable differences in the amount of apoB23-αHER2-coated versus apoB19.8-coated liposomes associated with the cells.

The lack of HER2 targeting with apoB23-αHER2-coated liposomes compared to apoB19.8-coated or uncoated liposomes can be attributed to at least three possible reasons: the association of multimeric as opposed to monomeric apoB23-αHER2 with the liposome membrane, the alteration of the antigen binding pocket on the HER2 antibody during the liposome binding procedure, or the lack of a sufficient coating density to demonstrate selectivity. As previously mentioned, characterization studies performed on an amino-terminal apoB fragment have demonstrated its tendency to form multimers in the absence of lipid, detergents, or denaturants [22]. Based on this study, the addition of 0.7-1 M GdnHCl to the concentrated apoB23-αHER2 stock solution should result in the disruption of multimers. However, it is unknown whether the

dilution of GdnHCl during the addition of liposomes results in the immediate reformation of these multimers rather than the association of monomeric apoB with the liposome membrane. An investigation into the oligomeric state of apoB23-αHER2 was conducted with FPLC, particle sizing and native PAGE analysis; however, the small difference in size between the monomeric and the predominantly hexameric state of apoB (7.2 nm vs. 12.8 nm, respectively [22]) led to inconclusive results. ApoB23-αHER2, as a FITC-conjugate, displayed preferential binding for SK-BR-3 and BT-474 cells. The loss of this specificity upon association with the liposome membrane suggests that the HER2 binding site was affected. Given the fact that the N-terminus of apoB-100 is highly globular, it is possible that this protrusion from the lipid membrane occludes the HER2 antibody fragment as has been seen with PEGylated liposomes with antibody conjugated directly to the liposome membrane [18, 25, 55]. This putative change in HER2 affinity could also be due to improper refolding of apoB23-αHER2 upon dilution of GdnHCl and introduction of liposomes, or to the association of multimers in such a way that the binding site is blocked.

In addition to the possibility of structure modification, the lack of HER2 specificity observed with apoB23-αHER2-coated liposomes could be due to the low coating density. As explained in Chapter 2, the manner in which apoB associates with the liposome membrane may plausibly limit the number of apoB molecules that can coat each liposome. A low coating density may, in turn, restrict an immunoliposome's avidity which may play a role in its apparent targetability. Kirpotin et al. found that a density of 10-15 Fab antibody fragments

per 100 nm liposome led to the internalization of the majority of cell-associated liposomes, while Lee et al. demonstrated selectivity with ~ 75 ligands per 130 nm liposome [8, 27]. Targeting to AKR-A cells has been demonstrated with as little as 1 IgG molecule per liposome [56]. The optimal coating density is dependent on the antibody or ligand type and can vary greatly. Thus, it is possible that apoB23 fused to a different antibody fragment with a low optimal coating density for liposomes might show selectivity.

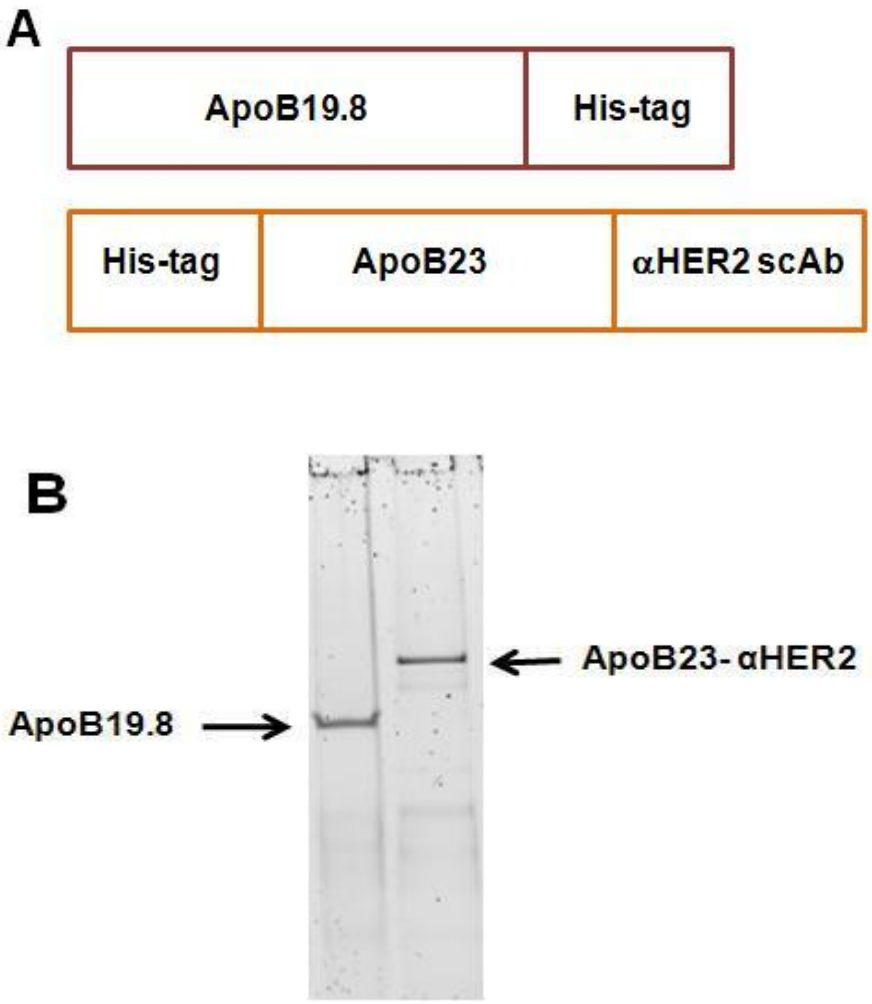

Figure 4.1: Expression of ApoB19.8 and ApoB23-αHER2

(A) Constructs of apoB19.8 and apoB23-αHER2 showing the presence of a hexahistidine (His)-tag at the C-terminus of apoB19.8 and N-terminus of apoB23-αHER2. The single chain anti-HER2 antibody (αHER2 scAb) lies at the C-terminus of apoB23-αHER2. (B) apoB19.8 and apoB23-αHER2 were expressed in SF9 cells and purified by nickel-affinity chromatography. Analysis of the purity in an SDS-PAGE gel revealed a major band for apoB19.8 and apoB23-αHER2 at around 100 kDa and 140 kDa, respectively.

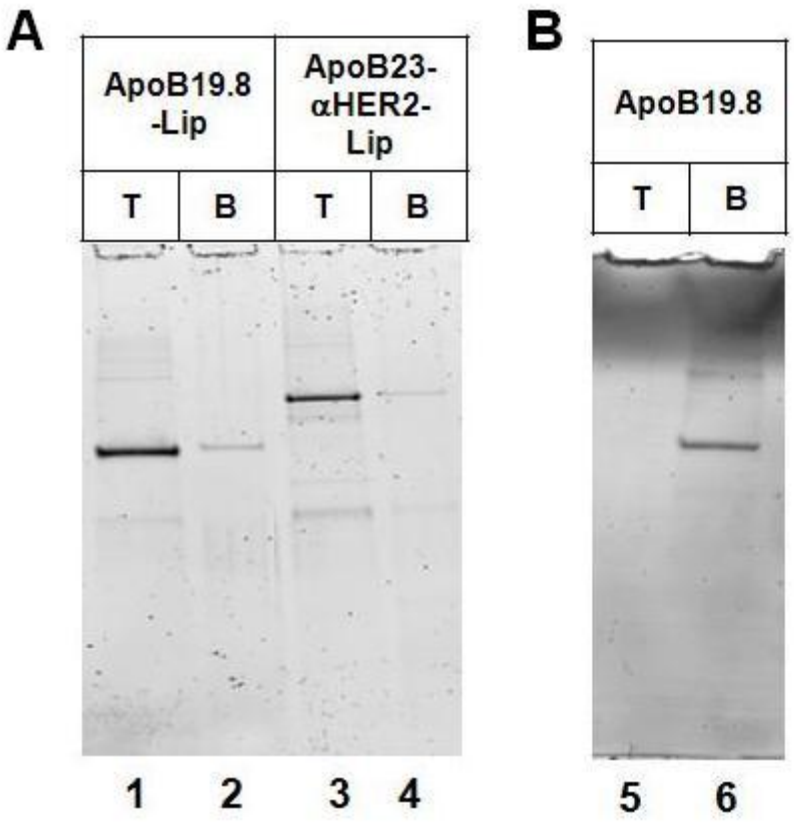

Figure 4.2: Binding of ApoB19.8 and ApoB23-αHER2 to DOPE:CHEMS Liposomes

DOPE:CHEMS liposomes were coated with apoB19.8 (ApoB19.8-Lip) or apoB23-αHER2 (ApoB23- αHER2-Lip) for 20 h followed by separation from free protein by Ficoll density gradient centrifugation. The lipid-containing top (T) and lipid-poor bottom (B) fractions were analyzed by SDS-PAGE. In the presence of DOPE:CHEMS liposomes, apoB19.8 (lanes 1-2) and apoB23-αHER2 (lanes 3-4) floated to the top fraction, signifying lipid association. When analyzed in the absence of lipid (as shown with apoB19.8 in lanes 5 and 6), apoB remained in the bottom fraction.

A

	Concentration of GdnHCl	% HPTS Released
Uncoated DOPE:CHEMS	0 M	5.9
	14 mM	5.6
	20 mM	5.4
	50 mM	6.1
	0.7 M	25.3
	1 M	27.1
	2 M	24.7

B

	Final Concentration of GdnHCl	% HPTS Released
ApoB19.8-coated DOPE:CHEMS	50 mM	5.9 (0.1)
ApoB23-αHER2-coated DOPE:CHEMS	50 mM	6.9 (3.4)

Figure 4.3: Leakage of Encapsulated HPTS Due to GdnHCl or ApoB Binding

(A) Uncoated DOPE:CHEMS with HPTS/DPX encapsulated were added to 0-2 M GdnHCl in HBSE 8.4 and the immediate release of HPTS detected by an increase in its fluorescence. (B) The leakage of HPTS from DOPE:CHEMS liposomes was measured before and after coating the liposomes with apoB19.8 or apoB23-αHER2. The '% HPTS Released' was calculated as described in the Materials and Methods section.

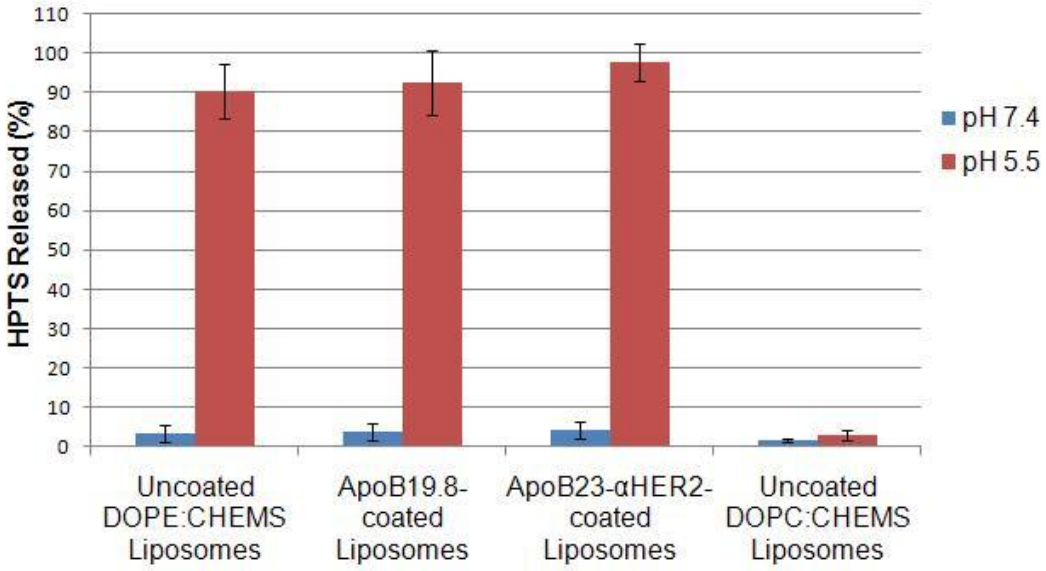

Figure 4.4: ApoB-Coated Liposomes Retain their pH-Sensitivity

The leakage of HPTS from coated or uncoated DOPE:CHEMS (pH-sensitive) or uncoated DOPC:CHEMS (pH-insensitive) liposomes in pH 7.4 or pH 5.5 buffer was detected by HPTS fluorescence as in Figure 4.3. Measurements were corrected for background and the % HPTS Released calculated. Data are the average of three independent experiments and are displayed as the mean ± SD.

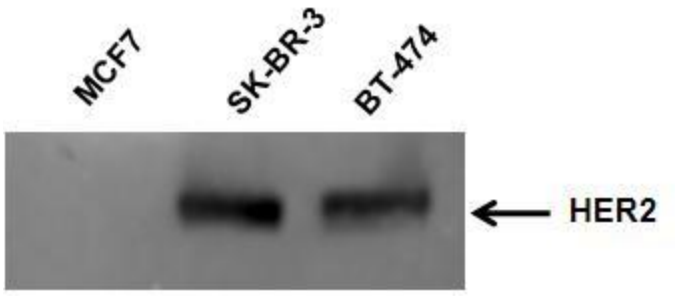

Figure 4.5: Analysis of HER2 Overexpression in Cell Lines

Cells were lysed in lysis buffer and 50 µg of total cellular protein was resolved in an 8 % SDS-PAGE Tris-glycine gel and transferred to a PVDF membrane. Detection of HER2 was performed with an anti-HER2 monoclonal antibody.

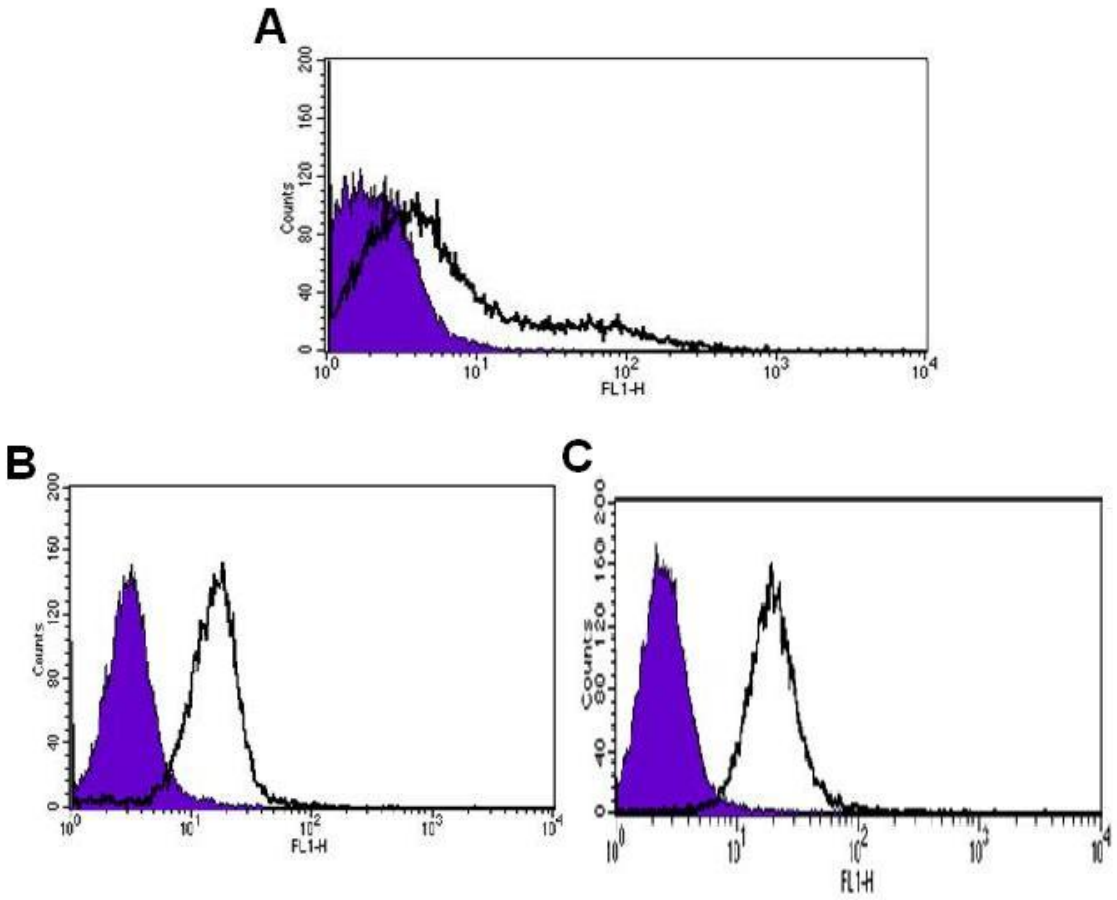

Figure 4.6: Selective Binding of ApoB23-αHER2-FITC Conjugate to HER2-Overexpressing Cells

The selectivity of FITC-labeled apoB23-αHER2 protein for target or control cells was evaluated by flow cytometry. MCF-7 (A), BT-474 (B), and SK-BR-3 (C) cells (500,000 cells total) were treated with 5 µg of apoB23-αHER2-FITC for 45 min. on ice. The cells were washed and the presence of bound apoB23-αHER2-FITC was detected using flow cytometry. *Open histograms*, cells treated with apoB23-αHER2-FITC conjugate. *Filled histograms*, untreated cells.

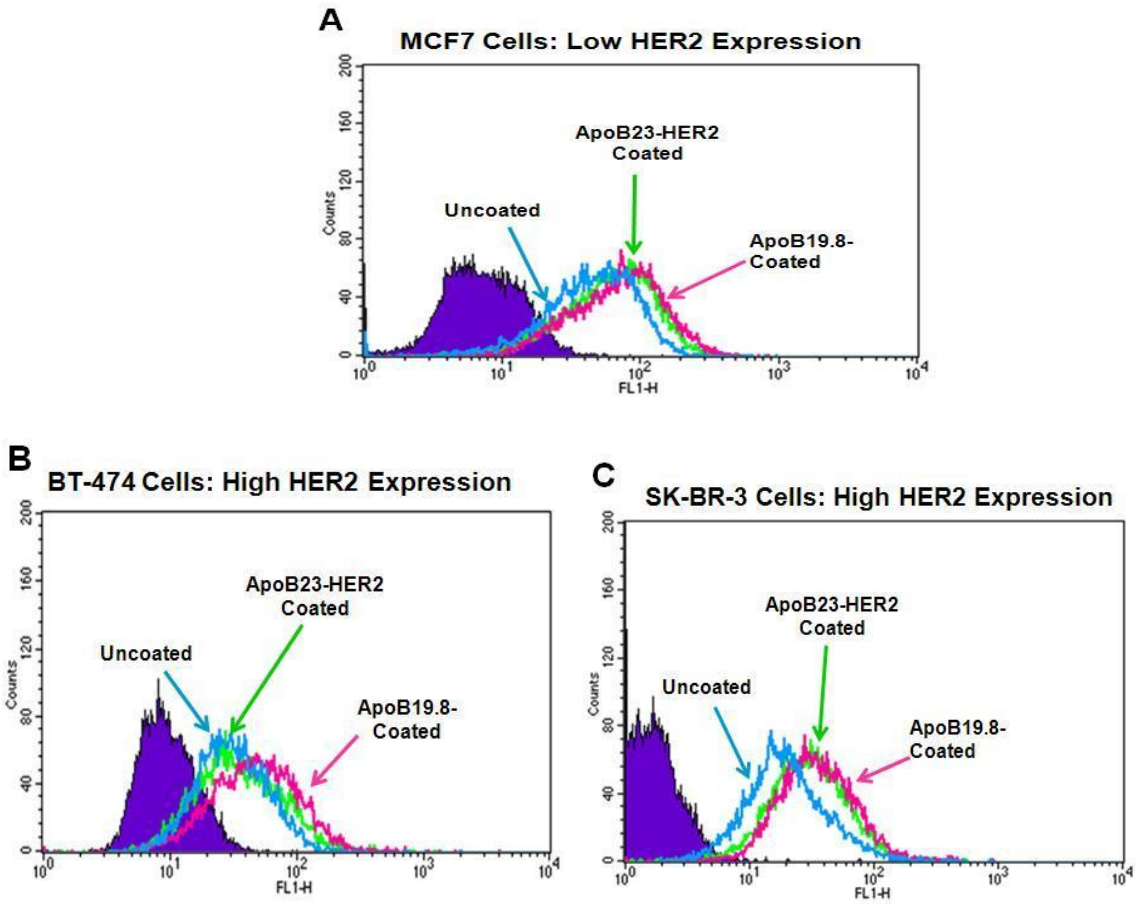

Figure 4.7: Targeting HER2-Overexpressing Cells with ApoB23-αHER2-Coated Liposomes

DOPE:CHEMS liposomes with encapsulated HPTS were formed and coated with apoB23-αHER2, apoB19.8 or left uncoated. A low HER2-expressing cell line (MCF7 (A)) and high HER2-expressing cell lines (BT-474 (B) and SK-BR-3 (C)) were pulsed with 50 μM of purified liposomes for 2 h at 37°C, washed, resuspended, and the cell-associated fluorescence detected by flow cytometry.

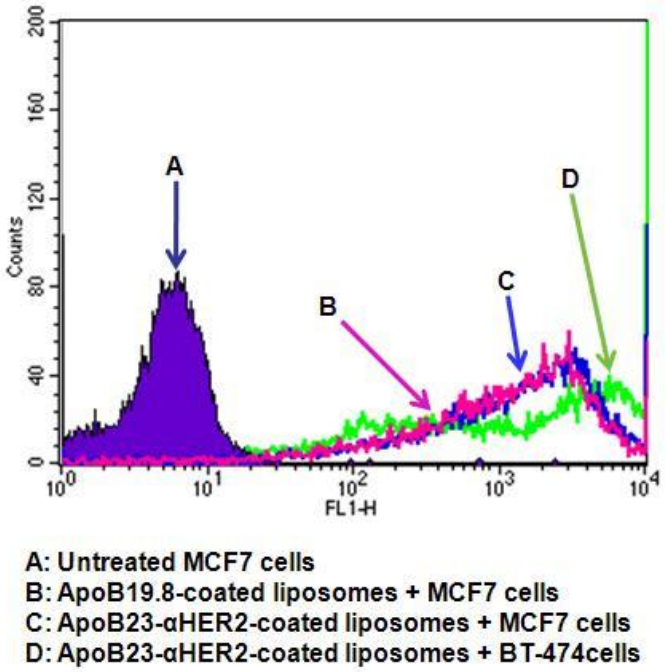

Figure 4.8: The Association of ApoB-Coated Liposomes with Cells in Suspension

MCF7 or BT-474 cells were washed in serum-free media and 0.5 x 10^6 cells were added to polypropylene tubes. Liposomes coated with apoB23-αHER2 or apoB19.8 were added at 100 µM and incubated with agitation for 2 h at 37°C. Treated cells were then washed with PBS and the cell-associated fluorescence measured via flow cytometry. Note that the histogram for untreated BT-474 cells overlapped that for untreated MCF7 cells. Similarly, the histogram for BT-474 cells treated with apoB19.8-coated liposomes overlapped that of BT-474 cells treated with apoB23-αHER2-coated liposomes (not shown).

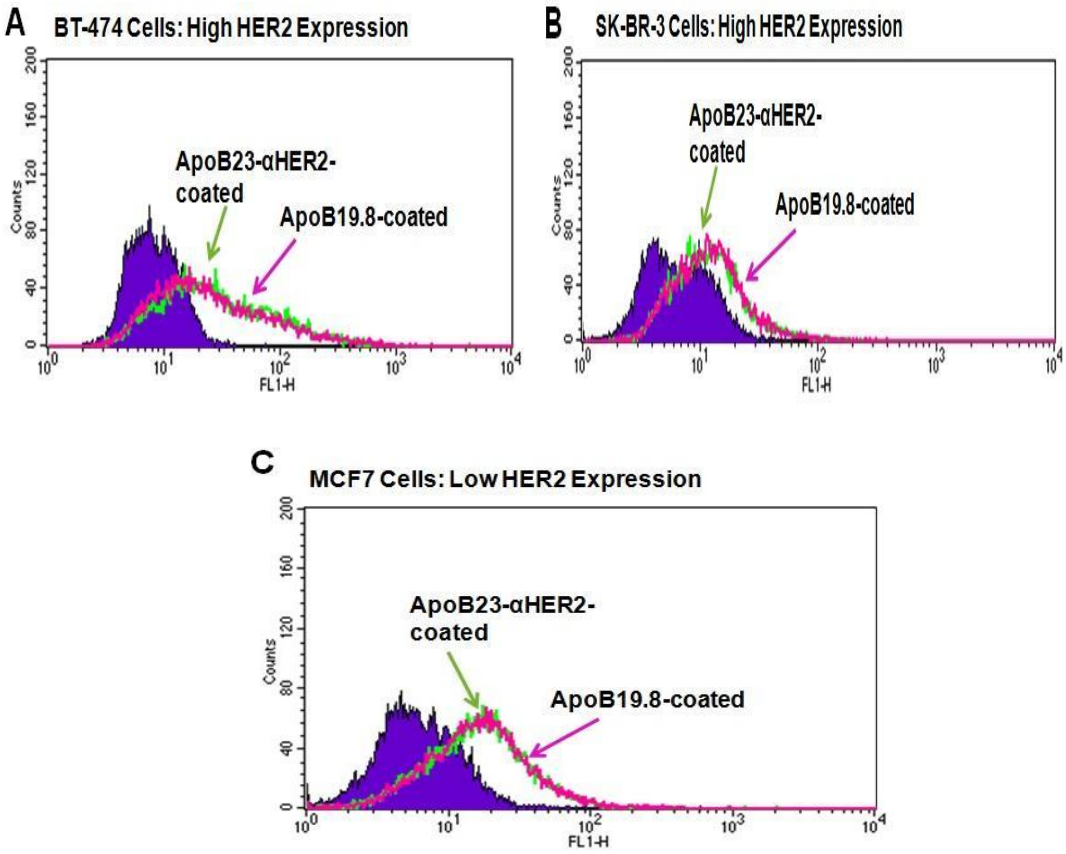

Figure 4.9: Targeting HER2-Overexpressing Cells with ApoB23-αHER2-Coated Neutral Liposomes

Liposomes composed of DOPC:Chol (2:1 molar ratio) were prepared with encapsulated HPTS and coated with apoB23-αHER2 or apoB19.8. The ability of the apoB23-αHER2-coated liposomes to selectively bind cells with high HER2 expression (BT-474 (A) and SK-BR-3 cells (B)) vs. cells with low HER2 expression (MCF7 cells (C)) was examined as described in Figure 4.7.

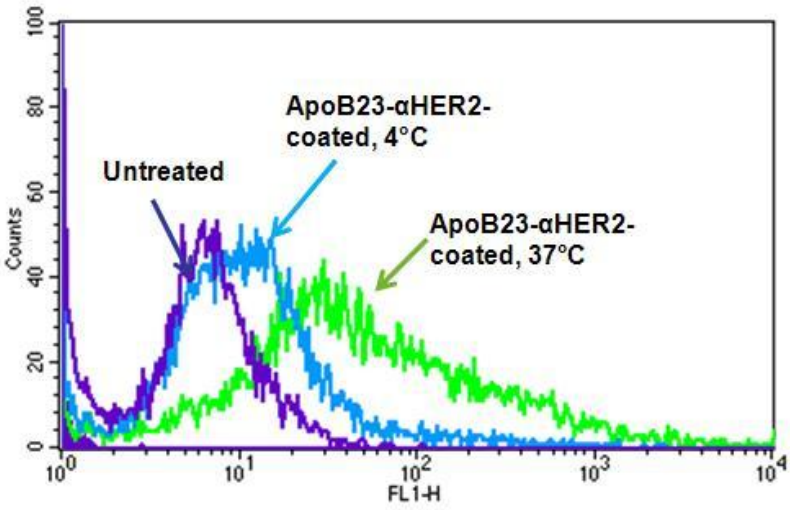

Figure 4.10: Cell-Associated Fluorescence Increases with Increased Temperature

Liposomes composed of DOPE:CHEMS with encapsulated HPTS were coated with apoB23-αHER2 or apoB19.8. Adherent BT-474 cells were pulsed with either liposome formulation for 2 h at 37°C or 4°C. After 2 h, the cells were washed, resuspended, and the HPTS fluorescence detected by flow cytometry.

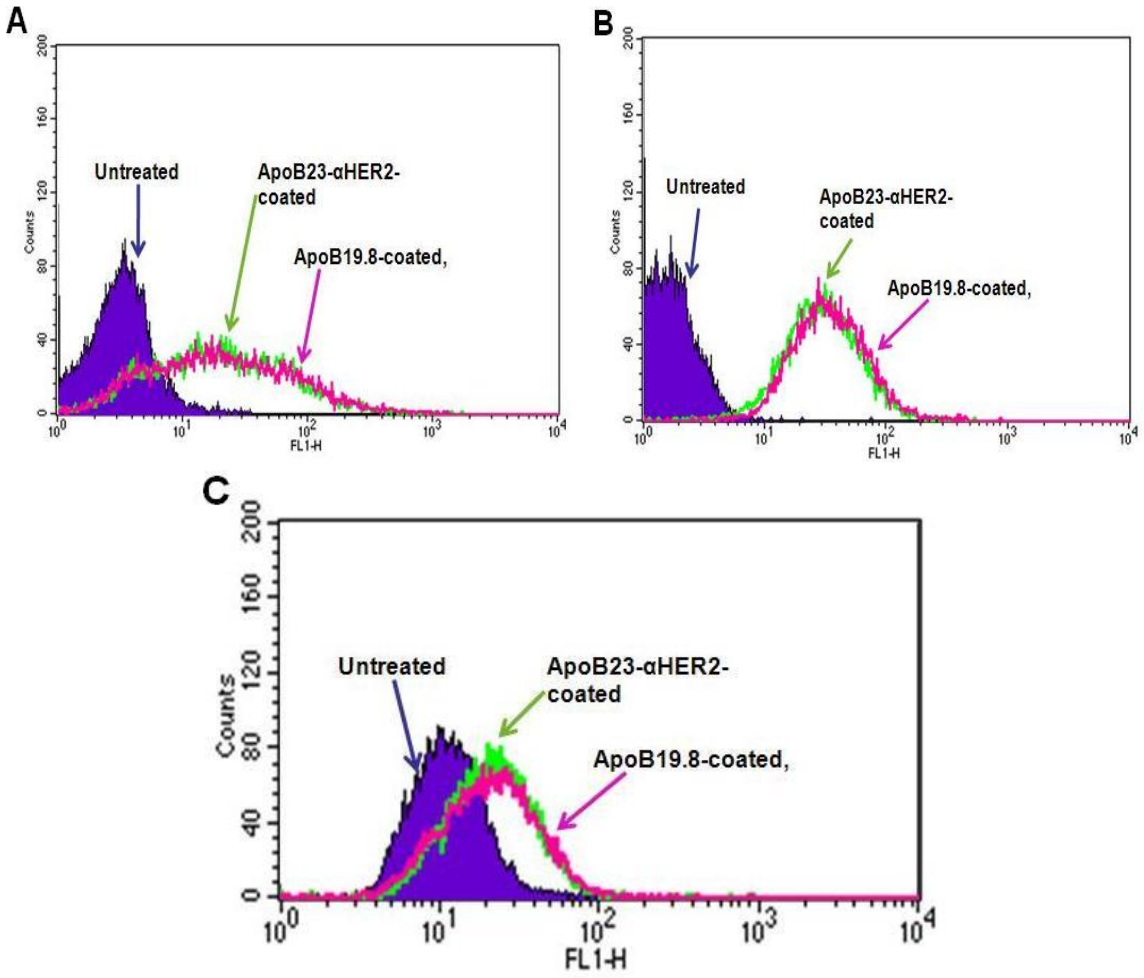

Figure 4.11: Modification of the Liposome Concentration Does Not Improve Selectivity

DOPE:CHEMS liposomes were prepared with HPTS encapsulated and coated with either apoB23-αHER2 or apoB19.8. BT-474 or SK-BR-3 cells were treated with either 5 μM or 100 μM of the different liposome formulations for 2 h at 37°C and cell-associated fluorescence detected as described in Figure 4.7. (A) BT-474 cells or (B) SK-BR-3 cells treated with 100 μM liposomes. (C) BT-474 cells treated with 5 μM liposomes.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

These studies demonstrated the feasibility of associating protein with the liposome membrane without adversely affecting the membrane integrity or the uptake of liposomes by macrophages through two relatively simple techniques: non-covalent conjugation of nickel-containing liposomes (Ni-Lip) with hexahistidine (His)-tagged proteins and hydrophobic interaction of a truncated form of the amphiphilic protein apolipoprotein B-100 (apoB) fused to a protein. The use of liposomes as delivery vehicles offers many potential advantages, including the delivery of a large payload with multiple components and the protection of encapsulated contents from degradation. Conventional, uncoated liposomes frequently exhibit non-ideal properties *in vivo*, including relatively short half-lives, and generally require the addition of macromolecules to the liposome surface for improved characteristics. However, some methods utilized to associate the macromolecules with the liposome can have a negative impact on the properties of the delivery system. The results presented in this dissertation demonstrate the feasibility of linking proteins to liposome membranes using Ni-His or apoB fusion proteins without compromising the liposomes' delivery characteristics.

The use of either Ni-His or the apoB fusion protein to couple protein to the liposome did not promote leakage of the liposome contents or increase its internalization by macrophages, suggesting that the stability of the liposome was maintained. Furthermore, His-tagged yellow fluorescent protein (YFP-His) attached to Ni-Lip enhanced the overall immunogenicity of the liposomal vaccine. Finally, the ability to associate a HER2 antibody fragment with liposomes via the apoB23-αHER2 fusion protein indicates the potential use of the apoB fusion protein in the production of immunoliposomes.

Our lab has previously shown that co-encapsulation of the endosomolytic protein listeriolysin O (LLO) and the model antigen ovalbumin (OVA) in pH-sensitive liposomes can stimulate the activation of both OVA-specific cytotoxic T lymphocytes (CTLs) and antibodies [1]. The addition of YFP-His to the surface of this liposomal vaccine was shown to maintain the relative level of OVA-specific CTLs while enhancing the OVA-specific and YFP-specific antibody responses. CTL and antibody activation are normally dependent on the delivery of antigen to the cytosol and endosome, respectively, of antigen-presenting cells (APCs) [2]. Thus, the results presented here are significant since they suggest that His-tagged protein and nickel-chelating lipid can be incorporated into liposome formulations without compromising the liposome's original delivery properties. Given these results, one could similarly expect that the addition of other antigens or APC-targeting ligands to liposomal vaccines using this procedure will likely not affect the delivery of its contents. The improvement in the original vaccine formulation is demonstrated through the enhanced antibody responses. It is also

possible that YFP-His stimulated CTL production; however, only the OVA-specific CTLs were measured in this study. Future studies in which YFP-specific CTLs are also measured may give a better appreciation for the efficacy of this liposomal vaccine.

For certain antigens and their associated liposome formulations, membrane-associated antigen was found to stimulate a more pronounced immune response in comparison to encapsulated antigen [3-5]. This enhanced response has also been shown for small molecules or peptides that are relatively non-immunogenic on their own and require conjugation to larger carriers such as liposomes to elicit a strong immune response [6, 7]. Finally, the Ni-His conjugation method was shown to have conjugation efficiencies nearing 100%. This finding may be of particular significance for antigens such as proteins and peptides that are costly or in short supply. With the increased development of both subunit (i.e. proteins, peptides) vaccines and multiple antigen delivery strategies due to enhanced immune responses, it seems fairly likely that liposomal vaccine formulations similar in design to ours (with both encapsulated and membrane-associated antigen) will be developed. A future study of this project should include the incorporation of biologically relevant antigens. It has been shown that multiple antigen delivery can result in enhanced protection [8]. One possible multiple antigen combination is the use of the influenza nucleoprotein (NP) along with the matrix 2 protein ectodomain (M2e) of influenza A. The highly conserved sequences of NP and M2e have led some to speculate that the incorporation of these antigens in an influenza vaccine formulation may

provide protection against multiple strains of influenza [9-11]. M2e was previously added to NP-containing liposomal vaccine formulations in our lab; however, only a weak M2e-specific antibody response was generated [12]. Since the M2e peptide used was only 16 amino acids in length, it is likely that the M2e antibody response could be greatly enhanced through conjugation of M2e to the liposome membrane with the Ni-His technique.

Although our results suggest that the Ni-His conjugation procedure can be used in a safe and effective manner, some potential limitations of this method must also be addressed. Upon intravenous injection, His-tagged proteins have been found to rapidly dissociate from Ni-Lip [13]. This finding might restrict this method from use in delivery systems where sustained liposome association in the presence of serum is needed, such as with tumor targeting. Platt et al. hypothesized that the relatively lower level of serum proteins in the subcutaneous space combined with the rapid uptake of liposomes by cells of the reticuloendothelial system may explain the success demonstrated with subcutaneously injected liposomal vaccines [13]. Relatively low molar amounts of nickel-chelating lipid were used in these studies for conjugation of YFP-His to the liposome surface. Although higher molar percentages were shown to increase the YFP-His coating density, they were also shown to alter the liposome membrane's properties as evident in their increased internalization by macrophages. In addition, the molar amounts of nickel-chelating lipid were kept low due to nickel toxicity concerns. Even though no overt signs of toxicity were observed in our mice and studies on nickel toxicity suggest that the greatest

threat lies with the inhalation of nickel compounds, further studies on the subcutaneous injection of Ni-Lip will need to be performed to properly evaluate the true potential of this procedure in drug delivery.

The second method for membrane association, utilizing the apoB fusion protein, was selected based on its ability to associate with liposome membranes through hydrophobic interaction and its relative biocompatibility due to its derivation from the endogenous protein apoB-100. We first investigated the apoB's ability to associate YFP with liposome membranes and found success in doing so. Although the full-length protein apoB-100 is believed to play an instrumental role in maintaining the circulation time of LDL at 2-3 days, the addition of the apoB fragment alone (apoB19.8) or as a fusion protein (apoB23-αHER2) to the liposome membrane did not suggest any enhanced stability, as investigated in serum stability and macrophage internalization assays. Reasons for this behavior may include a lack of adequate membrane coverage or the loss of stabilizing effects upon the alteration of apoB-100 to create the truncated protein. However, an encouraging result was that the addition of the truncated protein to the liposome membrane did not have a destabilizing effect.

To investigate the use of the apoB fusion protein to associate a targeting ligand with the liposome membrane, YFP was replaced with an antibody fragment with specificity for HER2 to create apoB23-αHER2. This fusion protein was also found to associate with liposomes without negatively affecting the membrane's properties (e.g. membrane integrity, pH-sensitivity), yet was not able to target the liposomes to HER2-overexpressing cells *in vitro.* We hypothesized

that this result may be a consequence of the relatively low coating density achievable with this coating method. As coating density can be directly related to specificity of binding, it is plausible that efficient targeting of HER2 may require a greater antibody density [14]. Since this relationship differs based on the choice of ligand and cell marker, the production of liposomes coated with apoB fused to a ligand other than the HER2 antibody fragment may be successful.

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APPENDIX A

THE INTRODUCTION OF A CYSTEINE AT LYSINE 397 OF CTLA4IG FOR SITE-SPECIFIC CONJUGATION TO LIPOSOMES

SUMMARY

The fusion of the ectodomain of the costimulatory molecule CTLA4 with the Fc segment of IgG has produced a soluble molecule (CTLA4Ig) that upon binding the B7 ligand expressed on antigen-presenting cells, exhibits immunosuppressive effects. Through increased avidity and accumulation in antigen-presenting cells, it has been proposed that CTLA4Ig conjugated to the surface of liposomes may inhibit T cell activity more effectively than free CTLA4Ig. Previous studies conducted with CTLA4Ig conjugated to the surface of liposomes in an undefined orientation revealed undesirable *in vivo* pharmacokinetics which were partly attributed to enhanced uptake via the Fc receptor on macrophages. In this study, we explored one way of conjugating CTLA4Ig to liposomes in a defined orientation. Lysine 397, on the C-terminus of the Fc IgG segment was mutated to cysteine for direct conjugation to maleimide-engrafted liposomes. We show that $CTLA4Ig_{K397C}$ liposomes exhibit enhanced inhibition of B7 binding sites in comparison to CTLA4Ig liposomes, suggestive of the increased avidity of the correctly oriented $CTLA4Ig_{K397C}$. In addition,

$CTLA4Ig_{K397C}$ liposomes inhibited T cell proliferation in an *in vitro* allogenic mixed lymphocyte reaction.

INTRODUCTION

The activation of T cells is dependent on the association of the T cell receptor with the major histocompatibility complex (MHC)-bound peptide as well as the generation of costimulatory signals [1]. The absence of a costimulatory signal can result in T cell inactivation [2]. CD28 and CTLA4 are two costimulatory molecules expressed on the surface of T cells with affinity for the B7 ligands (e.g. B7.1, B7.2) on antigen-presenting cells (APCs) [3, 4]. Upon B7 ligation, CD28 acts as a positive regulator of immune function through the production of various cytokines [5]. Conversely, CTLA4, a structural homolog of CD28, functions as a negative regulator of immune response by inhibiting T cell activation and proliferation [1].

The fusion of the ectodomain of CTLA4 with the Fc segment of IgG (CTLA4Ig) has been developed as a soluble molecule with affinity for the B7 ligands on APCs and has shown promise in its use in organ transplantation and various autoimmune disorders [4, 6-8]. However, it has been proposed that a more effective CTLA4Ig formulation would be desirable to decrease dosage amount and frequency [9]. Park et al. explored the ability of enhancing the efficacy of CTLA4Ig by conjugating the fusion protein to the surface of liposomes which, theoretically, would lead to increased avidity for B7-expressing cells [9]. The results of this study revealed enhanced binding to B7.1-expressing cells *in vitro* for CTLA4Ig liposomes compared to free CTLA4Ig, presumably due to the

multivalency of CTLA4Ig when conjugated to liposome membranes. Furthermore, *in vivo* studies using a mouse model showed increased accumulation of CTLA4Ig liposomes in the targeted engrafted tissue site. However, the conjugation of CTLA4Ig to liposomes resulted in a decreased half-life and increased accumulation in the liver and spleen. These results were attributed to enhanced uptake by macrophages via the Fc receptor due to the conjugation of CTLA4Ig on liposomes in the improper orientation [9].

In the present study, we explored an alternative method of conjugating CTLA4Ig to the liposome membrane in which the Fc segment theoretically was not exposed: the C-terminal lysine 397 of Fc was mutated to cysteine ($CTLA4Ig_{K397C}$) in order to form a site-specific thioether bond with maleimides on liposome membranes. The affinity of $CTLA4Ig_{K397C}$ liposomes for the B7 ligand was investigated in a binding study using B7.1-transfected P815 cells. The ability of $CTLA4Ig_{K397C}$ liposomes to block B7.1 binding was compared to that of CTLA4Ig liposomes. In addition, the inhibition of T cell proliferation was examined in an *in vitro* allogenic mixed lymphocyte reaction.

MATERIALS AND METHODS

Materials

All chemicals and reagents were analytical grade and purchased from Sigma-Aldrich (St. Lous, MO), unless otherwise noted. All tissue culture media and reagents were purchased from Invitrogen (Carlsbad, CA). L-α-phosphatidylcholine, (egg, chicken) (ePC) and maleimideparabenzoic-PE (MPB-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Liposome Preparation

All lipid films were prepared with a 2:1 molar ratio (10 μmol: 5 μmol) of ePC:cholesterol (Chol) with 2 mol% MPB-PE as described previously [9, 10]. Lipid films were resuspended with 500 uL HBSE 7.0 (10 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.0) and subjected to four freeze/ thaw cycles, followed by four cycles of extrusion through a 0.1 μm pore size filter (GE Water and Process Technologies). The liposomes were stored under argon at 4°C until the addition of CTLA4Ig.

Conjugation of CTLA4Ig and CTLA4Ig$_{K397C}$ to Liposomes

CTLA4Ig and CTLA4Ig$_{K397C}$ were provided by Dr. Chung-Gyu Park (Seoul National University, Seoul, South Korea). For CTLA4Ig-conjugated liposomes, CTLA4Ig in phosphate buffered saline (PBS) was first modified with SATA as previously described [9]. Briefly, 16 mg/mL SATA in dimethylformamide was prepared and added to CTLA4Ig at a molar and volume ratio of 20:1 and 1:100 (SATA:CTLA4Ig), respectively, for 25 min. at room temperature with gentle mixing. Excess SATA was then removed by dialyzing overnight at 1:500 (v/v) two times in HBSE 7.0. Upon removal of the protein from dialysis, the absorbance at 280 nm was measured and used to calculate the concentration: [CTLA4Ig/ CTLA4Ig$_{K397C}$ (mg/mL)] = (A_{280} x DF)/ ξ, where A_{280} = the absorbance of protein at 280 nm corrected for background and path length, DF = dilution factor, and ξ = extinction coefficient = 1.35 for CTLA4Ig and CTLA4Ig$_{K397C}$. Deacetylation buffer (0.5 M hydroxylamine, 25 mM EDTA, and 50 mM sodium phosphate, pH 7.0) was added at a 10:1 volume ratio of SATA-modified protein

solution:deacetylation buffer and incubated for 2 h under argon at room temperature with gentle mixing followed by the addition of 10 mM (final concentration) MES, pH 6.5.

For CTLA4Ig$_{K397C}$ (in PBS), cystines that might have formed were reduced by the addition of dithiothreitol (DTT) at a final concentration of 2 mM for 15 min. at room temperature with gentle mixing. Excess DTT was removed in a PD-10 desalting column (Sephadex G-25 medium, GE Healthcare) and the concentration of the CTLA4Ig$_{K397C}$ -containing fraction measured via absorbance as described above.

Liposomes were immediately added to deacetylated SATA-modified CTLA4Ig and reduced CTLA4Ig$_{K397C}$ at a 290:1 molar ratio of lipid:protein and incubated overnight under argon at 4°C with gentle mixing. Liposome-conjugated CTLA4Ig or CTLA4Ig$_{K397C}$ were separated from free protein by Sepharose CL-4B (GE Healthcare) gel filtration. Lipid concentration was determined by the method of Bartlett [11]. The concentration of conjugated protein was determined by running purified liposomes along with known quantities of CTLA4Ig to generate a standard curve in an SDS-PAGE gel, followed by detection with Krypton stain (Thermo Scientific) and quantification using a Typhoon 9200 Imager (GE Healthcare).

The Competitive Binding Assay and Allogenic Mixed Lymphocyte Reaction (MLR)

The binding assays and MLR assays were performed by Bongi Kim and Dr. Chung-Gyu Park (Department of Microbiology and Immunology, Seoul National University College of Medicine, Seoul, South Korea) as described in [9].

RESULTS

Affinity of CTLA4Ig$_{K397C}$ for B7.1

In a study performed by B. Kim, the affinity of soluble CTLA4Ig$_{K397C}$ for B7.1 was examined by treating B7.1-transfected P815 cells with different concentrations of CTLA4Ig or CTLA4Ig$_{K397C}$, washed, treated with FITC-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.), and analyzed. Flow cytometric results revealed that soluble CTLA4Ig$_{K397C}$ bound B7.1-expressing cells with similar affinity as soluble CTLA4Ig (Figure A.1) (B. Kim and C.-G. Park, *unpublished data*).

Conjugation of CTLA4Ig$_{K397C}$ to Liposomes

The production of monovalent CTLA4Ig has been shown to result in a bivalent molecule connected by disulfide bonds [4]. Thus, the conjugation of CTLA4Ig$_{K397C}$ to liposomes required the use of a sufficient amount of reducing agent to assure that the added cysteine is in its reduced form without reducing interchain disulfides. To investigate whether the reducing conditions used were appropriate, CTLA4Ig and CTLA4Ig$_{K397C}$ were reduced with 2 mM DTT for 15 min. at room temperature. After removal of excess DTT via gel filtration, the

proteins were run in a non-reducing gel; both fusion proteins migrated at ~ 100 kDa (Figure A.2A). For comparison, 5 mM of the reductant Tris (2-Carboxyethyl) phosphine hydrochloride (TCEP) was added to CTLA4Ig$_{K397C}$ and heated at 100°C for 5 min. to produce a fully reduced protein, ~58 kDa in size on a non-reducing gel (Figure A.2B). These results suggest that CTLA4Ig$_{K397C}$ remains a bivalent molecule under the milder reducing conditions. Furthermore, the incubation of reduced CTLA4Ig$_{K397C}$ and CTLA4Ig with liposomes resulted in the conjugation of CTLA4Ig$_{K397C}$, but not CTLA4Ig, to the liposome surface, suggesting that only the added cysteine was reduced (Figure A.2C).

For liposomes prepared for subsequent reactions, free sulfhydryls were produced using SATA modification for CTLA4Ig and DTT for CTLA4Ig$_{K397C}$ (as detailed under Materials and Methods), followed by liposome conjugation. The fusion protein-conjugated liposomes were purified from unbound fusion protein in a Sepharose CL-4B column. The presence of fusion protein in the liposome column fractions as detected by SDS-PAGE signified successful conjugation reactions (Figure A.3). The amount of fusion protein per liposome was quantified using a standard curve generated from known quantities of CTLA4Ig protein run in the same gel. Based on an expected average liposome size of 100 nm, the number of fusion protein molecules conjugated to the surface was estimated at approximately 150-200 molecules of CTLA4Ig$_{K397C}$ or CTLA4Ig per liposome.

CTLA4Ig$_{K397C}$ Liposomes Effectively Block the B7.1 Binding Site

The ability of CTLA4Ig$_{K397C}$ liposomes to block the B7.1 binding site was analyzed by B. Kim in a competitive binding assay with FITC-conjugated

CTLA4Ig (CTLA4Ig-FITC). P815 or B7.1-transfected cells were pre-incubated with the Fc receptor-specific monoclonal antibody, 2.4G2, to block Fc receptor binding. $CTLA4Ig_{K397C}$ liposomes or free CTLA4Ig were then incubated with B7.1-transfected P815 cells followed by a subsequent incubation with CTLA4Ig-FITC. The relative amount of CTLA4Ig-FITC that bound to free B7.1 ligands or competed off either the prebound $CTLA4Ig_{K397C}$ liposomes or free CTLA4Ig was analyzed by flow cytometry. Treatment of untransfected P815 cells and B7.1-transfected P815 cells with CTLA4Ig-FITC alone revealed the minimal and maximal amounts of bound CTLA4Ig-FITC, respectively (Figure A.4). The pre-incubation of cells with $CTLA4Ig_{K397C}$ liposomes resulted in a partial inhibition of B7.1 binding by CTLA4Ig-FITC. The leftward shift in fluorescence of the cells pre-incubated with $CTLA4Ig_{K397C}$ liposome in comparison to free CTLA4Ig suggests that $CTLA4Ig_{K397C}$ liposomes bind B7.1 with greater avidity than free CTLA4Ig (B. Kim and C.-G. Park, *unpublished data*).

The strength of the interaction between $CTLA4Ig_{K397C}$ liposomes and B7.1 in comparison to CTLA4Ig liposomes was explored by B. Kim in a competitive binding assay with varying concentrations of liposomes. Samples in which cells were treated with free CTLA4Ig were also run in parallel. The '% Staining of Control' was calculated at each protein concentration as a measure of the percentage of B7.1 binding sites that were blocked by the free or liposome-conjugated fusion protein in relation to the total number of B7.1 surface ligands (as detected by B7.1-transfected P815 cells directly treated with CTLA4Ig-FITC). Half-maximal inhibition was achieved with 0.0025 mg/mL $CTLA4Ig_{K397C}$

liposomes (Figure A.5). In comparison, higher concentrations of CTLA4Ig liposomes (0.05 mg/mL) and even higher concentrations of free CTLA4Ig (0.19 mg/mL) were required to block 50 % of the total B7.1 binding sites. These results suggest that CTLA4Ig$_{K397C}$ liposomes bind to B7.1 with greater avidity than CTLA4Ig liposomes (B. Kim and C.-G. Park, *unpublished data*).

CTLA4Ig$_{K397C}$ Liposomes Block Allogenic MLR

To investigate the ability of CTLA4Ig$_{K397C}$ liposomes to inhibit T cell activation, B. Kim performed an allogenic MLR by mixing the T cells from BALB/c mice with APCs from C57BL/6 mice and measured the incorporation of ^{3}H-thymidine in proliferating T cells. All fusion protein formulations inhibited T cell proliferation in a concentration-dependent manner (Figure A.6). CTLA4Ig$_{K397C}$ liposomes showed nearly complete inhibition at 10 µg/ mL CTLA4Ig$_{K397C}$ with 50% inhibition around 0.03 µg/ mL. In comparison, the half-maximal inhibitory response of free CTLA4Ig and CTLA4Ig liposomes was at ~0.4 µg/ mL CTLA4Ig (B. Kim and C.-G. Park, *unpublished data*).

DISCUSSION

In this study, we investigated the ability to create a CTLA4Ig mutant in which the C-terminal lysine-397 of the IgG segment was replaced with a cysteine. This cysteine provided a means of conjugating the CTLA4Ig$_{K397C}$ to the liposome membrane in a defined orientation. Previous studies in which the original form of CTLA4Ig was conjugated to liposomes after SATA modification revealed a liposome formulation with affinity for B7.1 *in vitro*, but a decreased

circulation time *in vivo* and enhanced accumulation in the liver and spleen [9]. These undesirable *in vivo* characteristics were attributed to the conjugation of some CTLA4Ig to the liposome membrane with the Fc segment exposed, resulting in enhanced internalization of CTLA4Ig liposomes by the Fc receptor on macrophages. In order to achieve a longer-lasting CTLA4Ig formulation, it was thus necessary to develop a method of conjugating CTLA4Ig to the liposome membrane that allows for greater control over CTLA4Ig's orientation. We show that $CTLA4Ig_{K397C}$ can bind to the liposome membrane, forming $CTLA4Ig_{K397C}$ liposomes; these liposomes bind B7.1 ligands with greater apparent strength than CTLA4Ig liposomes. In addition, $CTLA4Ig_{K397C}$ liposomes inhibit the proliferation of T cells as shown in the allogenic mixed lymphocyte reaction (B.Kim, C.-G.Park, *unpublished data*).

Part of the initial rationale for developing CTLA4Ig-conjugated liposomes, in which multiple copies of the protein are immobilized on the liposome membrane, was to create a delivery system with an avidity for B7.1 that is greater than that of soluble CTLA4Ig [9]. In comparison to free CTLA4Ig, enhanced B7.1 inhibition has been previously shown with CTLA4Ig liposomes and was demonstrated in this study with $CTLA4Ig_{K397C}$ liposomes (Figure A.4) [9]. Furthermore, our data show that $CTLA4Ig_{K397C}$ liposomes were able to block B7.1 binding sites more efficiently than CTLA4Ig liposomes (Figure A.5). $CTLA4Ig_{K397C}$ liposomes exhibited 50% inhibition of binding of CTLA4Ig-FITC to the B7.1-transfected cells at a concentration that was 20-fold less than that of CTLA4Ig liposomes. We interpret the greater apparent avidity of $CTLA4Ig_{K397C}$

liposomes for B7.1 as the consequence of CTLA4 being more accessible to B7.1 due to the favorable orientation of CTLA4Ig$_{K397C}$ on the liposome surface. The preliminary *in vitro* MLR assay results were also suggestive of the increased avidity of CTLA4Ig$_{K397C}$ liposomes, as T cell proliferation was predominantly inhibited at lower concentrations of CTLA4Ig$_{K397C}$ liposomes as compared to CTLA4Ig in its free or liposomal form (Figure A.6) (B.Kim, C.-G.Park, *unpublished data*).

CONCLUSIONS

We have shown that the mutation of lysine 397 to cysteine in the Ig portion of CTLA4Ig results in a fusion protein (CTLA4Ig$_{K397C}$) that, upon reduction, can bind maleimide-containing liposome membranes. As a free soluble protein, CTLA4Ig$_{K397C}$ displays affinity for B7.1-expressing cells similar to that of CTLA4Ig. Upon conjugation to liposomes, however, CTLA4Ig$_{K397C}$ liposomes show enhanced B7.1 binding relative to that of CTLA4Ig liposomes. Taken together, these results suggest that CTLA4Ig$_{K397C}$ liposomes have higher avidity for B7.1, which may be due to the conjugation of CTLA4Ig$_{K397C}$ to the liposome surface in a more favorable orientation. Furthermore, the ability of CTLA4Ig$_{K397C}$ liposomes to inhibit T cell activation in the *in vitro* MLR assay suggests that the CTLA4Ig$_{K397C}$ liposomes have the potential to be effective *in vivo* as well.

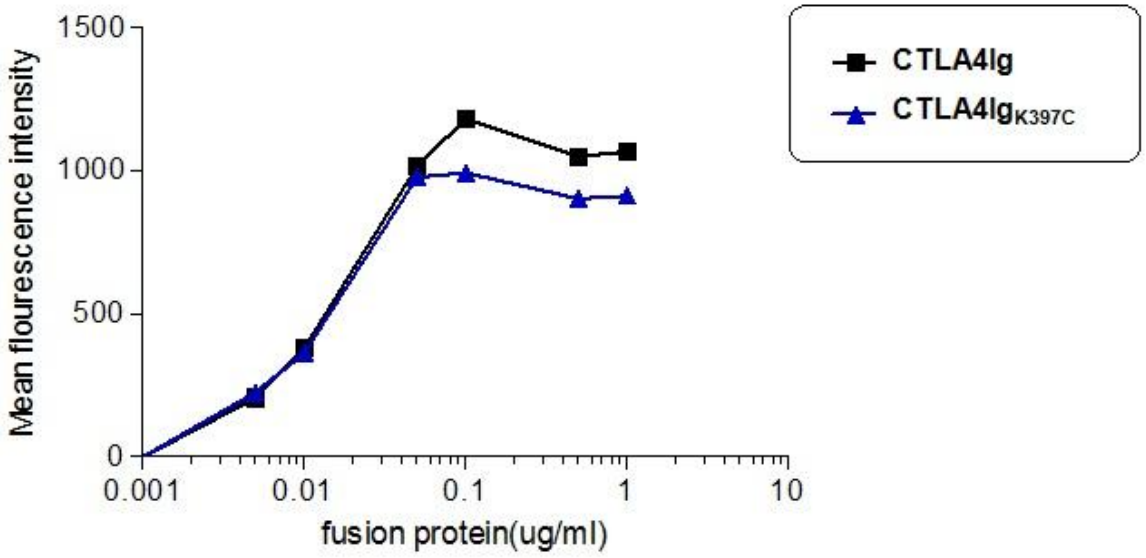

Figure A.1: Binding of Fusion Proteins to B7.1-Transfected Cells

B7.1-transfected P815 cells were treated with various concentrations of CTLA4Ig or CTLA4Ig$_{K397C}$, washed, treated with FITC-conjugated anti-mouse IgG, and analyzed via flow cytometry. (B. Kim and C.-G. Park, *unpublished data*).

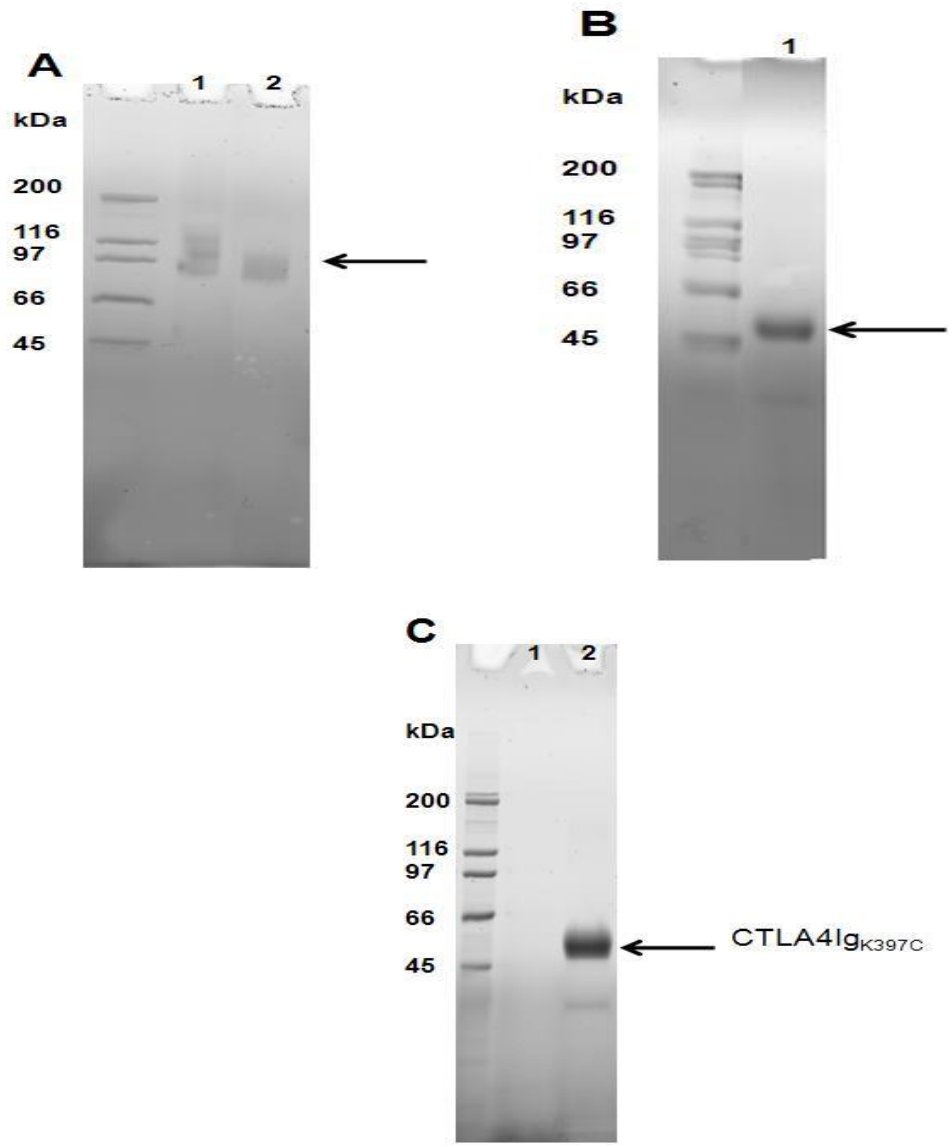

Figure A.2: Conjugation of CTLA4IgK397C to the Liposome Membrane

CTLA4Ig$_{K397C}$ and CTLA4Ig were reduced with 2 mM DTT for 15 min. at room temperature and excess DTT was removed by gel filtration. (A) Non-reducing gel of fusion proteins after treatment with DTT. Proteins migrated as a bivalent molecule with an apparent molecular weight of ~ 100 kDa. *1 = CTLA4Ig; 2 = CTLA4Ig$_{K397C}$* (B) CTLA4Ig$_{K397C}$ was reduced under stronger reducing conditions, yielding a monovalent molecule ~58 kDa in size. (C) Fusion proteins were incubated with maleimide-containing liposomes overnight and then purified in a Sepharose CL-4B gel filtration column and run in a reducing SDS-PAGE gel. *1 = CTLA4Ig liposomes; 2 = CTLA4Ig$_{K397C}$ liposomes*

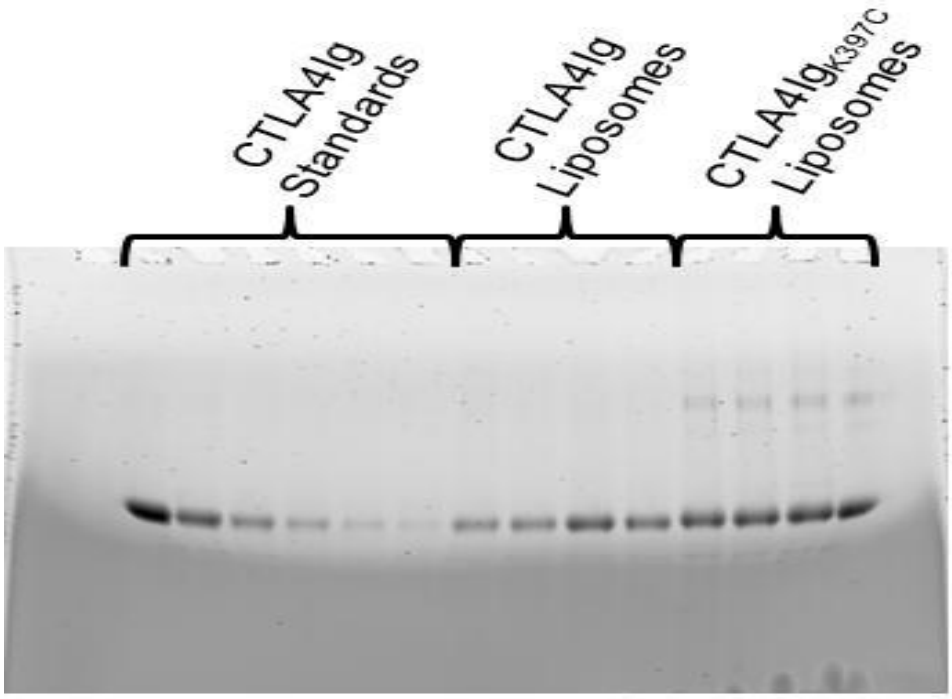

Figure A.3: Representative Gel of Purified CTLA4Ig or CTLA4Ig$_{K397C}$ Liposomes

After overnight conjugation, CTLA4Ig liposomes and CTLA4Ig$_{K397C}$ liposomes were purified in a Sepharose CL-4B column and run in an SDS-PAGE gel along with known quantities of CTLA4Ig to construct a standard curve.

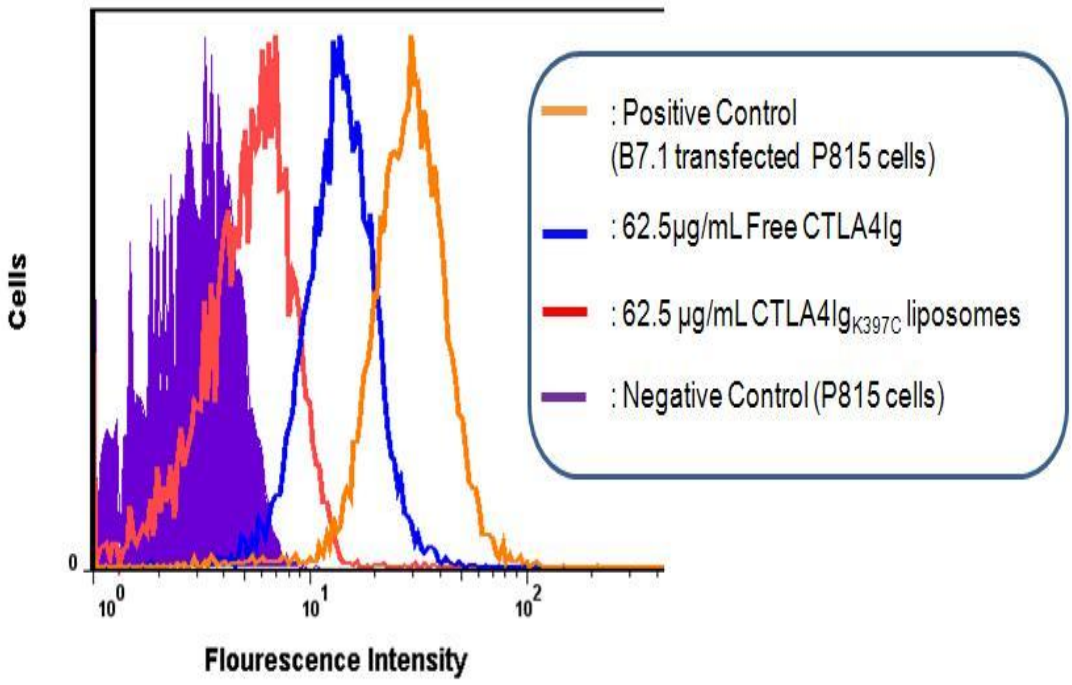

Figure A.4: Competitive Binding Assay with CTLA4Ig$_{K397C}$ Liposomes

P815 cells transfected with B7.1 were treated with free CTLA4Ig or CTLA4Ig$_{K397C}$--conjugated liposomes followed by FITC-labeled CTLA4Ig. The ability of free CTLA4Ig or CTLA4Ig$_{K397C}$-conjugated liposomes to block B7.1 binding by CTLA4Ig-FITC was analyzed using flow cytometry. For comparison, untreated B7.1 transfected cells and P815 cells were also labeled with CTLA4Ig-FITC. (B. Kim and C.-G. Park, *unpublished data*).

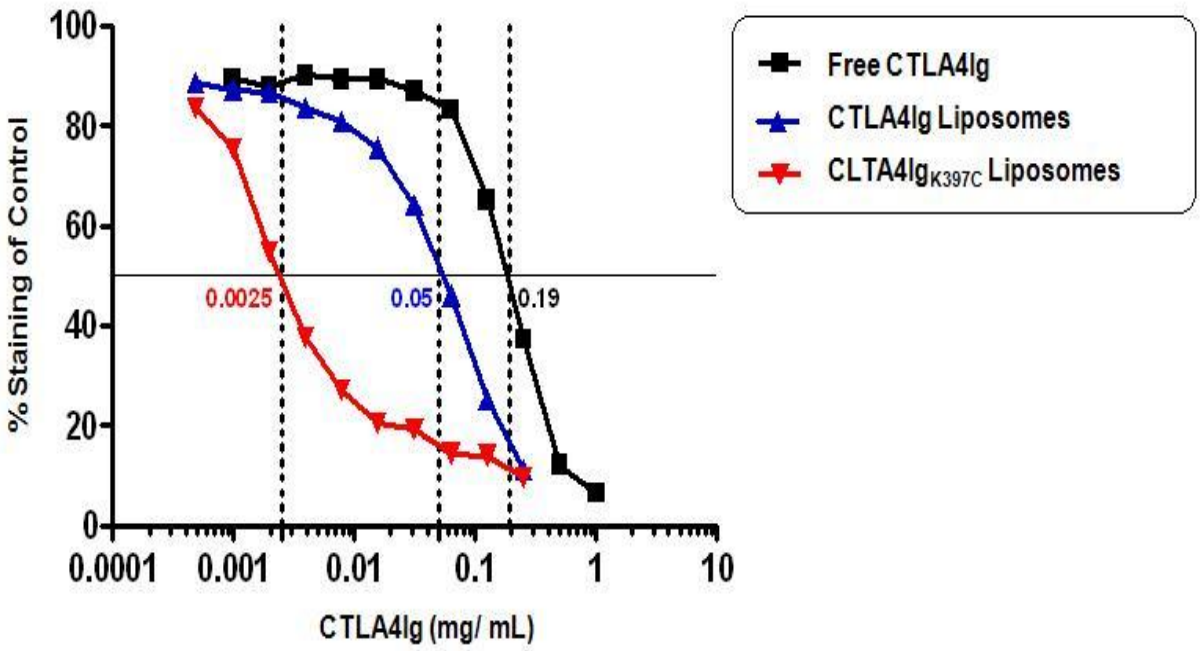

Figure A.5: CTLA4Ig$_{K397C}$ Liposomes Block B7.1 Binding More Effectively than CTLA4Ig Liposomes or Free CTLA4Ig

B7.1-transfected P815 cells were incubated with different concentrations of free CTLA4Ig, CTLA4Ig-liposomes, or CTLA4Ig$_{K397C}$-liposomes at 4°C followed by incubation with FITC-labeled CTLA4Ig and flow cytometry analysis. (B. Kim and C.-G. Park, *unpublished data*).

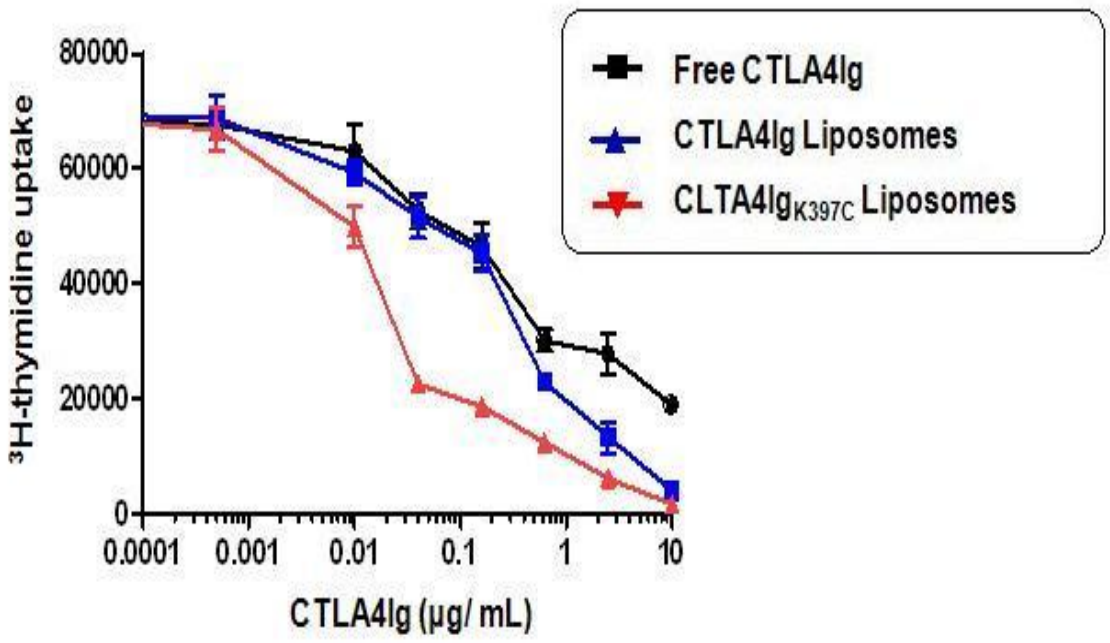

Figure A.6: CTLA4Ig$_{K397C}$ Liposomes Inhibit the Proliferation of T Cells

Lymph nodes harvested from BALB/c mice were mixed with splenocytes harvested from C57BL/6 mice that were depleted of T cells. Free CTLA4Ig, CTLA4Ig$_{K397C}$ liposomes, or CTLA4Ig liposomes were added and incubated for 80 h at 37°C, followed by a 16 h pulse with ^{3}H-thymidine. Upon completion of the total 96 h, the amount of ^{3}H-thymidine taken up by proliferating T cells was measured in a scintillation counter (B. Kim and C.-G. Park, *unpublished data*).

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