INCORPORATION OF CpG OLIGODEOXYNUCLEOTIDES INTO α2-MACROGLOBULIN:
DEVELOPMENT OF A NOVEL VACCINE ADJUVANT DELIVERY MECHANISM

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy
in the Department of Pathology in the
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ABSTRACT
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Abstract

Bacterial DNA is immunostimulatory, and the motifs responsible for this activity are unmethylated CpG dinucleotides. Following cellular uptake, CpG-containing oligodeoxynucleotides (CpG ODN) are trafficked to the endosome where they bind Toll-like receptor 9 (TLR9) to initiate a signaling cascade that culminates in the release of numerous pro-inflammatory cytokines. Because of their immunostimulatory properties, CpG ODN are being clinically evaluated as treatments and vaccine adjuvants for infectious diseases, cancer, and allergic disorders.

α2-Macroglobulin (α2M) is a human plasma protein that binds and modulates the activity of a variety of cytokines, growth factors, enzymes, and antigens. Upon proteolytic activation, α2M is converted to its receptor recognized form, α2M*, and rapidly binds to and is internalized by immune competent cells expressing the α2M* endocytic receptor, LRP, and is then trafficked to the endosome. Based on these interactions, α2M seems to play an important role at sites of infection and inflammation by controlling the level of proteinase activity, modulating cytokine signals, and enhancing antigen processing for the adaptive immune response.

Here, we report the first evidence that α2M* binds and forms stable complexes with nucleic acids. We have characterized the mechanisms and stoichiometry of this interaction, examined the pH and temperature stability of these complexes, and identified structural variables in the nucleic acids, namely length, base composition, and chemical modifications, that affect the nature of this interaction. We hypothesized that CpG ODN incorporation into α2M* may alter their immunostimulatory properties. Murine
macrophages (MΦs) treated with α2M*-ODN complexes respond more rapidly and produce a greater cytokine response than those treated with free CpG ODN alone. Treating human PBMCs with α2M*-ODN complexes likewise demonstrated their enhanced ability to elicit immune responses. This was due to more rapid uptake and CpG ODN protection from degradation by extracellular nucleases. Co-incorporation of both protein ligands and CpG ODN into α2M* yields ternary complexes; these may permit the simultaneous delivery of both protein antigens and adjuvants to immune competent cells, potentially greatly enhancing the adaptive immune response and protective immunity.

Based on the findings that incorporation into α2M* confers enhanced immunostimulatory activity of CpG ODN, this technology may be exploited to improve CpG ODN-based therapeutics by increasing efficacy, minimizing side effects, reducing dosing requirements, and reducing cost.
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List of Abbreviations

$\alpha_2$M, $\alpha_2$-Macroglobulin

$\alpha_2$M*, $\alpha_2$-Macroglobulin, receptor-recognized/activated/fast-form

$\alpha_2$M*-ODN, $\alpha_2$-Macroglobulin-oligodeoxynucleotide complex

Ab, antibody

Ag, antigen

APC, antigen presenting cell

BHV-1, bovine herpes virus 1

BSA, bovine serum albumin

CD, cluster of differentiation

CpG, 5’-CG-3’ dinucleotide

CpG ODN, CpG-containing oligodeoxynucleotide

Cys$_{949}$, cysteine residue 949

DC, dendritic cell

DMEM, Dulbecco’s Modified Eagle Media

DNA, deoxyribonucleic acid

ds, double-stranded

EDTA, ethylene diaminetetraacetic acid

ELISA, enzyme linked immunosorbent assay

FACS, fluorescence-activated cell sorting

FGF, fibroblast growth factor

FPLC, fast protein liquid chromatography
Glx$_{952}$, glutamic acid residue 952
GRP78, glucose response protein 78
HEL, hen egg lysozyme
HIV gp120, human immunodeficiency virus glycoprotein 120
HNE, human neutrophil elastase
IFN$\alpha$, interferon-$\alpha$
IFN$\gamma$, interferon-$\gamma$
Ig, immunoglobulin
IL-1, interleukin 1
IL-4, interleukin 4
IL-5, interleukin 5
IL-6, interleukin 6
IL-10, interleukin 10
IL-12, interleukin 12
IRAK1, interleukin-1 receptor-associated kinase
K$_d$, dissociation constant
kDa, kilodalton
LAL, limulus amebocyte lysate
LPS, lipopolysaccharide
LRP, low-density lipoprotein receptor related protein
M$\Phi$, macrophage
MAPK, mitogen-activated protein kinase
MARCO, macrophage receptor with collagenous structure
MCP-1, monocyte chemotactic protein-1
MHC, major histocompatibility complex
MIP-1, macrophage inflammatory protein-1
MyD88, myeloid differentiation factor 88
NF-κB, nuclear factor κB
NGF, neural growth factor
NK, natural killer
ODN, oligodeoxynucleotides
ODN #1826, 5' - TCCATGACGTTCTGACGTT - 3'
ODN #2395, 5' - TCGTCGTTTTCGGCGCGCGCCG - 3'
PEG, polyethylene glycol
PGE₂, prostaglandin E₂
P=O, phosphodiester
P=S, phosphorothioate
PAGE, polyacrylamide gel electrophoresis
PAMP, pathogen associated molecular pattern
PBMC, peripheral blood mononuclear cells
PBS, phosphate buffered saline
pDC, plasmacytoid dendritic cell
PDGF, platelet-derived growth factor
Pu, purine
Py, pyrimidine
RNA, ribonucleic acid
RPM, revolutions per minute

RPMI, Roswell Park Memorial Institute

SDS, sodium dodecyl sulfate

ss, single stranded

TBE, tris/boric acid/EDTA

TG, thiolglycollate

TGF-β1, transforming growth factor-β1

TGF-β2, transforming growth factor-β2

TLR, toll-like receptor

TLR3, toll-like receptor 3

TLR4, toll-like receptor 4

TLR9, toll-like receptor 9

TNFα, tumor necrosis factor α

TRAF6, TNF-receptor-associated factor-6

VLP, virus-like particles
Chapter 1: Introduction
1.1 CpG Oligodeoxynucleotides

The ability to discriminate self from non-self is vital to the function of the immune system. To do this, its cells have developed Pattern Recognition Receptors (PRR) specific to molecular structures that are expressed by pathogens but not by cells of the body. Examples of such Pathogen-Associated Molecular Patterns (PAMP) include lipopolysaccharide (LPS), peptidoglycan, and flagellin. A family of homologous receptors, termed Toll-like receptors (TLR), recognize these PAMP; to date, thirteen have been characterized [2-4]. Each receptor in the family is specific for a particular PAMP; TLR3 binds double-stranded RNA [5], TLR4 recognizes LPS [6], and the ligand for TLR9 is bacterial DNA [7], which is the focus of this study.

It has been known for two decades that bacterial DNA is immunostimulatory [8-11]. In 1995, the biologically active structure was identified as the CpG dinucleotide [12]. The immunostimulatory properties of bacterial DNA and its synthetic mimic, CpG oligodeoxynucleotides (CpG ODN), have been studied extensively. Both have been shown to induce the production of reactive oxygen species [13] and activate signaling pathways, including mitogen-activated protein kinases (MAPK) and NF-κB [14]. CpG ODN activate a number of immune competent cells, including B cells, monocytes, macrophages (MΦs), dendritic cells and NK cells and induce them to proliferate, upregulate both MHC classes and co-stimulatory molecules, and produce a multitude of pro-inflammatory cytokines, including IL-1, IL-6, IL-10, IL-12, IFNα, IFNγ and TNFα [15-19]. When mice are treated with CpG ODN, there is an increase in IL-12 and IFN
as well as enlarged draining lymph nodes, which have a large population of activated dendritic cells [22].

CpG ODN stimulate innate immune responses. Treatment with CpG ODN prior to infectious challenge protects mice against *Listeria monocytogenes* [20], *Francicella tularensis* [23], malaria [24] and herpes simplex virus [25, 26]. Treatment with CpG ODN protects mice from Leishmania infection and can even cure chronic Leishmania infection [27]. In addition, CpG ODN enhance the adaptive immune response. Cattle immunized with bovine herpes virus 1 glycoprotein gD and CpG ODN develop greater immune responses and protection against BHV-1 [28]. Pigs show an enhanced antigen-specific antibody response to the OmlA antigen of *Actinobacillus pleuropneumoniae* when CpG ODN is added to the vaccine formulation [29]. Orangutans, which normally have a seroconversion rate of less than 15% when vaccinated against Hepatitis B, show 100% conversion with the addition of CpG ODN to the vaccine [30].

### 1.2 Mechanism of CpG ODN Immunostimulation

An initial question was how the immune system distinguishes between DNA of foreign sources from the body’s own DNA, which is constantly released from cells that sustain damage or undergo apoptosis. This can be explained by key intrinsic differences between prokaryotic and eukaryotic DNA. Bacterial DNA exhibits the expected frequency of the CpG dinucleotide, which is 1:16. However, genomic sequence analysis of vertebrate DNA has shown that this dinucleotide is present at a quarter of the expected frequency [31]. Furthermore, bacterial CpG dinucleotides are not methylated, while
roughly 70% of vertebrate cytosines are methylated on carbon five [32]. When the cytosines in the CpG dinucleotide of immunostimulatory CpG ODN are replaced with 5-methyl cytosine, their immunostimulatory properties are lost [12]. The sequences surrounding the CpG motif are also important; sequences that enhance and those that blunt the activity of the CpG motif have been identified [12, 33]. With the combination of CpG under-representation, the predominance of cytosine methylation, and the necessity for specific surrounding sequence contexts, the immunostimulatory CpG motif is sufficiently rare to prevent immune stimulation by vertebrate DNA.

Researchers have uncovered much of the mechanism by which CpG DNA stimulates immune competent cells. CpG DNA is taken up by cells via pinocytosis and receptor-mediated endocytosis [34, 35]. These mechanisms are still under investigation, but many proteins involved in the process have been identified [36-40]. Upon internalization, nucleic acids are trafficked to the endosome, which undergoes maturation and recruits endoplasmic reticulum-derived vesicles containing TLR9, the signaling receptor for CpG DNA [13, 41-43]. These vesicles merge, and TLR9 binds CpG DNA in a CpG-dependent manner [44]. On binding, TLR9 signals through the adaptor protein myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase (IRAK1) and TNF-receptor-associated factor-6 (TRAF6) [45]. This cascade culminates in activation of NF-κB [33, 46, 47] and AP-1 transcription family members to produce cytokines that heavily favor a Th1-biased response [17].

Each immune competent cell type responds differently to CpG ODN. B cells are induced to proliferate and secrete IL-6 and IL-10, which leads to increased production of IgM [48, 49]. B cells also upregulate Fcγ receptor and costimulatory molecules, MHC II,
CD80, and CD86 [12, 50]. CpG ODN activate dendritic cells to secrete IL-6, TNFα, and IFNα and to upregulate their expression of MHC II, ICAM-I and costimulatory molecules CD40, CD54, CD80, and CD86 [51-54]. In mice, MΦs express TLR9 and are activated to secrete TNFα, IL-12 and PGE₂ and to increase expression of cyclooxygenase-2 [55, 56]. Human MΦs do not express TLR9 but are indirectly activated to produce IL-6 and TNFα and to upregulate CD40 and CD69 [57, 58].

While the cytokine burst induced by bacterial DNA affects a large variety of immune competent cells, only those cells expressing TLR9 are directly responsive to CpG DNA [59]. The cell types that express TLR9 differ across species; murine cell types that have TLR9 include MΦs, B cells and dendritic cells [7, 60]. TLR9 expression in humans is limited to B cells, plasmacytoid dendritic cells (pDC), and NK cells [7, 9, 59, 61].

1.3 Therapeutic Applications of CpG ODN

CpG ODN have received a great deal of attention for their numerous potential therapeutic applications. CpG ODN exhibit a number of characteristics that make them promising candidates for use as a new vaccine adjuvant. They induce strong responses from B and T cells and strongly promote a T_{H}1-biased inflammatory response [17], which is of particular interest because alum, the only adjuvant approved for clinical use, induces a T_{H}2-biased response, severely limiting its efficacy against intracellular infections [62]. CpG ODN activate APC when administered by injection as well as by oral and mucosal delivery [63]. CpG ODN are also under investigation for their potential
use in the treatment of cancer. By activating lymphocytes and inducing the production of IFN\(\gamma\), CpG ODN improve immunosurveillance of cancer and prevent tumor growth [64]. Others have put forth the strategy using CpG ODN as a treatment for allergic diseases [65-68]. As an example, asthma develops as the result of an immune response to harmless environmental antigens. Typically, the response is characterized by the predominance of T cells that produce T\(\text{H}_2\) cytokines IL-4 and IL-5, causing B cells to aberrantly differentiate and produce IgE, which binds its receptor on mast cells and basophils. Subsequent exposure triggers degranulation of mast cells and inflammation. However, since a balance tends to exist between T\(\text{H}_1\) and T\(\text{H}_2\) cytokine profiles, enhancing the T\(\text{H}_1\) response should reduce the strength of the T\(\text{H}_2\) profile. CpG ODN have such a strong bias toward producing T\(\text{H}_1\)-type responses, they may be able to treat disorders that arise from T\(\text{H}_2\) responses [65]. Based on their potential pharmaceutical applications, a variety of strategies have been employed to enhance the biological activities of CpG ODN. Sequence variations have been evaluated in high throughput systems to determine the optimal ODN length, number of CpG motifs, and sequences surrounding the CpG dinucleotide for immune stimulation [69, 70]. Three classes of CpG ODN have been identified; they are distinguished by differences in sequence and backbone modification, and each stimulates a different subset of cells to produce qualitatively distinct responses. Class A (D type) ODN, composed of a palindromic P=O middle segment capped with P=S-modified ends, activate NK cells and pDC to produce IFN\(\gamma\) and IFN\(\alpha\), respectively [69, 71, 72], but fail to stimulate B cells. Class B (K Type) ODN are wholly P=S sequences with multiple CpG motifs and a poly(G) tail at the 3’ end; this class triggers IgM and IL-6 production from B cells, activates pDC to produce
TNF rather than IFNα, and stimulates strong cytolytic activity in NK cells [70, 71, 73].

Class C ODN is a hybrid of the first two classes with a completely P=S-modified backbone, palindromic sequences, but no poly(G) tail; this class has the ability to induce both type I IFN production as well as B cell activation[70].

1.4 Barriers to CpG ODN Efficacy

Despite promising results, studies have revealed potential obstacles to CpG ODN clinical use. Depending on the method of administration, there are numerous obstacles to CpG ODN stimulating their target cells, including degradation by nucleases, uptake by non-responsive cells, and non-specific binding to plasma proteins. CpG ODN composed of the naturally occurring phosphodiester (P=O) backbone are rapidly degraded in vivo by nucleases, often preventing the desired immune response [51, 74, 75]. Efficacy can be improved with higher doses and repeated dosing [76]; however, this can cause systemic side effects, including splenomegaly and lethal toxic shock [77, 78]. The potency of CpG ODN can be greatly enhanced by chemical modification of the backbone.

Substitution of a non-bridging oxygen with sulfur, known as phosphorothioate (P=S) modification, confers nuclease resistance [77, 79]. This modification, however, is not without its own drawbacks. P=S-modified ODN have numerous CpG motif-independent effects that result from increased non-specific binding to plasma proteins [76, 80] as well as cell surfaces, which can lead to the disruption of normal cellular functions. P=S CpG ODN interact with essential protein tyrosine receptors Flk-1 and EGFR, causing inhibition of ligand-receptor activation and disruption of cellular metabolic activities.
[81]. There also appears to be CpG motif-independent immune activation that can have deleterious results like lymphadenopathy [22], lymphoid follicle destruction [82], granuloma formation [83], and exacerbation of autoimmune diseases [84]. In addition to these side effects, P=S CpG ODN are taken up preferentially by a subset of immune competent cells that differs from the cells that take up P=O CpG ODN. Furthermore, the responses that each type of CpG ODN elicit are qualitatively different, with altered cytokine profiles, cellular maturation and proliferation, and antibody production [70, 71]. These effects do not preclude their clinical utility, but they are described here to highlight that they are implicit in the use of P=S ODN. The clinical trials that are underway employ the use of P=S CpG ODN because of their superior potency, but there are still potentially serious side effects that will define the limits of dosing amounts and frequency, thus potentially limiting efficacy. It is conceivable that there are certain disorders- cancer, infectious diseases, allergic disorders- that would be treated more effectively by the type of responses elicited by P=O rather than P=S ODN. Given the poor pharmacokinetic profile of P=O ODN, their efficacy remains low. If, however, P=O CpG ODN could be protected from nuclease digestion and taken up rapidly by immune competent cells, it is likely that their efficacy would be greatly improved.

The ultimate utility of CpG ODN may still rest on the ability to reduce these side effects by altering the pharmacokinetics of CpG ODN. To this end, numerous investigators have packaged CpG ODN into discrete units that exhibit enhanced uptake thereby reducing the harmful effects resulting from off-target immune stimulation and non-specific protein binding. P=O CpG ODN incorporated into cationic liposomes elicit strong immune responses in vivo. However, effects are CpG motif-independent [85], and
the use of liposomes is limited by their toxic effects [86]. CpG ODN have also been packaged into virus-like particles (VLP), which improve efficacy and reduce systemic side effects [87]. However, the high cost of production and the potential for multiple exposures to break tolerance of VLP may pose significant barriers to their widespread use.

A potential solution to these problems is to package CpG ODN into a molecule or microparticle that is taken up specifically by immune competent cells. Ideally, this would protect CpG ODN from non-specific binding to plasma proteins and cell surfaces as well as prevent digestion by nucleases. Preventing the inactivation of CpG ODN by these mechanisms could increase their potency enough to reduce dosing requirements, thus reducing side effects. Likewise, specific delivery to responsive cells could improve their overall efficacy, increasing their clinical utility.

1.5 $\alpha_2$-Macroglobulin

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a plasma protein that was first identified as a proteinase inhibitor and later shown to play a role in both antigen presentation and neuronal growth cone development. In its native form, this 718 kDa tetrameric protein is composed of two cage-like structures, which are linked by disulfide bonds. Like other members of the thiolester superfamily, complement proteins C3 and C4, $\alpha_2$M is converted by proteolysis to an activated form, denoted as $\alpha_2$M* [88, 89]. Proteolytic cleavage induces a conformational change whereby the cages close, capturing the
proteinase and potentially other molecules within the $\alpha_2$M* binding pockets (Figure 1A). Upon activation, the receptor binding site is exposed, and $\alpha_2$M*, along with the molecules trapped inside, is rapidly taken up into cells by receptor-mediated endocytosis and delivered to the endosome [90].

### 1.6 $\alpha_2$M Mechanism of Activation and Uptake

$\alpha_2$M has a unique mechanism by which it inhibits proteinases (Reviewed in [91]). The four identical subunits form a cage-like structure with two large pockets on either side of the central base plate of the molecule. Deep within each pocket is a sequence of 20-25 amino acids, the “bait region”, that is highly reactive to proteinases. Proteolytic cleavage causes a minor conformational change that exposes a thiolester bond, making it highly susceptible to nucleophilic substitution by primary amines. This substitution reaction cleaves the thiolester bond (Figure 1B), which causes a major conformational change in $\alpha_2$M; the arms of the cage close, entrapping the proteinase within the pocket. The resulting “activated” conformation, $\alpha_2$M*, is more compact than its native structure, conferring greater mobility in an electrophoretic field. The change can be observed as more rapid migration on PAGE run under native conditions (Figure 1C).

Upon activation of $\alpha_2$M, the long arms of each subunit come into close proximity with each other and form the receptor recognition site [92]. The molecule may then be taken up by its high affinity receptor, the low-density lipoprotein receptor-related protein (LRP/CD91), which is expressed by a number of cell types, including MΦs, B cells, fibroblasts, dendritic cells and hepatocytes [93, 94]. Once the protein takes on its
receptor-recognized form, it has a half-life in plasma of 2-5 min [92]; this rapid uptake is evidence of the efficiency with which $\alpha_2\text{M}^*$ is taken up. Following endocytosis, $\alpha_2\text{M}^*$ and its contents are trafficked to the endosome, which becomes acidified. This destabilizes $\alpha_2\text{M}^*$, which breaks apart and releases its contents for processing by the cell.

Figure 1: $\alpha_2\text{M}$ can be proteolytically converted to its receptor recognized form, which has an altered electrophoretic mobility. A) Diagram of $\alpha_2\text{M}$ undergoing activation and conformational change from slow to fast form. B) The mechanism of covalent incorporation by nucleophilic substitution at the thiolester bond. C) $\alpha_2\text{M}$ (slow; Lane 1) and $\alpha_2\text{M}^*$ (fast; Lane 2) separated by native PAGE.
1.7 The Role of $\alpha_2$M in Inflammation and Immunity

There has been much speculation as to the functions of $\alpha_2$M. It was first thought to protect the body from uncontrolled proteolysis. Ordinarily, the concentrations of proteinases are very tightly regulated, but at sites of infection and inflammation, the quantity of proteinases released by pathogens and by the large populations of cells that infiltrate an area can overwhelm the regulation mechanisms. To limit proteolytic damage to tissues, $\alpha_2$M binds and rapidly clears proteinases from the blood. Supporting this claim are data showing that the concentration of $\alpha_2$M increases at sites of inflammation [95-97], possibly in response to the need for proteinase inhibition.

$\alpha_2$M binds to and modulates the activity of numerous cytokines, including TGF-$\beta$1, TGF-$\beta$2 [98, 99], TNF$\alpha$ and IFN$\gamma$ [100], PDGF [101], FGF [102], IL-1$\beta$ [103], IL-6 [104], and NGF [105]. There are also important differences in the interactions between cytokines and $\alpha_2$M, which indicates the complexity of the physiological role that $\alpha_2$M plays. Some molecules, PDGF, TGF-$\beta$1, TGF-$\beta$2, and IL-6, bind the native form of $\alpha_2$M. In this case, $\alpha_2$M may function as a carrier protein by protecting these molecules from proteolytic degradation [104] or preventing their filtration through the glomerulus. Other molecules, including TNF$\alpha$ and IL-1$\beta$, preferentially bind to the activated $\alpha_2$M*. Given that $\alpha_2$M* is taken up so rapidly, this interaction may provide a means for rapid clearance of these active compounds from the circulation.

The role of $\alpha_2$M became more complex with the discovery that $\alpha_2$M* activates cellular signaling through a second receptor, GRP78. Upon binding GRP78, $\alpha_2$M* induces changes in calcium levels, cyclic AMP and inositol phosphate [106, 107]. Rather
than functioning solely as a sink for excess proteinases, α2M may provide a mechanism by which cells can acquire feedback from the extracellular milieu. α2M interacts with a multitude of macromolecules and can incorporate them irreversibly on proteolytic activation. Once activated, α2M* binds and is internalized by cells that express LRP. α2M* can also bind its signaling receptor, GRP78, to induce a cascade of molecular signaling events. The modified concept of α2M is that this molecule serves as a “sensor of proteolysis” [108].

A series of antigen presentation experiments showed that α2M* has a role in the adaptive immune response. MΦs were pulsed with either hen egg lysozyme or lysozyme incorporated into α2M*, and antigen presentation was measured by the ability of APCs to stimulate T hybridoma clones that were responsive to lysozyme. To successfully activate T cells, MΦs pulsed with α2M*-lysozyme complexes require 250 times less antigen than those pulsed with free lysozyme [109]. α2M*-antigen complexes have been tested in vivo for their ability to induce an antibody response. α2M*-lysozyme complexes yield antibody titers that are 10 to 500 times greater than those from free lysozyme alone [110]. Immunization studies demonstrating the beneficial effects of α2M* have also been performed. Mice immunized with an HIV envelope peptide require 100-fold lower doses of peptide to produce a response when the peptide is incorporated in α2M* compared to peptide mixed with complete and incomplete Freund’s adjuvant (CFA/IFA) or in combination with monophosphoryl lipid/granulocyte-colony stimulating factor (MPL/G-CSF) [111]. α2M*-antigen complexes also produce antibody responses in mice that are four to five orders of magnitude greater than those elicited by antigen alone [112].
1.8 $\alpha_2M$ and CpG ODN

Given the ability of $\alpha_2M^*$ to bind and modulate the activity of numerous biologically active molecules and its ability to delivery molecules to immune competent cells, we hypothesized that CpG ODN would incorporate into $\alpha_2M$ and the resulting complexes will possess enhanced immunostimulatory properties that improve the clinical efficacy of CpG ODN-based therapeutics. $\alpha_2M^*$ can bind a large variety of molecules with drastically different physical properties. It can inhibit enzymes from all four major classes of proteases [1] and peptides only a few residues in length or proteins as large as 120 kDa [111].

In addition to the molecular promiscuity that $\alpha_2M$ exhibits, molecules bound within are targeted for delivery to immune competent cells: MΦs, DCs, and B cells. Studies have demonstrated the significantly enhanced immune responses that result from treating cells and animals with $\alpha_2M^*$-bound antigens compared to antigens alone or mixed with the strongest known adjuvants. This increased biological activity results from the protection and directed delivery, as well as the receptor-mediated uptake, that packaging into $\alpha_2M^*$ provides.

In light of these findings, we predicted that incorporation into $\alpha_2M^*$ would enhance the activity of CpG ODN as well. Given the barriers that have been encountered in the process of developing CpG ODN based therapeutics, $\alpha_2M^*$-aided delivery may offer distinct advantages. $\alpha_2M^*$ may protect them from nuclease digestion, non-specific protein binding, and renal clearance. Targeted delivery to immune competent cells could increase potency, thus reducing systemic side effects. $\alpha_2M^*$-aided delivery may improve
the overall efficacy of CpG ODN, thereby improving clinical utility. In light of the numerous clinical applications for which CpG ODN are currently being evaluated, improved efficacy could have widespread benefits in terms of preventing and reducing disease.

### 1.9 Experimental Objectives

Aim 1: Evaluate α₂M*-ODN complexes for therapeutic applications

A. Demonstrate immunostimulatory properties of α₂M*-ODN complexes using murine and human cells.

B. Characterize mechanisms by which α₂M*-ODN complexes are taken up by cells.

C. Determine whether CpG ODN and protein antigens can co-incorporate into α₂M, forming an α₂M*-Ag-ODN ternary complex.

Aim 2: Characterize the chemical nature of the interaction between α₂M and CpG ODN

A. Identify optimal conditions for the incorporation of CpG ODN into α₂M*.

B. Determine the stoichiometry and mechanism of incorporation, and measure the stability of α₂M*-ODN complexes.

C. Identify CpG ODN structural variables that affect incorporation.
Chapter 2: Development of an $\alpha_2$M*-ODN Complex Synthetic Process
2.1 Purification of $\alpha_2$M from Human Plasma

This protocol, which is adapted from previous versions published [1, 92], yields $\alpha_2$M in its native conformation and is free of contamination by LPS and other proteins, including activated $\alpha_2$M*. The yield is roughly 100 mg of protein per unit of human plasma at a final concentration of 3-4 mg/ml. The end product from the preparation is stable for up to several months when stored at either 0° C or -80° C.

2.1.1 Resin Preparation

The following solutions should be prepared using pyrogen-free water, freshly opened reagents, and sterile spatulas. Glassware, stir bars, and aluminum weigh boats should be baked at 250° C for at least 4 h to eliminate contamination with LPS prior to use. To adjust the pH of solutions, aliquots of each should be taken using pyrogen-free pipette tips or sterile pipets, aliquots should be transferred to test tubes for pH measurement, and the acid and base solutions used to adjust the pH should be freshly made. Amounts of reagents are listed here but should be verified to yield the given concentrations because of varying formula weights due to differences in hydration levels of some salts. Sterile filter all solutions.

1) 50% PEG-8k; 500 ml

Transfer 250 g PEG-8k to a large Erlenmeyer flask, and bring volume to 500 ml with the addition of water. Final volume may be slightly higher than 500 ml in
order to dissolve PEG completely. This solution will become very viscous.

2) Buffer A: 0.05 M EDTA, 0.5 M Sodium Chloride, pH 7.0; 300ml
   4.38 g EDTA, 8.77 g NaCl
   Adjust pH with NaOH

3) Buffer B: 3 mg/ml Zinc Chloride; pH does not matter; 300ml
   900 mg ZnCl₂

4) Buffer C: 0.25 M Sodium Acetate, 0.15 M NaCl, pH 5.0; 300 ml
   6.15g NaAc, 2.63g NaCl
   Adjust pH with HCl

5) Buffer D: 0.1 M Sodium Phosphate, 0.8 M NaCl, pH 6.5; 2.5 L
   1 L of 0.1 M monobasic sodium phosphate: 15.56 g NH₂PO₄, 56.1g NaCl
   1.6 L of 0.1 M dibasic sodium phosphate: 28.5 g N₂HPO₄, 74.8 g NaCl
   Add mono- to dibasic while monitoring pH

6) Buffer E: 0.02 M Sodium Phosphate, 0.15 M Sodium Chloride; pH 6.0; 2.0 L
   1.5 L of 0.02 M monobasic sodium phosphate: 4.14 g NH₂PO₄, 13.15 g NaCl
   500 ml of 0.02 M dibasic sodium phosphate: 1.25 g N₂HPO₄, 4.38 g NaCl
   Add di- to monobasic while monitoring pH
7) Buffer F: 0.01 M Sodium Acetate, 0.15 M Sodium Chloride, pH 5.0; 300ml
   0.246g CH₃COONa, 2.63g NaCl
   Adjust pH with HCl

All volume transfers should be done using sterile pipets or pyrogen-free pipette tips with aerosol filters. Dispense 15 ml of zinc resin (Chelating Sepharose Fast Flow; Amersham Biosciences) into two 50 ml conical tubes (30 ml total). Numerous buffer changes and wash cycles are needed to prepare the resin; all procedures should be performed in the tissue culture hood. In each step, add 25 ml of the solution indicated to each tube, which already contains 15 ml of resin. Invert the tubes several times for thorough mixing. Centrifuge tubes at 500 rpm for 1 min at 4°C. Remove supernatant, and repeat.

1. To each tube add 25 ml of 1 M sodium hydroxide, mix, centrifuge, remove, and add 25 ml of 1 M sodium hydroxide. Soak resin for 1 h to remove LPS contamination.

2. Rinse twice with sterile water and then begin washes with Buffer A; use all 300 ml of Buffer A. Test pH of supernatant to make sure NaOH has been neutralized.

3. Wash with 1L of sterile water, repeating cycles described above.

4. Wash with 300ml of Buffer B, repeating cycles described above.

5. Wash with 300ml of Buffer C, repeating cycles described above.

6. Wash with 300ml of Buffer D, repeating cycles described above.
2.1.2 Protein Fractionation

1. Sterilize ceramic Buchner funnel and stir bars in advance by baking over night at 250°C; all other supplies are packaged sterile and disposable. All steps should be performed in a tissue culture hood, and all solutions should be ice cold.

2. Quickly thaw 2 units of human fresh frozen plasma, obtained from the American Red Cross of Durham, NC, using warm water from faucet in 4 L bucket and crushing frozen chunks manually. Proteases become active once thawed, so cleavage of α₂M by proteases is minimized by thawing quickly and storing on ice thereafter.

3. In tissue culture hood, place a large ceramic filter on top of a sterile 1 L media bottle. Being careful not to contaminate gauze, use sterile spatula to place 3-4 sterile 4x4 gauze in filter. Spray outside of plasma bag with alcohol and wipe dry. Cut one corner with sterile disposable scalpel and pour plasma into ceramic filter. Gauze will serve to remove any clots. Do not use glass filter because of high protein binding to glass.

4. Measure the volume of filtered plasma with graduations on media bottle. Bring plasma to 4% PEG-8k by addition of 8.7 ml of 50% PEG solution per 100 ml of plasma.

5. Add a sterile stir bar and 1 tablet of Complete Protease Inhibitor Cocktail (Rocche). Stir for 1 h at 4°C.

6. Transfer solution to sterile 250 ml conical tubes using sterile pipets to minimize risk.
of contamination. Centrifuge at 3800 g for 45 min at 4°C.

7. Pool all supernatants into a sterile 1 L media bottle. Pellets are no longer needed but should be saved in case protein cannot be detected in the supernatant (SN) fraction.

8. Bring SN to 16% PEG 8k by addition of 35.25 ml of 50%PEG per 100 ml of SN. Add a sterile stir bar, and stir for 1 h at 4°C.

9. Transfer to 250 ml conical tubes, centrifuge at 3800 g for 45 min at 4°C.

2.1.3 Affinity Purification

1. Remove SN. Using a sterile spatula, scoop out the bulk of each pellet and pool them in a sterile 500 ml media bottle. To the pooled pellets add 200 ml of ice cold Buffer D, the fully charged and equilibrated sepharose resin, and one tablet of protease inhibitor cocktail. The pellets will be too thick to stir, so place the bottle on a shaker in the cold room overnight to allow pellets to go into solution. To collect the remnants of pellets from each 250 ml conical tube from previous centrifugation, add 10 ml of Buffer D to each tube. Put tubes in rack and place on shaker at 4°C overnight.

2. The next morning, transfer the dissolved remnants from each conical tube to the media bottle. Add a sterile stir bar, and stir at 4°C for 2 h.
3. Transfer the mixture to 50 ml conical tubes. Rinse the bottle with Buffer D, and collect rinses into conical tubes. Centrifuge the tubes at 500 rpm for 1 min at 4°C. Remove supernatant, re-suspend the resin in Buffer D, and combine them into two 50 ml conical tubes.

4. Wash with Buffer D. With each wash step, add ice cold Buffer D to bring the volume up to 40 ml in each tube, invert the tubes to ensure mixing, and centrifuge at 500 rpm for 1 min. Collect washes in a sterile 1 L media bottle. After 1 L of Buffer D has been used, measure the protein concentration in each wash using absorbance at 280 nm. Continue washing with Buffer D until absorbance drops below 0.05.

5. Wash with ice cold Buffer E by the same methods as the Buffer D washes, pooling washes in a sterile 500 ml media bottle. After using 200 ml of Buffer E, collect fractions separately, and monitor the protein concentration in each wash by UV absorbance. Once protein concentrations level off, wash twice more, and analyze the fractions by PAGE (see next section) to determine whether they contain α₂M.

6. Elute with Buffer F. To elute at the highest concentration possible, add small volumes of ice cold Buffer F to the sample; to each tube add a volume equal to that of the resin. Re-suspend the resin, centrifuge at 500 rpm for 1 min, collect the supernatant, and repeat. As fractions are collected, add 1 M HEPES (tissue culture grade, non-pyrogenic) in a ratio of 100 µl HEPES per 5 ml of fraction (1:50 dilution) to adjust the pH from 5.0 to approximately 7. This is done to minimize the period that α₂M is maintained at a pH of
5.0, which will cause significant protein degradation over time. The final concentration of HEPES will be 20 mM.

7. Measure protein concentration in each fraction spectrophotometrically based on $A_{280} = 8.93$ for a 1% solution and a path length of 1 cm. Analyze fractions containing protein using PAGE under native conditions to screen for contaminations. Any fractions with impurities or significant amounts of activated $\alpha_2M^*$ should be discarded. Combine the remaining fractions containing pure $\alpha_2M$ and measure the protein concentration. Calculate the total protein yield from the volume and concentration of the sample.

8. Concentrate the protein using Centriprep YM-50 filtration units (Amicon). Centrifuge at 2k rpm for 15 min at 4°C, remove the filtrate, and repeat. The filtrate fractions should be saved until the sample is recovered and quantified to prevent product loss through failure of the filtration units. Based on the total protein yield calculated in the previous step, concentrate the protein to a volume that will yield a final protein concentration of 3-4 mg/ml. If this concentration is exceeded, the protein will begin to aggregate and precipitate.

9. Using a syringe, filter the solution through a 0.45 micron filter to remove any possible bacterial contamination. Determine the final protein concentration spectrophotometrically. Transfer aliquots to LPS-free microcentrifuge tubes, seal tubes with parafilm, and store in 0°C water bath or at -80°C.
2.1.4 Analysis of Product

Prior to combining elution fractions, the purity of each fraction should be determined using PAGE under native conditions. Include slow and fast standards. The most likely contaminant will be $\alpha_2M^*$, which is a result of proteolysis. This can be minimized by avoiding the introduction of contaminants, keeping reagents and samples ice cold, working quickly, and using a protease inhibitor cocktail. Measure LPS contamination using the QCL assay (BioWhittaker Inc).

2.2 Analysis of $\alpha_2M$ by PAGE

To distinguish between $\alpha_2M$ and $\alpha_2M^*$ (slow and fast forms, respectively), samples are analyzed by PAGE under native conditions. Load 2-5 µg of sample into a 5% TBE gel (Bio-Rad), and electrophorese the gel at 150 V for 1 h with running buffer containing 43 mM Imidazole and 35 mM HEPES. If the sample is pure and the protein is intact, a single band will be visible on staining with Coomassie brilliant blue or Fairbanks solutions (Figure 1C). Fast form $\alpha_2M^*$ will also appear as a single band but is distinguished by its higher rate of migration, which is the origin of it “fast form” distinction. $\alpha_2M^*$ can be converted by proteolysis or nucleophilic substitution by primary amines, typically ammonium or methylamine. Regardless of the mechanism of activation, $\alpha_2M^*$ has the same appearance on PAGE analysis under native conditions. Under denaturing conditions, the different mechanisms of activation can be distinguished by the characteristic banding patterns of the $\alpha_2M^*$ heat fragments, but these details go beyond the scope of this thesis.
2.3 Receptor-Recognized $\alpha_2$M* Binds CpG ODN

$\alpha_2$M has the ability to bind a vast array of biologically active macromolecules exhibiting a range of masses, charges, surface functional groups, and other physical properties. Based on these findings, we hypothesized that $\alpha_2$M would bind CpG ODN. It has previously been shown that both the native and receptor-recognized forms, $\alpha_2$M and $\alpha_2$M*, respectively, bind different molecules; the types of molecules that each binds has not yet demonstrated a predictable pattern of binding and must therefore be identified empirically. To determine whether nucleic acids can bind to $\alpha_2$M, fluorescently labeled CpG ODN were mixed with $\alpha_2$M, which was converted to its receptor-recognized form with human neutrophil elastase (HNE). The samples were then analyzed by PAGE under native conditions; CpG ODN were detected by fluorescent imaging using a Storm 860 Phosphorimager® (Molecular Dynamics) and ImageQuant software, and $\alpha_2$M* was detected by Coomassie brilliant blue staining. As controls, samples of CpG ODN and $\alpha_2$M were analyzed separately. As shown in Figure 2A, CpG ODN only associate with $\alpha_2$M* that has been proteolytically converted to its receptor-recognized form (Lane 3). The vast majority, 87.3%, of CpG ODN bound to $\alpha_2$M*, while only a minute portion of CpG ODN, 3.4%, bound to $\alpha_2$M in its native form (Lane 2). The CpG ODN that remained unbound migrated at the same rate as CpG ODN loaded separately (Lane 1). Run as controls, lanes 4 and 5 contained $\alpha_2$M with HNE and $\alpha_2$M alone, respectively. Figure 2B shows the location of $\alpha_2$M on PAGE analysis. It should be noted that while $\alpha_2$M and $\alpha_2$M* can be distinguished from each other by PAGE analysis under native
conditions, such analysis cannot be done on this gel because the conditions used were chosen to optimize the separation of free CpG ODN from both forms of $\alpha_2$M while remaining on the gel. Separation of $\alpha_2$M from $\alpha_2$M* requires a longer duration in the electrophoretic field, which results in the loss of CpG ODN from the gel. However, these samples were also analyzed under conditions that separate $\alpha_2$M from $\alpha_2$M* to confirm that the $\alpha_2$M had undergone complete activation by HNE (data not shown).

Figure 2: $\alpha_2$M binds CpG ODN only when converted to its receptor-recognized form, $\alpha_2$M*. CpG ODN were mixed with $\alpha_2$M, which was proteolytically activated with HNE. Samples were analyzed by PAGE. A) CpG ODN were detected by fluorescent imaging. B) $\alpha_2$M samples were detected by Coomassie brilliant blue staining. Lane 1: CpG ODN alone; 2: $\alpha_2$M + CpG ODN; 3: $\alpha_2$M + CpG ODN + HNE; 4: $\alpha_2$M + HNE; 5 $\alpha_2$M alone.
2.4 Determination of Optimal Conditions for ODN Incorporation

2.4.1 Selection of Buffer and pH Conditions

Fundamental to this project was the ability to generate $\alpha_2$M*-ODN complexes in a manner that optimizes the use of reagents and yields complexes that are free of contaminations. While much is known about the interaction between $\alpha_2$M and protein ligands, this thesis is the first to document the incorporation of ODN into $\alpha_2$M* through the use of the intrinsic ability of $\alpha_2$M to incorporate macromolecules. An earlier study described the conjugation of nucleic acids to $\alpha_2$M through the use of chemical linking molecules [113]. Rather than employ similar synthetic methods, however, we chose methods based on those used to generate $\alpha_2$M*-Ag complexes because of their demonstrated enhanced biological activity. We reasoned that if protein antigens bound to $\alpha_2$M* displayed enhanced delivery and presentation, the likelihood of maintaining or improving the biological activity of CpG ODN on incorporation would be maximized through the use of similar methods to generate the complexes. Therefore, to develop methods for the generation of $\alpha_2$M*-ODN complexes, we began with the procedures outlined by the reports on $\alpha_2$M*-enhanced antigen presentation [109].

To generate $\alpha_2$M*-Ag complexes, human $\alpha_2$M in its native form was mixed with the antigen of choice in a molar ratio of 1 mole of $\alpha_2$M to anywhere from 10 to 50 moles of antigen. Enough protease was added to activate $\alpha_2$M, the proteolytic reaction proceeded at room temperature, and the antigen was incorporated into fast-form $\alpha_2$M*.
Previously, the reaction had been performed with minor variations in the conditions with respect to buffers, pH levels, and salt concentrations, which was done to accommodate for the stability and solubility properties of the antigens used. To determine the combination of these variables that would be optimal for the incorporation of ODN into α₂M*, the incorporation reaction was performed repeatedly with the systematic alteration of each of these variables. The relative amount of incorporation under each condition was determined by measuring the amount of ODN bound to α₂M* by analyzing samples by PAGE under native conditions and detecting labeled ODN by fluorescent imaging using a Storm 860 Phosphorimager® (Molecular Dynamics) and ImageQuant software.

The buffers tested were PBS and Tris, and the salt concentrations with Tris were 50 mM and 200 mM. As shown in Figure 3, the amount of ODN that incorporated into α₂M* was relatively consistent regardless of buffer and salt concentrations tested. Therefore, PBS was chosen as the buffer to use for the incorporation reaction based on availability and superior thermal stability. Once the buffer was established, the optimal pH level was determined by the same methods; the incorporation reaction was carried out over a range of pH levels, from 7.0 to 10.0. This experiment was performed using both P=O and P=S ODN, and, somewhat unexpectedly, the optimal pH was different for ODN composed of different backbones. The highest amount of incorporation of P=O ODN was observed at a pH of 7.0, while a pH of 9.0 was optimal for the incorporation of P=S ODN. The Coomassie brilliant blue stain and fluorescent image of the gel with P=S ODN are shown in Figure 4A and B, respectively, but the quantified fluorescent signals from both P=O and P=S ODN samples are shown in Figure 4C. Inspection of Figure 4A reveals incomplete activation of α₂M at pH levels from 7.0 to 8.5, as evidenced by the
double bands corresponding to both the fast and slow forms of α₂M. The preference that ODN exhibit for binding activated but not native form α₂M is demonstrated again here by the appearance of single bands in the fluorescent image, corresponding to the location of activated α₂M*.

Figure 3: Optimal CpG ODN incorporation is achieved using PBS to buffer the incorporation reaction. The incorporation reaction was performed using Tris and PBS as buffers, and with Tris, salt concentrations of 50 mM and 200 mM. Following the incorporation reaction, samples were analyzed by PAGE, and bound ODN was measured by fluorescent imaging; the strongest signal was assigned a value of 100, and the remaining data were adjusted accordingly. Data shown here are means of the experiment performed in triplicate ± s.d.
Figure 4: P=O and P=S CpG ODN exhibit optimal incorporation at different pH levels. Fluorescently labeled CpG ODN were mixed with α2M, followed by activation with HNE. Samples were analyzed by PAGE. A) Detection of α2M by Coomassie brilliant blue staining. B) Detection of CpG ODN by fluorescent imaging of the same gel. C) Quantification of the fluorescent signals shown in Panel B. The strongest signal of P=O and P=S CpG ODN were each assigned a value of 100, and the remaining data were adjusted accordingly. Lane 1: α2M slow form; 2: α2M* fast form; 3-9: CpG ODN mixed with α2M and activated using HNE at pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, respectively. ■ P=O ODN; □ P=S ODN. Data shown here are means of the experiment performed in triplicate ± s.d.

2.4.2 ODN Molar Excess

The next condition tested was the molar ratio of ODN to α2M. A similar procedure was followed for this experiment; α2M was incubated with increasing concentrations of P=O or P=S ODN, HNE was added for proteolytic activation, and samples were analyzed by PAGE under native conditions. Figure 5 shows that incorporation increases with increasing amounts of ODN until the molar ratio reaches...
tenfold molar excess, at which point, the incorporation plateaus for both P=O and P=S ODN. Based on these experiments, α2M*-ODN complexes were prepared under the following conditions. The incorporation reaction was conducted in PBS with the pH adjusted to 7.0 and 9.0 for P=O and P=S ODN, respectively. For both P=O and P=S forms, the ODN were present in a tenfold molar excess, with respect to α2M.

Figure 5: The amount of CpG ODN that incorporates into α2M* is dependent on the amount of CpG ODN present. α2M was incubated with increasing concentrations of CpG ODN, and following proteolytic activation, samples were analyzed by PAGE. A) α2M* was detected by Coomassie brilliant blue staining; B) CpG ODN were detected by fluorescent imaging. C) Signals from Panel B were quantified and are displayed graphically. The strongest signal for P=O and P=S ODN incorporation were each assigned a value of 100, and the remaining signals were adjusted accordingly. Lane 1: α2M slow form; 2: α2M* fast form; 3-8: α2M mixed with CpG ODN added in the following molar ratios with respect to α2M: 1, 2, 5, 10, 25, and 50, respectively. ■ P=O ODN; □ P=S ODN. Data shown here are means of the experiment performed in triplicate ± s.d.
2.5 Analysis of ODN Fluorescent Label

2.5.1 Effects on Biologically Activity

A large portion of the data obtained in this thesis was based, in part, on quantification of ODN incorporated within α2M*. To compare the biological activity of free ODN and α2M*-ODN complexes, cells had to be treated with equal amounts of ODN, whether free or α2M*-bound. Central to the chemical analyses of α2M*-ODN complexes—mechanism and stoichiometry of ODN incorporation, complex stability, and competition studies—is the ability to detect and quantify the ODN. To this end, the ODN employed in these experiments were fluorescently labeled on the 3’ end of the molecule with Bodipy 630/650 (Molecular Probes). The 3’ end was chosen as the site for labeling because the biological activity of CpG ODN is abrogated by 5’ labeling [114]. Biological studies, therefore, required the use of ODN with a 3’ label, and for consistency, all other experiments described in this thesis used ODN with the same labeling site. A control experiment was performed to determine any biological impact of the label. TG-elicited murine MΦs were treated with equal concentrations of unlabeled CpG ODN or 3’ labeled ODN, and, after 24 h, TNF production was measured by ELISA. No difference was observed in the immunologic response elicited by labeled versus unlabeled ODN (data not shown).

2.5.2 Stability

Conclusions drawn from these studies are based on the accurate quantification of
ODN bound to $\alpha_2$M*. Complexes were incubated under relatively harsh conditions for extended periods, and it was possible that the diminution of signal resulted from breakdown of the Bodipy label used to detect the ODN. To determine the effects of altered pH levels and temperatures on the signal strength of Bodipy, labeled ODN were subjected to the same incubation conditions as the $\alpha_2$M*-ODN complexes, namely pH levels from 5.0 to 10.0 and temperatures from 0$^\circ$ C to 50$^\circ$ C. Samples were then analyzed by PAGE and quantified by fluorescent imaging using a Storm 860 PhosphorImager® (Molecular Dynamics) and ImageQuant software. No loss of signal was observed under the conditions tested; even the samples incubated at the highest temperature, 50$^\circ$ C, and the extreme pH levels, 5.0 and 10.0, showed no loss of signal strength (data not shown). These results confirm that changes in the Bodipy signal strength do not contribute to the changes observed in the studies on $\alpha_2$M*-ODN complex stability.

### 2.5.3 Signal Quenching

Also essential to the accurate quantification of ODN bound to $\alpha_2$M* is the consistency of the signal measured from the Bodipy label. Some reports have documented quenching of the Bodipy signal when bound to various proteins [115], raising concern that the signal of Bodipy bound to ODN in complex with $\alpha_2$M* could likewise be affected. If this quenching entailed a fixed, proportionate loss of signal strength, the results from experiments comparing relative amounts of ODN incorporation would not be affected. These include measurement of ODN bound by covalent versus non-covalent mechanisms, ODN incorporation as a function of the molar excess of
competing ligands, binding and uptake studies, and stability studies. In each of these studies, the absolute amount of ODN is irrelevant, but rather, conclusions are drawn from differences in the relative amounts of ODN bound to α₂M* under each condition. Bodipy quenching would, however, alter results obtained from experiments that hinge on measuring the absolute quantity of ODN bound to α₂M*. These include determination of the molar incorporation ratio and all biological assays in which cells are treated with a known amount of ODN. If α₂M* quenches the Bodipy signal, then the amount of ODN bound to α₂M* and the amount with which cells were treated would be consistently underestimated, thus eliminating our ability to draw conclusions on the effects of α₂M* incorporation on the activity of ODN.

To determine whether incorporation into α₂M* alters the signal strength of Bodipy bound to ODN, α₂M*-ODN complexes were generated and purified by the same methods described later in this thesis. Samples of α₂M*-ODN complexes were diluted in Tris-glycine (25 mM Tris, 192 mM glycine, pH 8.3) buffer to a range of concentrations that spanned those concentrations used in the biological assays described in this thesis. α₂M*-ODN samples with identical concentrations were also prepared using Tris-glycine-SDS buffer (25 mM Tris, 192 mM glycine, 0.1% w/v SDS, pH 8.3). The SDS was added to denature α₂M* so that incorporated ODN would dissociate from the protein, thus eliminating the possibility of signal quenching by α₂M*. The fluorescent signal of each sample was measured using a Shimadzu spectrofluorophotometer. For each pair of samples with equal concentrations of α₂M*-ODN complexes, there was no difference observed in the signal strength of samples in the presence or absence of SDS. This confirms that the signal strength of Bodipy conjugated to ODN is not altered by
incorporation into $\alpha_2\text{M}^*$, which supports that our previous methods of quantitating ODN bound to $\alpha_2\text{M}^*$ are accurate.

2.6 Purification of $\alpha_2\text{M}^*$-ODN by FPLC

When $\alpha_2\text{M}^*$-ODN complexes are produced from the incorporation reaction, there is a heterogeneous mixture of molecules in solution. ODN is present in a tenfold molar excess, with respect to $\alpha_2\text{M}$; a portion of ODN gets incorporated into $\alpha_2\text{M}^*$, and a significant portion remains unbound. Likewise, human neutrophil elastase (HNE) is present in a twofold molar excess, with respect to $\alpha_2\text{M}$; the majority of HNE gets incorporated into complexes, but there is a portion that does not incorporate but might remain proteolytically active. There is also a mixture of buffers present; low pH sodium phosphate used as an elution buffer in the purification of $\alpha_2\text{M}$, HEPES added to adjust the pH for the long-term storage of $\alpha_2\text{M}$ after purification, and Tris added to optimize the pH level during the incorporation reaction. Also in solution are any salts present in the lyophilized ODN and HNE, which are reconstituted prior to use. Separation by gel electrophoresis is effective for analytical purposes, but this technique is not applicable for large scale preparations of pure $\alpha_2\text{M}^*$-ODN complexes.

Since these complexes would eventually be used to elicit responses from immune competent cells, it was necessary to develop a purification procedure that would remove all these contaminating agents without introducing any new ones so that valid conclusions about the immunostimulatory properties of $\alpha_2\text{M}^*$-ODN complexes could be drawn. Of central importance was the need to separate $\alpha_2\text{M}^*$-ODN complexes from
unbound ODN. The significant molar excess of ODN is necessary to drive the incorporation reaction forward and achieve the highest possible molar incorporation ratio, but since an aim of this work is to demonstrate that incorporation into $\alpha_2$M* enhances the biological activity of ODN, purified $\alpha_2$M*-ODN complexes must be free of contamination with unbound ODN. Likewise, to determine cellular binding and uptake as well as thermal stability and dissociation rates of the complexes, it was necessary to remove unbound ODN. Removal of unbound HNE and transferring $\alpha_2$M*-ODN complexes into an optimal buffer were additional reasons to develop a technique with which to purify $\alpha_2$M*-ODN complexes following the incorporation reaction.

The ODN used in these experiments have a molecular weight of 6 kDa, while that of $\alpha_2$M is 718 kDa. With such a large disparity in molecular weights, separation by size was likely to be the most efficient means of purification. Attempts at separation of free ODN from $\alpha_2$M*-ODN complexes were made using various types of dialysis. Centrifugal filtration devices with size-exclusion porous membranes were employed, as were centrifugal filtration devices designed to bind free nucleic acids. In each case, a certain degree of separation was achieved, but there was still a significant portion of free ODN contaminating the $\alpha_2$M*-ODN sample. The difficulty of separation likely arises from the large molar excess of unbound ODN compared to the molar quantity of $\alpha_2$M*-ODN complexes; the techniques listed above removed the majority of free ODN, but the small portion that remained was still significant in comparison to the amount bound to $\alpha_2$M*.

Successful purification is achieved with the use of Fast Protein Liquid Chromatography (FPLC). The system used was a BioLogic FPLC (BioRad) with a
Superdex 200 10/300 G/L size exclusion column (Amersham Biosciences). To demonstrate that free ODN can be separated from $\alpha_2$M* with essentially no residual contamination, ODN and $\alpha_2$M* were loaded separately on the column and observed to elute in different fractions. Each sample was eluted under similar FPLC conditions and fractions were collected. The UV absorbance of each fraction was measured to detect $\alpha_2$M*, and ODN were detected by measuring the fluorescence using a Shimadzu RF-5301 PC spectrofluorophotometer. Figure 6 demonstrates the effective separation of ODN and $\alpha_2$M* through the use of FPLC. Prior to loading samples onto the column, the entire system was cleaned with 0.5 M NaOH for 4 hours, which prevents the introduction of LPS contamination into the sample. Data presented elsewhere in this thesis show that $\alpha_2$M*-ODN complexes prepared using this method are free of LPS contamination as determined by both QCL assay and the absence of response from immune competent cells treated with the purified complexes. This step also provides a means by which the $\alpha_2$M*-ODN complexes could be transferred into a buffer suited to the assays for which they will later be used.
Figure 6: Free CpG ODN can be separated from \( \alpha_2\)M*-ODN complexes by FPLC. A) Following the incorporation reaction, the mixture of \( \alpha_2\)M*, ODN, and HNE was eluted through a Superdex 200 10/300 GL column by FPLC. The absorbance at 280 nm was measured continuously as the samples eluted off the column, and two major peaks were observed. B) \( \alpha_2\)M* and ODN were loaded separately and eluted through the column. \( \alpha_2\)M* (■; left Y axis) was measured by UV absorbance and ODN (□; right Y axis) were detected by fluorescence.
Chapter 3: Characterization of the Biological Activity of $\alpha_2$M*-ODN Complexes
3.1 Murine in vitro Response

3.1.1 Dose Dependency and Response Kinetics

We next sought to determine how incorporation into α2M* affects the immunostimulatory properties of CpG ODN. MΦs, which play a key role in the murine response to CpG ODN by secreting pro-inflammatory cytokines that recruit and activate other immune cells [78, 116], were selected as the model in which to first measure the biological activity of α2M*-ODN complexes. Murine MΦs were treated with either free or α2M*-bound CpG ODN over a range of concentrations, and biological activity was initially measured by quantifying TNF production. Over the entire dose range tested, CpG ODN bound to α2M* showed an enhanced ability to stimulate MΦs compared to free CpG ODN (Figure 7). α2M* increased the potency of CpG ODN by four to sixfold, with the most pronounced enhancement occurring at the lower concentrations of CpG ODN tested. From concentrations of 0.13 µM to 1.0 µM, the average increase in efficacy was greater than fivefold for α2M*-bound versus free CpG ODN.

In control studies, cells were treated with α2M* complexes that contained a GpC ODN control sequence and with α2M* alone at the same concentrations as those used in the previous experiment; both failed to elicit any response that could be detected at all concentrations tested (data not shown). These negative results demonstrate that α2M*-ODN complexes stimulate cells through the same CpG-dependent mechanism through which free CpG ODN stimulate cells. These results further demonstrate that α2M* alone is not responsible for the immune system activation. The α2M*-ODN complexes used
were all free of LPS contamination, as determined by the QCL assay; negative results obtained with the control materials confirm that both α₂M* alone and α₂M*-ODN complexes are free of possible contaminants that might elicit an immune response.

Another potential mechanism for the enhanced response to α₂M*-ODN complexes is α₂M*-induced signaling. In addition to the α₂M* endocytic receptor LRP/CD91, MΦs also express on their surface the α₂M*-signaling receptor, glucose-regulated protein-78 (GRP78/BiP). On binding GRP78, α₂M* initiates changes in calcium levels, cyclic AMP, and inositol triphosphate [106]. It is possible that such signaling, induced by α₂M*, might prime cells to respond, making them more responsive to CpG ODN that are present. If GRP78 signaling were involved in the response, pretreatment with α₂M* alone, followed by treatment with CpG ODN, should demonstrate similar enhancement to that previously observed with α₂M*-ODN complexes. To determine whether GRP78-signaling affects the activity of α₂M*-ODN complexes, MΦs were pretreated with α₂M* for 1 h and washed; CpG ODN were then added with fresh media; TNF production was measured by ELISA. The α₂M* concentrations used were 500 pM, which is tenfold greater than the K_d of GRP78 for α₂M* and induces maximal signaling [117], and 500 nM, the α₂M* concentration occurring in the highest dose of α₂M*-ODN complexes tested. Neither concentration of α₂M* had an effect on the CpG ODN-induced response (Figure 7), indicating that the α₂M*-GRP78 signaling pathway does not contribute to the augmented responses elicited by α₂M*-ODN complexes.
Figure 7: Incorporation in $\alpha_2$M* enhances the biological activity of CpG ODN. TG-elicited MΦs were treated with free CpG ODN or CpG ODN incorporated into $\alpha_2$M. Cells were incubated for 24 h, and TNF was measured by ELISA. ■ $\alpha_2$M*-ODN; □ free CpG ODN; ■ 500 nM $\alpha_2$M* pre-treatment, then CpG ODN; □ 500pM $\alpha_2$M* pre-treatment, then CpG ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).

The stability of $\alpha_2$M*-ODN complexes was measured to verify that they remained intact long enough to be taken up by cells and exert the enhanced biological activity observed. Both P=O and P=S $\alpha_2$M*-ODN complexes were purified and incubated at 37$^\circ$ C and 0$^\circ$ C and analyzed by PAGE; $\alpha_2$M*-bound and dissociated CpG ODN were measured by fluorescent imaging. At 37$^\circ$ C, 80% of both P=O and P=S $\alpha_2$M*-ODN complexes remained intact after 24 h, and they exhibited a half-life of 96 h. When samples were incubated at 0$^\circ$ C, 95% of both types of complex remained intact after a period of 18 months. Based on these findings, the vast majority of complexes remain intact long enough to be taken up by cells and exert their effects within the time span of these experiments.

After finding that $\alpha_2$M*-bound CpG ODN exhibit both enhanced potency and
efficacy for immunostimulation, the kinetics of the responses induced by free and bound CpG ODN were compared by measuring immune stimulation over time. MΦs were treated with equal amounts of free or α2M*-bound CpG ODN, and TNF production was measured over five time points (Figure 8). The most pronounced differences between the responses occurred early; the response to α2M*-ODN complexes was detected after 2 h, the first time point tested, while that induced by free CpG ODN was not detectable until after 8 h, by which time the response to α2M*-ODN was reaching its peak. The peak response to α2M*-ODN was not only twice that induced by free CpG ODN, but it also occurred 16 h earlier.

![Figure 8: CpG ODN bound to α2M* induce a stronger response that begins and peaks earlier than free CpG ODN.](image)

Figure 8: CpG ODN bound to α2M* induce a stronger response that begins and peaks earlier than free CpG ODN. TG-elicited MΦs were treated with free or α2M*-bound CpG ODN at a concentration of 0.5 µM. At the times indicated, media was collected, and TNF was measured by ELISA. ■ α2M*-ODN; □ free CpG ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).
3.1.2 Multiplex Analysis of Response Kinetics

CpG ODN stimulate MΦs to produce several cytokines and chemokines in addition to TNF, including IL-1, IL-6, IL-12, and monocyte chemotactic protein-1 (MCP-1) [118, 119]. These effector molecules are crucial to the development of the T_{H1}-biased response that is characteristic of CpG ODN stimulation. To more fully characterize the immune response elicited by α_{2}M*-ODN complexes, the production of these cytokines over time was measured using multiplex analysis (Figure 9). Again, the differences in cytokine production between free and α_{2}M*-bound CpG ODN stimulation are most pronounced at the earlier time points. Complex-induced responses begin and peak earlier and, with the exception of IL-6, reach levels that are at least twofold greater than those induced by free CpG ODN. α_{2}M*-bound CpG ODN demonstrate an enhanced ability to stimulate the production of numerous cytokines that are central to a T_{H1}-biased immune response. Similar to results obtained with respect to TNF responses, there were no responses to the negative controls α_{2}M* alone or the control sequence ODN bound to α_{2}M*, and there was also no enhancement by pre-treatment with α_{2}M* followed by CpG ODN (data not shown).
Figure 9: CpG ODN bound to α₂M* induce more rapid and robust production of numerous pro-inflammatory cytokines. TG-elicited MΦs were treated with free or α₂M*-bound CpG ODN at a concentration of 0.5 µM, and media was collected over numerous time points. Multiplex analysis was used to measure the following cytokines: A) IL-1α; B) IL-6; C) IL-12 (p70); D) MCP-1. ■ α₂M*-ODN; □ free CpG ODN. Data are a representative example from one of three experiments, each performed in triplicate; error bars indicate ± s.d

3.2 Mechanisms for Enhanced Biological Activity

CpG ODN stimulate immune competent cells to produce an inflammatory response by binding its receptor, TRL9, which is located intracellularly [39, 114]. Because the ligand-receptor interaction occurs within the cell, CpG ODN must be internalized in order to elicit a response; therefore, a key determinant of the biological activity of CpG ODN is its ability to enter cells [12, 13, 42]. We hypothesize that incorporation within α₂M* facilitates uptake of CpG ODN by immune-competent cells,
and that this enhanced delivery is, in part, responsible for the increased biological activity.

3.2.1 Rate of Uptake

Once converted to its receptor-recognized form, $\alpha_2$M* binds to its uptake receptor, LRP, and is taken up very rapidly; the circulating half-life of activated $\alpha_2$M* is 2-5 min in plasma. The primary receptor involved in plasma clearance is LRP on Kupffer cells and hepatocytes of the liver [92, 120]. Following endocytosis, LRP dissociates from $\alpha_2$M* and is recycled to the cell membrane [121]. Due to the high surface expression of LRP and its rapid recycling, large amounts of $\alpha_2$M* can be cleared quickly and efficiently from the extracellular environment.

CpG ODN enter cells by pinocytosis and receptor-mediated endocytosis. At concentrations below 1 µM, receptor-mediated endocytosis dominates because of the high sensitivity of the receptors involved, which include include Mac-1 [36], MARCO [37], and CXCL16 [38]. Many other surface proteins that bind CpG ODN non-specifically have also been identified on immune competent cells [122-124]. At higher concentrations, these mechanisms become saturated, and pinocytosis becomes the predominant mechanism by which CpG ODN enter cells [34, 35, 123]. Using FACS, CpG ODN have been detected in cells within as little as 15 min from the time of exposure [39, 125]. However, based on the high capacity that LRP-expressing cells have for taking up $\alpha_2$M* complexes, we hypothesized that ODN incorporation into $\alpha_2$M* will increase the rate at which CpG ODN are endocytosed and that this rapid uptake is at least
partially responsible for the enhanced biological activity of α₂M*-bound CpG ODN.

To measure the rates of uptake of α₂M*-ODN complexes and free CpG ODN, murine MΦs were treated with 0.5 µM of either free or α₂M*-bound CpG ODN and incubated at 37°C. After periods from 1 min up to 2 h, cells were washed to remove unbound ligands. Bound and internalized CpG ODN were measured directly by fluorescent imaging; signal obtained were quantified and are expressed graphically in Figure 10. Uptake of all four ligands, both free and α₂M*-bound P=O and P=S CpG ODN, follows a linear pattern over time, but those bound to α₂M* are taken up significantly faster. Both P=O and P=S ODN bound to α₂M* are taken up at a sixfold greater rate than free P=S CpG ODN and at a 50-fold greater rate than free P=O CpG ODN. These results show that the rate of α₂M*-ODN uptake is not affected by the backbone composition of the incorporated CpG ODN, and that CpG ODN bound to α₂M* are internalized by immune competent cells significantly faster than free CpG ODN.
Figure 10: α₂M*-bound CpG ODN are endocytosed at a greater rate than free CpG ODN. TG-elicited MΦs were dosed with equal amounts of ODN either free or bound to α₂M* and incubated at 37°C. After the specified durations, cells were washed to remove ODN that remained in the media. ODN that had been taken up by cells were measured by fluorescent imaging; the strongest signal was assigned a value of 100, and the remaining data were adjusted accordingly. ■ α₂M*-ODN (P=S); ▲ α₂M*-ODN (P=O); □ free P=S ODN; △ free P=O ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).

3.2.2 Rate of Cellular Stimulation

Based on the high rate of α₂M*-ODN complex uptake compared to that of free ODN, we hypothesized that CpG ODN-responsive cells would be stimulated more rapidly by CpG ODN bound to α₂M* than by free CpG ODN. TG-elicited murine MΦs were treated with 0.5 µM of either free CpG ODN or CpG ODN bound to α₂M*. After periods of exposure ranging from 15 min to 4 h, the cells were washed to remove unbound ligands. Fresh media was added, and TNF production was measured 24 h after the initial exposure to CpG ODN. As Figure 11 shows, cells are stimulated with as little as 15 min of exposure to α₂M*-ODN, the shortest duration tested, while it takes 2 h of exposure to free CpG ODN to elicit a response, by which time cells exposed to α₂M*-
ODN have produced a response that is eightfold greater than that induced by free CpG ODN. Based on these results, rapid uptake is one mechanism by which incorporation into α2M* enhances the biological response to CpG ODN. As shown in Figure 7, there is graded production of cytokines in response to increasing amounts of CpG ODN. It follows, then, that increased cellular uptake of CpG ODN will result in a greater immune response, to a certain limit. Since CpG ODN bound to α2M* are endocytosed at a greater rate than free CpG ODN, it is likely that the minimum amount of internalized CpG ODN necessary to elicit an immune response will be reached more quickly when CpG ODN are bound to α2M*.

![Figure 11: Macrophages are stimulated by a shorter period of exposure to α2M*-ODN than to free CpG ODN.](image)

TG-elicited Mφs were dosed with free CpG ODN or CpG ODN-α2M*-ODN complexes at an ODN concentration of 0.5 µM for a period of 15 min up to 4 h. The cells were then washed three times, and fresh media was added. After 24 h from the addition of CpG ODN, TNF was measured by ELISA. ■ α2M*-ODN; □ free CpG ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).
3.2.3 Mechanism of Binding

We have demonstrated that $\alpha_2$M*-ODN complexes are taken up by cells more rapidly and that cells will produce an immune response after a shorter period of exposure to $\alpha_2$M*-ODN complexes than to free CpG ODN. This rapid and efficient uptake of $\alpha_2$M*-ODN complexes occurs through LRP-mediated endocytosis [92, 120, 126]. All reports to date have shown that $\alpha_2$M* complexes are taken up exclusively through LRP-mediated endocytosis. This has been shown regardless of the mechanism by which $\alpha_2$M is converted to its receptor-recognized form, whether $\alpha_2$M* has a ligand bound to it, and the identity and binding site of the ligand bound to $\alpha_2$M* [120, 126]. Based on an extrapolation of prior data from studies on $\alpha_2$M*-protein complexes, we hypothesize that the $\alpha_2$M*-LRP interaction is likewise unaffected by the incorporation of CpG ODN.

Binding studies were performed to determine whether the interaction between $\alpha_2$M* and LRP is affected by the incorporation of CpG ODN.

To demonstrate that binding and uptake of $\alpha_2$M*-ODN complexes occur through LRP, the binding of labeled $\alpha_2$M* was measured in the presence of increasing concentrations of competing ligands, $\alpha_2$M*-ODN and free CpG ODN. $\alpha_2$M* was IR-labeled using the IR Dye 800CW Protein Labeling Kit (Li-Cor). TG-elicited murine MΦs were harvested and plated following methods described above. After incubating the cells at $37^\circ C$ for 18 h, the plates were moved to $4^\circ C$ and incubated there for 1 h to allow for temperature equilibration; the remainder of the experiment was performed at $4^\circ C$. Cells were washed three times and then cultured with ice cold isotonic buffer, 150 mM...
NaCl, 25 mM HEPES, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1% BSA, pH 7.4 (Buffer A). The binding of $\alpha_2$M* to LRP is dependent on both Ca$^{2+}$ and Mg$^{2+}$ [92], so to determine non-specific binding, a portion of cells were washed and cultured in a similar solution except with 5 mM EDTA and without Ca$^{2+}$ and Mg$^{2+}$ (Buffer B). Cells were treated with 1.0 nM IR-labeled $\alpha_2$M*, and increasing concentrations of either free ODN or $\alpha_2$M*-bound ODN. Cells were incubated at 4$^\circ$C for 18 h and then washed four times with ice cold Buffer A to remove unbound $\alpha_2$M*. Bound $\alpha_2$M* was measured by IR imaging using an Odyssey infrared imaging system (Li-Cor).

As predicted, both P=O and P=S $\alpha_2$M*-ODN complexes demonstrate the ability to compete with $\alpha_2$M* for surface binding, while even 100-fold molar excess of free CpG ODN has no effect on $\alpha_2$M* binding (Figure 12). Based on the similar binding affinities of $\alpha_2$M* and $\alpha_2$M*-ODN, it is evident that CpG ODN incorporation, regardless of the CpG ODN backbone composition, does not alter the receptor-mediated endocytosis of $\alpha_2$M* by LRP.
Figure 12: Incorporation of CpG ODN does not alter the affinity of α2M* for LRP. α2M*-ODN complexes are taken up through LRP. MΦs were treated with a fixed concentration of labeled α2M* and increasing amounts of either free ODN or α2M*-ODN. After incubation, cells were washed to remove unbound α2M*, and bound α2M* was measured by fluorescent imaging; the amount bound in the absence of competing ligand was assigned a value of 100. The symbols indicate the following ligands: ■ α2M*-ODN (P=S); ▲ α2M*-ODN (P=O); □ free P=S ODN; Δ free P=O ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).

3.2.4 Inhibition of Binding by Nickel

A second method was employed to confirm that α2M*-ODN complexes are taken up by LRP. The surface binding of these ligands was measured in the presence of increasing concentrations of Ni^{2+}, a metal ion that inhibits the interaction between α2M* and its receptor LRP [127]; it should be noted that no studies to date have reported the effects of Ni^{2+} on binding of free CpG ODN. TG-elicited murine MΦs were harvested and plated following the methods described above. After incubating the cells at 37°C for 18 h, the plates were moved to 4°C and incubated there for 1 h to allow for temperature equilibration. The cells were washed three times and cultured in ice cold Buffer A; to measure non-specific binding, a portion of cells were washed and cultured in Buffer B.
Murine MΦs were treated with 0.5 µM of free or α2M*-bound CpG ODN and increasing concentrations of NiSO₄. Cells were incubated at 4°C for 18 h and washed to remove unbound ligands. Surface-bound ODN were measured by fluorescent imaging.

As previously reported, α2M* binding to cells is inhibited by the presence of Ni²⁺, but CpG ODN surface binding is actually enhanced by Ni²⁺ (Figure 13). Similar to the binding studies discussed earlier, these results demonstrate that the incorporation of CpG ODN into α2M* does not alter the cellular binding of α2M*. These α2M*-ODN complexes bind LRP with the same affinity as α2M* alone, and this binding is inhibited by Ni²⁺ in the same manner. While the purpose of this experiment was to further characterize the surface binding of α2M*-ODN complexes, we also discovered that free CpG ODN binding is affected by the presence of Ni²⁺, a phenomenon that had not previously been described.
Figure 13: Ni\textsuperscript{2+} inhibits the surface binding of α\textsubscript{2}M*-ODN complexes while it enhances the surface binding of CpG ODN. TG-elicited MΦs were incubated with free ODN or α\textsubscript{2}M*-ODN and increasing amounts of Ni\textsuperscript{2+}, incubated at 4°C for 18 h, and washed to remove unbound ligands. Free ODN and α\textsubscript{2}M*-ODN were measured by fluorescent imaging; the amounts bound in the absence of Ni\textsuperscript{2+} were assigned a value of 100. ■ α\textsubscript{2}M*-ODN; □ free ODN. Data shown here are a representative example of three experiments; error bars indicate ± s.d. (n = 3).

The uptake studies described here offer a direct comparison of the uptake rates between free and α\textsubscript{2}M*-bound CpG ODN, and they demonstrate that α\textsubscript{2}M* significantly enhances the rate of CpG ODN uptake. Despite the uptake of free CpG ODN by both receptor-mediated endocytosis and pinocytosis, α\textsubscript{2}M*-aided delivery still increases the uptake of CpG ODN by sixfold. The high rate of α\textsubscript{2}M*-ODN complex uptake is the product of high levels of LRP expression, rapid uptake, as well as receptor recycling to the surface following uptake. The combination of these events culminates in a high capacity to endocytose α\textsubscript{2}M* complexes.
3.2.5 Nuclease Protection

One of the barriers limiting the clinical efficacy of P=O CpG ODN is their very short \textit{in vivo} half-life. Due to their susceptibility to nucleases, they are rapidly degraded, rendering them inert. Solving this problem with P=S modification [75], however, creates a new set of issues. In addition to side effects like lymphadenopathy [22], splenomegaly [128], granuloma formation [83], and lethal toxic shock [78], P=S-modified ODN induce CpG-independent immunostimulation, indicating that the synthetic structures are stimulating the immune system by a pathway distinct from CpG-TLR9 ligation. Given the qualitatively different responses that CpG ODN of different backbone compositions induce, it is reasonable to postulate that different CpG ODN may be more effective at treating or preventing different diseases. Therefore, we may increase the therapeutic potential of CpG ODN by testing the larger library of CpG ODN sequences and backbone structures.

In the interest of reviving P=O CpG ODN as potential therapeutic agents, we sought to determine how their incorporation into $\alpha_2$M* would affect their susceptibility to nucleases. Proteins incorporated within $\alpha_2$M* are blocked from interacting with other large molecules in solution. Interactions are blocked between antibodies and specific ligands, signaling molecules and their receptors, and enzymes and substrates [120, 126], a quality that first characterized $\alpha_2$M as a proteinase inhibitor. Based on the characteristics of $\alpha_2$M* complexes, we hypothesized that $\alpha_2$M* may confer protection from nuclease digestion to incorporated CpG ODN. Free and $\alpha_2$M*-bound P=O CpG ODN were
incubated with the nucleases Benzonase and DNase I and then added to murine MΦs. Media was collected after incubation for 24 h, and cytokine responses were measured by ELISA. The raw data were normalized so that responses to the CpG ODN not pre-incubated with nucleases were assigned values of 100, and the remaining responses were adjusted accordingly (Figure 14). While both nucleases reduce the activity of free CpG ODN by 75%, they have no effect on the activity of CpG ODN bound within α2M*, indicating that α2M* protects CpG ODN from inactivation by nuclease digestion.

These data are the first to establish the CpG ODN binding site on α2M*. This nuclease protection is likely the result of steric hindrance between the bound CpG ODN and nucleases present in solution. As α2M* undergoes proteolytic conversion to its receptor-recognized form, there is a major conformational change that reduces the size of the binding pocket openings, thus preventing molecules larger than 20 kDa from diffusing into or out of the binding pocket [120, 126]. Since CpG ODN are not digested by nucleases, they are likely trapped within the α2M* binding pocket, so named for its tendency to bind numerous protein ligands [126]. Small nucleases that are still capable of diffusing into the binding pocket may still be inhibited since CpG ODN may incorporate into α2M* in a manner that shields the backbone, making it inaccessible to nucleases. Likewise, the dimensions of the pocket may limit the movement and orientation of nucleases, precluding proper alignment of the enzyme active site with the CpG ODN backbone. In the following chapter, we identify two mechanisms by which ODN incorporate into α2M*, by covalent and non-covalent binding. Results from this experiment demonstrate complete conservation of the biological activity of incorporated CpG ODN, indicating that, regardless of the mechanism by which CpG ODN are bound,
they are fully protected from enzymatic degradation, indicating that both covalent and non-covalent fractions are bound within the binding pocket of α2M*

Due to the synthetic nature of the *in vitro* cell culture system, there are likely few nucleases present that could inactivate P=O CpG ODN, which is why P=O CpG ODN are only slightly less potent than their P=S counterparts. Based on the extremely short half-life and efficacy of P=O CpG ODN compared to P=S equivalents *in vivo*, however, natural biological situations likely have a much higher level of nuclease activity. This suggests that α2M*-bound P=O CpG ODN will demonstrate even greater enhancement *in vivo* than that observed through *in vitro* testing. Preliminary murine *in vivo* studies have been conducted, and they demonstrate even greater biological activity enhancement than observed using in vitro models; these results will be discussed in Chapter 5.

![Graph](image)

**Figure 14: CpG ODN bound to α2M* are protected from degradation by nucleases.** Free or α2M*-bound CpG ODN were incubated with nucleases Benzonase or DNase I and then added to cells. After 24 h, TNF production was measured by ELISA; responses in the absence of nuclease treatment were both assigned a value of 100. Data are a representative example from one of three experiments, each performed in triplicate; error bars indicate ± s.d
3.3 Human in vitro Response

The enhanced biological activity of α2M*-ODN complexes demonstrated by activation of murine MΦs might not occur with human cells, which show numerous differences in the biological activity of CpG ODN. The TLR9 proteins expressed by human and murine cells share a large portion of sequence identity, but each recognizes different CpG motifs that optimally stimulate responses [129-131]. In mice, the motif shown to be most active is 5’-Pu-Pu-CpG-Py-Py-3’, such as those found in ODN #1826. In humans, there are three classes of CpG ODN that stimulate distinctly different subsets of cells to generate qualitatively different responses. Class A (D type) ODN, composed of a palindromic P=O middle segment capped with P=S-modified ends, activate NK cells and pDC to produce IFNγ and IFNα, respectively [69, 71, 72], but fail to stimulate B cells. Class B (K Type) ODN are wholly P=S sequences with multiple CpG motifs and a poly(G) tail at the 3’ end; this class triggers IgM and IL-6 production from B cells, activates pDC to produce TNF rather than IFNα, and stimulates strong cytolytic activity in NK cells [70, 71, 73]. Class C ODN is a hybrid of the first two classes with a completely P=S-modified backbone, palindromic sequences, but no poly(G) tail; this class has the ability to induce both type I IFN production as well as B cell activation [70].

Another key difference is the cellular expression of TLR9; human MΦs do not express TLR 9 and are therefore unresponsive to CpG ODN. Rather, the human cells activated by CpG ODN include pDC, B cells, and NK cells [132].

To demonstrate the ability of α2M*-ODN complexes to stimulate an immune response in humans, PBMC were treated with CpG ODN #2395, a Class C ODN that
exhibits optimal activity in stimulating PBMC [70]. Cells were treated with equal amounts of either free or α₂M*-bound CpG ODN over a range of concentrations and incubated for 24 h; media was collected, and cytokines were measured by ELISA. The production of IFNα, IL-6, and TNF are shown in Figure 15A, C, and E, respectively, which demonstrate that α₂M* incorporation enhances both the potency and efficacy of CpG ODN in human PBMC. The amount of IFNα produced in response to 0.25 uM α₂M*-ODN is tenfold greater than that induced by the same amount of free CpG ODN. The maximum amount of IFNα in response to α₂M*-ODN is nearly twice that of the maximal response to free CpG ODN. While the peak IL-6 responses are similar, the maximal response to α₂M*-ODN is elicited at a fourfold lower dose than free CpG ODN. TNF production elicited by complexes is greater at every concentration tested and is twofold greater at all but the highest concentration. As controls, PBMC were treated with α₂M* alone and α₂M* carrying an ODN control sequence with no biological activity. At concentrations similar to those used in the previous experiment, responses to both α₂M* alone and control sequence α₂M*-ODN complexes were undetectable (data not shown). These findings confirm that the α₂M* itself is not responsible for the immune response and that cellular activation is dependent on the CpG motif of the ODN incorporated within α₂M*. The data further confirm that the complexes used to elicit immune responses were free of contamination by other agents that might cause an immune response, such as LPS.

The response kinetics of PBMC were also examined. PBMC were treated with 0.5 µM of free or α₂M*-bound CpG ODN and incubated for varying durations; cytokines were measured by ELISA. Figure 15B, D, and F show the production of IFNα, IL-6, and
TNF, respectively, over a period of 48 h. Like the previous dose-response curves, these figures show the superior efficacy of α₂M*-ODN over free CpG ODN; the peak production of IFNα is enhanced by twofold, that of IL-6 is enhanced by sixfold, while the maximum TNF production is increased by 15-fold. In addition to greater peak responses, the responses elicited by α₂M*-ODN begin much earlier than those induced by free CpG ODN stimulation. IFNα production reached a level of 700 pg/ml just 8 h after stimulation with α₂M*-ODN; this level was not reached by free CpG ODN activation until 24 h after treatment. Similarly, α₂M*-ODN stimulated the production of 750 pg/ml of IL-6 within 4 h, while free CpG ODN treatment did not reach that level of IL-6 production until after 48 h. The differences in TNF production may be the most striking; the responses to both free and α₂M*-bound CpG ODN peaked after 8 h, but with 15-fold greater production of TNF from those cells treated with α₂M*-ODN, the response to free CpG ODN is barely visible on the same scale.

These data expand our knowledge of the immunostimulatory properties of α₂M*-ODN complexes. In addition to their similarity to our findings with murine MΦs, these results confirm that α₂M* greatly enhances the ability of CpG ODN to stimulate immune responses in human cells. Despite numerous differences in TLR9 biology between mice and humans, α₂M*-bound CpG ODN consistently demonstrate enhanced potency and efficacy for activation of immune competent cells, with the responses both beginning and peaking earlier. Given the numerous clinical applications of CpG ODN currently being studied, these results offer a promising new delivery system for improving the clinical efficacy of CpG ODN while reducing their side effects.

In both the human and murine biological assays described here, human α₂M* was
used for the incorporation of CpG ODN. There is enough sequence identity between human and mouse α2M* that both proteins exhibit similar behavior with respect to cellular binding and uptake by the α2M* endocytic receptor, LRP, of both species. However, the differences are significant enough that murine tolerance to the human protein will be broken as a result of repeated exposure. Therefore, human and murine α2M* can be used interchangeably for short term experiments, such as the biological assays described in this thesis. Murine cells can be treated with human α2M* without concerns of immunogenicity of foreign proteins; this is evidenced by the lack of response when murine cells were treated with α2M* alone as a negative control. However, since long term exposure to human α2M* eventually breaks tolerance, murine α2M must be used for experiments that span greater durations, such as those measuring antibody titers, tumor rejection, or protection from infectious agents; future experiments such as these will be discussed in Chapter 5.
Figure 15: α₂M*-ODN complexes exhibit an enhanced ability to stimulate human PBMC. PBMC were treated with either free or α₂M*-bound CpG ODN, and inflammatory cytokines were measured in the media after 24 h. Dose-response curves were obtained measuring A) IFNα; C) IL-6; and E) TNF. Cells were treated with 0.5 µM of free or α₂M*-bound CpG ODN, and cytokines were measured in the media after the stated durations; B) IFNα; D) IL-6; and F) TNF. ■ α₂M*-ODN; □ free CpG ODN. Data are a representative example from one of three experiments, each performed in triplicate; error bars indicate ± s.d.
Chapter 4: Chemical Analysis of $\alpha_2$M*-ODN Complexes
The remainder of studies described in this thesis focuses on the chemical nature of the interaction between ODN and $\alpha_2$M*. For clarity, the meanings of abbreviations are reiterated. “ODN” refers to oligodeoxynucleotides, single-stranded nucleic acids composed of 10-25 bases. “CpG ODN” specifically refers to ODN possessing at least one CpG dinucleotide. Throughout this thesis, “ODN” and “CpG ODN” have been used synonymously, but to be accurate, the latter is a subset of the former. This distinction is made because the chemical analyses that follow were performed, in part, with the use of ODN free of CpG dinucleotides; with respect to the interaction between ODN and $\alpha_2$M*, no differences were observed to result from the presence or absence of the CpG dinucleotide. Results are therefore applicable to ODN rather than the subset, CpG ODN; abbreviations used in this chapter reflect this with the use of ODN rather than CpG ODN.

4.1 Mechanism and Stoichiometry of ODN Incorporation

The mechanism by which ligands incorporate into $\alpha_2$M* has been well characterized (reviewed in [91]). Molecules either associate non-covalently through hydrophobic binding, charge-charge interactions, and Van der Waals forces, or form covalent linkages. When the structure of $\alpha_2$M* is disrupted by the denaturing conditions of SDS-PAGE analysis, molecules that are bound non-covalently dissociate from $\alpha_2$M*, while those that are covalently bound remain attached to the fragments of $\alpha_2$M*.

Molecules bind covalently to one of the two residues that make up the thiolester bond: Cys$_{949}$ and Glx$_{952}$. If bound to the Cys residue, ligands will dissociate under reducing conditions but will remain bound to the Glx. To determine the mechanism by which
ODN incorporate, complexes were analyzed in a series of PAGE experiments. Prior to electrophoresis, complexes were purified by FPLC to remove unbound ODN. These gels were run for short durations so that unbound ODN would remain on the gel and could be detected by fluorescent imaging.

PAGE analysis under native conditions revealed that the entire portion of incorporated ODN remained bound to α2M* (Figure 16), indicating that once free ODN have been removed by FPLC purification, there is minimal loss of ODN from α2M*, even when samples are exposed to an electrophoretic field. When the same α2M*-ODN samples were analyzed by SDS-PAGE, there was significant loss of ODN from the complexes. The amount that remains bound following non-reducing SDS-PAGE analysis represents the entire fraction of covalently bound ODN. Reducing agents will cleave the bond formed by ligand binding to the Cys residue but leave intact the bond formed with the Glx residue. The amount of ODN that remains bound under reducing conditions, therefore, represents the fraction that is Glx-bound. The difference between these two amounts represents that portion bound to the Cys residue. Samples were analyzed by SDS-PAGE in the presence and absence of the reducing agent β-mercaptoethanol, and the amounts of bound ODN were measured by fluorescent imaging (Figure 16 C and E), and the locations of α2M* fragments were detected by Coomassie brilliant blue staining of the same gels (Figure 16 D and F). The amounts of ODN bound in each conditions were compared; Arrow #1 indicates the total amount of ODN incorporated within α2M*, Arrow #2 shows the entire covalent fraction, and Bracket #3 identifies the portion that is covalently bound to the Glx residue. These analyses were performed using both P=O and P=S ODN; the results shown in Table 1 list the stoichiometry of incorporation by all
three mechanisms. These experiments were performed in quadruplicate; values represent experimental means ±s.d. (n = 4).

Figure 16: ODN incorporate into α2M* through both covalent and non-covalent mechanisms. Complexes were purified by FPLC and analyzed by PAGE under native conditions. A) α2M*-bound ODN were detected by fluorescent imaging, and B) α2M* was detected by Coomassie brilliant blue stain of the same gel. Similar images were obtained when the same samples were analyzed by PAGE under denaturing conditions; C) and D) non-reducing conditions; and E) and F) reducing conditions, respectively. Arrows #1-3 identify the signals that were quantified to determine the relative amounts of α2M*-bound ODN under these conditions.

Table 1: The Stoichiometry and Mechanisms of ODN Incorporation into α2M*

<table>
<thead>
<tr>
<th>ODN Backbone</th>
<th>Mechanism of Binding</th>
<th>Moles ODN per mole α2M*</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>P=O</td>
<td>Total</td>
<td>0.56 (±0.08)</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Covalent</td>
<td>0.42 (±0.07)</td>
<td>75.0%</td>
</tr>
<tr>
<td></td>
<td>Glx952</td>
<td>0.33 (±0.05)</td>
<td>58.9%</td>
</tr>
<tr>
<td></td>
<td>Cys949</td>
<td>0.09 (±0.02)</td>
<td>16.1%</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1.8 (±0.22)</td>
<td>N/A</td>
</tr>
<tr>
<td>P=S</td>
<td>Covalent</td>
<td>0.73 (±0.14)</td>
<td>40.5%</td>
</tr>
<tr>
<td></td>
<td>Glx952</td>
<td>0.44 (±0.11)</td>
<td>24.8%</td>
</tr>
<tr>
<td></td>
<td>Cys949</td>
<td>0.29 (±0.09)</td>
<td>15.7%</td>
</tr>
</tbody>
</table>

Both P=O and P=S ODN used in these studies were ODN #1826, the sequence used in the murine in vitro assays. This was the model sequence used in all investigations of α2M*-ODN complexes, with the exception of those studies aimed at determining how variations in ODN length and base composition affect incorporation.
As discussed later, the stoichiometry and mechanisms by which ODN incorporate into α2M* are dependent on more variables than just the ODN backbone composition, which is the only one identified here. Incorporation is also affected by ODN length, base composition, methylation, and the presence of other incorporating ligands. The stoichiometry and distribution by which other sequences of ODN incorporate into α2M* will deviate from those stated in this table, but the analysis of ODN #1826 serves as the foundation against which other ODN stoichiometries will be compared.

### 4.2 Stability of α2M*-ODN Complexes

To determine the stability of α2M*-ODN complexes, they were synthesized and purified by FPLC. Unbound ODN were removed because ODN incorporation may be reversible, creating a state of equilibrium between bound and unbound ODN. Removal of unbound ODN allows us to determine the rate at which ODN dissociate from α2M in the absence of the molar excess of unbound ODN. Complexes were incubated at temperatures ranging from 0°C to 50°C. Over five time points, samples from each temperature were analyzed by PAGE under native conditions to quantify the ODN that remained incorporated. The amount of ODN bound in the sample incubated at 0°C was assigned a value of 100, and the remaining data were adjusted accordingly.

Figure 5 shows the relative quantities of ODN that remained incorporated over the span of 120 h. We had previously found that complexes stored at 0°C were stable for extended periods of time; native PAGE analysis after 18 months showed approximately
5% ODN dissociation from the complexes (data not shown). As Figure 17 shows, compared to the samples incubated at 0°C, there was essentially no difference in the amount of both P=O and P=S ODN that remained incorporated in the sample incubated at 25°C. At 37°C, there was a measurable difference; after 72 h, complexes with both P=O and P=S ODN had lost 40% of the bound ODN. The largest differences between P=O and P=S ODN dissociation occurred at the higher temperatures. At 42°C, 60% of the P=O ODN was lost after 24 h, while it took 120 for P=S ODN to reach the same degree of dissociation. As expected, the highest rate of dissociation occurred at 50°C, the highest temperature tested; after 5 h, 80% of the P=O ODN and 60% of the P=S ODN had been lost. However, at this temperature α2M* begins to degrade, which is confirmed by Coomassie brilliant blue staining, which indicate that the protein is breaking down at 50°C in as little as 5 h (data not shown). The quantification of ODN signals for samples incubated at 50°C is therefore unreliable. This temperature was included only for the purpose of defining the upper temperature limit; if these complexes were developed as therapeutics, they would never be stored at such a temperature, so the instability at 50°C does not reduce the potential clinical utility of these complexes. As a control, fluorescently labeled ODN were incubated at similar temperatures, and the fluorescence of samples was measured at similar time points to verify that reduction in signal strength was not the result of degradation of the label itself; there was no loss of signal strength at any temperature over the entire duration of the experiment (data not shown).
Figure 17: $\alpha_2$M*-ODN complexes are stable for several days over a range of temperatures. ODN were incorporated into $\alpha_2$M via HNE activation. The complexes were incubated at temperatures from 0\textdegree\,C to 50\textdegree\,C. At the given time points, aliquots were analyzed by PAGE under native conditions, and the amount of ODN that remained bound was measured. A) $\alpha_2$M*-ODN (P=O), and B) $\alpha_2$M*-ODN (P=S) complexes. Samples incubated at 0\textdegree\,C were assigned values of 100, and the remaining data were adjusted accordingly. Temperatures in legend are listed in \textdegree\,C. Data shown here are means of the experiment performed in triplicate ± s.d.

The stability of complexes stored under various pH levels was also measured.

The methods were similar to those described in the previous experiment; after complexes were generated and purified by FPLC, the pH of samples was adjusted to levels between
4.0 and 7.4; samples were stored at 0° C. After durations of 1 h, 4 h, and 24 h, samples were analyzed by PAGE under native conditions, and the amounts of ODN that remained bound were measured by fluorescent imaging; results are shown in Figure 18. There was significant loss of both P=O and P=S ODN among the samples that were stored at pH levels below 5. This was due to the breakdown of α2M* itself, which was confirmed on detection of α2M* with Coomassie brilliant blue staining (data not shown). At pH levels between 5 and 7.4, the stabilities of P=O and P=S ODN bound to α2M* differed slightly. Complexes with P=O ODN exhibited the greatest stability at a pH of 5.0, and ODN dissociation progressively increased as the pH increased to 7.4. Complexes with P=S ODN, however, demonstrated similar stabilities at all pH levels between 5 and 7.4 over all the durations tested.

Loss of ODN at pH levels below 5.0 is due to the breakdown of α2M*, but the exclusive loss of P=O ODN from α2M* at pH levels above 5.0 is not well understood at this point. It is possible that the complexes had been contaminated with nucleases prior to proteolytic conversion of α2M and incorporation of ODN within the binding pockets. If contamination occurred early, the nucleases would potentially be trapped within α2M*, allowing access to the ODN that is also trapped within. If such nucleases were more active at pH levels near neutrality, the rate of P=O ODN degradation would increase under these conditions. ODN cleavage would permit the fluorescent label to dissociate from the complexes, leading the decrease in signal that was observed. P=S ODN, however, are impervious to nuclease digestion, a similar loss of signal would not be observed, which was the case. However, the studies were conducted on three separate occasions with separate preparation of α2M*-ODN complexes, so the likelihood of
nuclease contamination prior to complex formation of all preparations is low.

Alternatively, the binding mechanism of P=O ODN may differ slightly from P=S ODN and may occur in part through amino acid chains that can accept or donate protons. As the solution becomes more basic, residues may become deprotonated, altering their conformation and producing a negative charge, which would then repel the negatively charged ODN. More work is needed to determine why these stability trends are observed, but, for the purposes of developing methods to produce and store these complexes for therapeutic use, the optimal pH conditions under which the complexes may be stored have been characterized.
Figure 18: $\alpha_2$M*-ODN complexes are stable for over a broad pH range. ODN were incorporated into $\alpha_2$M* via HNE activation. The complexes were incubated in solutions with pH levels from 4.0 to 7.4. After the stated durations, samples were analyzed by PAGE under native conditions, and the amount of ODN that remained bound was measured. A) $\alpha_2$M*-ODN (P=O), and B) $\alpha_2$M*-ODN (P=S) complexes. For each set of data, the strongest signal measured was assigned a value of 100, and the remaining values were adjusted accordingly. Data shown here are means of the experiment performed in triplicate ± s.d.

4.3 Effects of ODN Chemical Modification on Incorporation

Studies of how incorporation into $\alpha_2$M* affects the biological activity of CpG ODN have been discussed earlier in this thesis. These preliminary characterizations demonstrate that the biological activity is enhanced and that $\alpha_2$M*-ODN complexes show
promise as therapeutic agents. A potential limiting factor is the maximum concentration of ODN bound within $\alpha_2M^*$ that can be achieved. The solubility limit of $\alpha_2M^*$ is 6-8 $\mu$M [1]; above this concentration, the protein begins to aggregate and precipitate out of solution, which could irreversibly alter the biology of these complexes. This solubility defines the upper limit of the concentration of CpG ODN that is bound to $\alpha_2M^*$ because the ODN incorporate into $\alpha_2M^*$ in a fixed, predictable molar ratio. As discussed earlier in this chapter, that ratio is dependent on the backbone composition of the ODN; $P=S$ ODN incorporate in a ratio that is threefold higher than $P=O$ ODN. The maximum ODN concentration using ODN #1826 was 12 $\mu$M, a value consistent with $\alpha_2M^*$ solubility (6 $\mu$M) and the molar incorporation ratio of that particular ODN (2.0 moles/mole $\alpha_2M^*$).

However, in the process of evaluating the ability of these complexes to stimulate immune responses from both murine and human cells, different sequences were incorporated into $\alpha_2M^*$. Analysis of products revealed that ODN of different sequences incorporated at different ratios. As stated, the stoichiometric ratios listed in Table 1 were obtained using ODN #1826. When ODN #2395 was incorporated, ratios as high as 4.5 moles/mole $\alpha_2M^*$ were obtained. This highlighted that there were more influences on the molar incorporation ratio than just the backbone composition. In the interest of increasing the upper limit of ODN concentration in a formulation of $\alpha_2M^*$-ODN complexes for clinical use, it will be important to identify the ODN variables that increase the incorporation ratio; characterizing the influences of this interaction may also yield greater understanding of the mechanisms involved. Previous studies have attempted to identify variables of protein ligands that will predictably affect the molar incorporation ratio of ligands into $\alpha_2M^*$; such variables include molecular mass, Stokes radius, and isoelectric...
point. After comparison of numerous protein ligands and their ratios of incorporation, there have yet to be any variables identified that affect incorporation in a predictable fashion (unpublished data, Pizzo lab). Despite these discouraging findings, a systematic characterization of the effects of ODN variation on the molar ratio of incorporation was performed. The following sections will address the variables studied and how they affect the interaction between ODN and α₂M*.

4.3.1 Backbone Composition

In the sections that follow, ODN incorporation was measured by fluorescent imaging. In the development of the biological assays described in the previous chapter, the fluorescent label was attached at the 3’ end to avoid abrogation of biological activity [114]. All analyses described here employed the same labeling methods. To determine whether the interaction between ODN and α₂M* was dependent on the label, the first experiment performed was a competition between labeled and unlabeled ODN. Labeled ODN and α₂M were mixed in a molar ratio of 5:1 with increasing concentrations of unlabeled ODN up to 100-fold molar excess ODN, with respect to the labeled ODN. Following activation with HNE, the samples were analyzed by PAGE under native conditions, and bound ODN was measured by fluorescent imaging. This experiment was performed using both P=O and P=S ODN. As shown in Figure 19, increasing amounts of unlabeled ODN compete with labeled ODN for binding. At fivefold excess, incorporation of labeled ODN drops by 60%, and at 50-fold molar excess, it is reduced by 95% for both P=O and P=S ODN. These results indicate that the unlabeled ODN can
compete with labeled ODN for binding and that the label is not responsible for the interaction. Furthermore, it demonstrates that ODN bind to α2M* through a specific and saturable mechanism.

![Diagram showing ODN binding α2M* in a specific, saturable manner.](image)

**Figure 19: ODN bind α2M* in a specific, saturable manner.** Labeled ODN and α2M were mixed with a fixed amount of labeled ODN and increasing amounts of unlabeled ODN. Numbers 0-100 indicate the molar ratio between unlabeled and labeled ODN. A) Coomassie brilliant blue stain; and B) Fluorescent image of the same gel. C) The signals from A were quantified and represented here graphically; the amounts bound in the absence of competing ligand were assigned a value of 100. Data shown here are means of the experiment performed in triplicate ± s.d.

As described earlier in this chapter, the molar ratios with which P=O and P=S ODN bind are quite different; P=S ODN incorporate at a ratio that is threefold that of the P=O ODN incorporation. To determine whether P=O and P=S ODN share a binding site
on $\alpha_2M^*$, competition studies were performed. Conditions were similar to the experiment described above except that the competing ligands had alternate backbone compositions; Figure 20 shows the results of these experiments. The presence of unlabeled P=O ODN does not affect the incorporation of P=S ODN; even at 100-fold excess P=O ODN, the amount of P=S ODN that incorporates into $\alpha_2M^*$ is unaltered. However, the presence of equimolar unlabeled P=S ODN completely inhibits P=O ODN incorporation, demonstrating that the two forms of ODN share a common binding site on $\alpha_2M^*$. It further shows that the affinity with which P=S ODN binds is much greater than that of P=O ODN.
Figure 20: P=O and P=S ODN share a binding site on α₂M*, but P=S ODN have a greater affinity. α₂M was mixed with labeled ODN of either P=O or P=S backbone composition and increasing amounts of unlabeled ODN with the alternate backbone composition, followed by activation with HNE and analysis of products by PAGE under native conditions. Numbers 0-100 indicate molar ratio of unlabeled to labeled ODN. A) and B) are Coomassie brilliant blue stain and fluorescent imaging, respectively, of PAGE analysis of unlabeled P=O ODN competing with labeled P=S ODN. C) and D) are Coomassie brilliant blue stain and fluorescent imaging, respectively, of PAGE analysis of unlabeled P=S ODN competing with labeled P=O ODN. E) The signals from Panels B and D were quantified and are displayed graphically; the amounts bound in the absence of competing ligands were assigned a value of 100, and remaining data were adjusted accordingly. ■ Bound P=O ODN in the presence of competing P=S ODN; □ Bound P=S ODN in the presence of competing P=S ODN. Data shown here are means of the experiment performed in triplicate ± s.d.
A possible explanation for this finding is that the backbone modification alters the hydrophobicity of the ODN molecule. Previous studies on ligand incorporation into $\alpha_2M^*$ have shown that the $\alpha_2M^*$ binding pocket is lined with hydrophobic residues [133-135]. P=S modification is the replacement of a non-bridging oxygen with a sulfur atom, which has a lower electronegativity than oxygen. This reduces the polarity of its bond with phosphorus, thereby reducing the partial charges on both atoms. This increases the hydrophobicity of the entire molecule, which may account for the significantly increased affinity with which P=S ODN incorporate into $\alpha_2M^*$. In addition, studies comparing the behaviors of P=O and P=S ODN have shown that the P=S modification increases non-specific binding to proteins in general; P=S ODN bind to a larger number of proteins and do so with a greater affinity [76, 81, 136].

Despite this disparity in binding affinities, the stability of complexes is quite similar. As discussed earlier in this chapter, P=S ODN bound to $\alpha_2M^*$ exhibit slightly greater stability at elevated temperatures and at higher pH conditions. When these parameters are adjusted to optimize stability, however, both P=O and P=S ODN bound to $\alpha_2M^*$ are stable for extended durations. So while the P=S ODN affinity for binding is greater than that of P=O ODN, both form complexes that are stable enough to be stored for long durations with minimal break down of the complexes. This is an important characteristic in the context of determining the potential therapeutic utility of these complexes; they must be stable long enough to be packaged and distributed for clinical use.
4.3.2 Methylation

Bacterial DNA and CpG ODN are distinguished from endogenous mammalian DNA by the frequency of unmethylated CpG dinucleotides. The CpG dinucleotides present in these DNA fragments must be unmethylated in order to elicit an immune response [12]. This demonstrates that the CpG-TLR9 interaction is dependent not only on the nucleic acid sequence but also on chemical modifications of the nucleotides involved.

The chemical composition of the nucleic acid backbone has been shown earlier in this thesis to influence the interaction between ODN and α2M*. Based on the observations that chemical modification of the ODN affects binding to α2M* and that the immunostimulatory properties of CpG ODN are likewise influenced by chemical modification, we sought to determine whether methylation of ODN would affect its association with α2M*. To determine whether incorporation is affected, a methylated ODN was incorporated into α2M*, and the mechanism and stoichiometry of the interaction were measured. ODN #1826 was methylated at the fifth carbon on the cytosine base of both CpG dinucleotides. α2M*-ODN complexes were generated by the methods described earlier. Samples were analyzed by PAGE under native and denaturing conditions, and the amounts of bound ODN were measured by fluorescent imaging. Under native conditions, the amount of methylated ODN that incorporate into α2M* is 50% compared to the amount of unmethylated ODN that binds (Table 2). Under denaturing conditions, both reducing and non-reducing, the amount of methylated ODN that was associated with α2M* was again half that of unmethylated ODN. These results indicate that the total amount of ODN that incorporates into α2M* is cut in half when the
ODN is methylated, but the relative distribution among the different mechanisms of binding is unchanged compared to the incorporation of unmethylated ODN.

For the purposes of optimizing the generation of α₂M*-ODN complexes that elicit immune responses, reduced incorporation by ODN methylation has no impact since the ODN used must be unmethylated to remain biologically active. However, this observation raises the possibility that α₂M* has the innate ability to distinguish between native and foreign DNA. α₂M* has a diverse set of observed activities, making it difficult to identify a single unifying function of this protein. One theory is that α₂M serves as a sensor of proteolysis, allowing cells to sample the extracellular environment. Macromolecules such as foreign proteins or nucleic acid fragments diffuse into the α₂M binding pocket and become irreversibly trapped within α₂M* upon proteolytic activation. This reaction converts α₂M to its receptor-recognized form, which is endocytosed quickly by immune competent cells: MΦs, DCs and B cells. This series of events distills down to a mechanism by which a diverse variety of molecules, whether endogenous or foreign, may be taken up by immune competent cells when proteases are present in the extracellular space, which is characteristic of infections and inflammation. In the setting of an infection, bacterial expansion as well as the resulting inflammation would lead to the release of cellular debris from the breakdown of both host and bacterial cells. The finding that methylation reduces incorporation into α₂M* demonstrates that α₂M* exhibits some selectivity for DNA fragments of bacterial origin over those of the mammalian host. The experiments conducted to date have only measured the amounts of methylated ODN that bound; the ability of α₂M* to demonstrate selectivity in binding to foreign or endogenous DNA could be assessed by competition studies to measure the
relative binding affinities of methylated and unmethylated ODN. While a twofold increase in incorporation is not overwhelming, it is possible that unmethylated ODN possess a binding affinity for $\alpha_2$M* that greatly exceeds that of methylated ODN. Data are currently too limited to make any further conclusions about the selectivity of $\alpha_2$M* for unmethylated nucleic acids, but the effects of methylation on incorporation remain an interesting finding.

### Table 2: Effects of Methylation on the Stoichiometry and Mechanism of ODN Binding to $\alpha_2$M*

<table>
<thead>
<tr>
<th></th>
<th>Moles ODN per mole $\alpha_2$M*</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P=O ODN</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0.56 (±0.08)</td>
<td>N/A</td>
</tr>
<tr>
<td>Covalent</td>
<td>0.42 (±0.07)</td>
<td>75.0%</td>
</tr>
<tr>
<td>Glx</td>
<td>0.33 (±0.05)</td>
<td>58.9%</td>
</tr>
<tr>
<td>Cys</td>
<td>0.09 (±0.02)</td>
<td>16.1%</td>
</tr>
<tr>
<td><strong>Me-ODN</strong></td>
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<tr>
<td>Total</td>
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<tr>
<td>Covalent</td>
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<tr>
<td>Glx</td>
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<tr>
<td>Cys</td>
<td>0.04 (±0.01)</td>
<td>14.3%</td>
</tr>
</tbody>
</table>

### 4.4 Effects of Nucleic Acid Structural Variations on Incorporation

In addition to determining how chemical modifications alter the interaction between $\alpha_2$M* and ODN, we sought to investigate how other ODN variables affected binding. The variables assessed in this thesis include nucleic acid base composition, length, single- versus double-stranded states, and RNA versus DNA. In each case, the method of investigation was similar. Fluorescently labeled ODN #1826 (5’-TCCATGACGTTCCTGACGTT - 3’) was mixed with $\alpha_2$M in a ratio of 5:1. Increasing
amounts of the competing ligand were added to the mixture so that the ratio of ligands (unlabeled:labeled) increased from 0:1 to 100:1. $\alpha_2$M was activated with the addition of HNE, and the samples were analyzed by PAGE under native conditions. $\alpha_2$M*-bound ODN #1826 was detected by fluorescent imaging using a Storm 860 Phosphorimager® (Molecular Dynamics) and ImageQuant software. Gels were stained with Coomassie brilliant blue to confirm that all $\alpha_2$M present had been converted to its receptor-recognized form, $\alpha_2$M*.

### 4.4.1 Base Composition

As noted earlier, ODN of different sequences incorporate into $\alpha_2$M* at different ratios. To identify the mechanisms responsible for this variability, we sought to determine whether the different nitrogen bases of the nucleic acid fragments could influence binding. The role that each nitrogen base has in ODN incorporation was examined by measuring the incorporation of P=O ODN #1826 in the presence of increasing concentrations of P=O poly-N nucleotides: G$_{20}$, C$_{20}$, T$_{20}$, and A$_{20}$. By measuring the amount of ODN #1826 incorporation in the presence of these competing ligands, we were able to assess the affinity with which each poly-N ODN binds. The results, shown in Figure 21, reveal base-specific differences in binding affinity. G$_{20}$, C$_{20}$, and T$_{20}$ all exhibit the ability to compete with ODN #1826 for binding. At equal concentrations of ODN #1826 and T$_{20}$, C$_{20}$, and G$_{20}$, the incorporation of ODN #1826 is reduced by 67%, 72%, and 92%, respectively. A$_{20}$, however, does not displace ODN #1826 from binding even at 100-fold molar excess. These differences indicate that
binding is at least partially dependent on the nitrogen base portion of the ODN and that binding may occur specific structures on the nitrogen bases. It is also possible that poly-A ODN have the ability to incorporate but have a distinctly different binding site, thus eliminating the ability to compete with ODN #1826 for binding. If this were the case, binding of poly-A ODN may be exclusively through non-covalent means because ODN #1826 binds covalently to the only two sites available for covalent incorporation. Binding of poly-A could be assessed by the direct measurement of labeled poly-A ODN rather than employing unlabeled poly-A as a competing ligand, although this experiment was not performed.

On inspection of the nitrogen base structures, the most obvious difference between adenine and the other nitrogen bases is that adenine lacks a carbonyl group (Figure 22). The mechanism of covalent incorporation into $\alpha_2M^*$ has been well characterized; nucleophilic attack of the thiolester bond between Cys$_{949}$ and Glx$_{952}$ causes cleavage of this bond and covalent attachment to one of these two residues [126]. For proteins to undergo nucleophilic attack, surface lysines must be present. The lysine side chain has a primary amine that acts as the nucleophile and breaks the $\alpha_2M$ thiolester bond. Without primary amines, proteins do not readily become covalently bound but rather incorporate through non-covalent means. The carbonyl oxygen on G, C, and T may serve as the ODN equivalent of the protein primary amine by undergoing nucleophilic attack on the thiolester bond. Covalent binding would then occur through the carbonyl oxygen. Alternatively, the nitrogen bases may alter the non-covalent interactions with $\alpha_2M^*$, thus resulting in the observed differences. Further investigations are needed to strengthen or disprove this hypothesis, but that goes beyond the scope of 83
Figure 21: ODN incorporation is affected by the presence of thymine, cytosine, and guanine, but not adenine. Labeled ODN seq #1826 was mixed with α2M and increasing concentrations of unlabeled 20-mer ODN, each composed entirely of T, C, G, or A (N20). Following activation with HNE, the samples were analyzed by PAGE under native conditions, and ODN was measured by fluorescent imaging; the amounts bound in the absence of competing ligands were assigned a value of 100. ■ = T; ▲ = C; □ = G; ∆ = A. Data shown here are means of the experiment performed in triplicate ± s.d.
4.4.2 Length of ODN

The next parameter that was examined was the length of the incorporating ODN. The size range of proteins that have been incorporated into \( \alpha_2 \)M* extends from approximately 1 kDa up to 120 kDa (HIV gp120). Despite the large number of proteins that have been incorporated into \( \alpha_2 \)M*, a predictable pattern of how size affects the molar ratio of incorporation has not emerged. These ratios must be determined empirically for each new protein examined, and the same holds true for a new class of macromolecules.
such as nucleic acids. ODN #1826 has a molecular mass of 6.6 kDa, but due to the linear
structure of nucleic acids, it likely has a much larger Stokes radius than proteins of a
comparable mass. Since α₂M* can incorporate proteins as large as 120 kDa, it is unlikely
that the size of ODN #1826 approaches the upper limit of nucleic acid incorporation.

As a preliminary investigation, binding competitions using ODN of different
lengths were performed to determine the relative affinities with which they bind. To
prevent sequence variations from affecting the binding affinities, poly-N ODN were used
as ligands. The affinities of G₂₀, C₂₀, and T₂₀ were compared to those of G₁₀, C₁₀, and
T₁₀, respectively. Adenine was excluded because, as previously shown, it does not
exhibit the ability to compete with ODN #1826 for binding. ODN #1826 were mixed
with α₂M and increasing concentrations of the competing ligands. Complexes were
formed by proteolytic activation, and samples were analyzed by PAGE under native
conditions; the amounts of bound ODN #1826 were measured by fluorescent imaging.
Relative affinities were determined by comparing the ability of each N₂₀ to compete with
the ability of its 10-mer counterpart. For example, the amount of ODN #1826 bound in
the presence of 6.25 molar excess T₁₀ was compared to the amount bound in the presence
of 6.25 molar excess T₂₀. These calculations were performed for each pair of poly-T, -C,
and -G ODN to determine their relative binding affinities; results are shown in Table 3.
The ratios shown are the product of the amount of bound ODN #1826 in the presence of
N₁₀ divided by the amount bound in the presence of N₂₀. Under all conditions, this ratio
was between 2 and 3, indicating that the amount of bound ODN #1826 was consistently
two- to threefold higher in the presence of N₁₀.

Under these conditions, the larger ODN consistently exhibited a greater binding
affinity, but this is merely a preliminary study of the influence of ODN length. To obtain a more complete understanding, future studies will be performed with a larger number of permutations; multiple sizes of competing ligands as well as direct measurement of poly-N incorporation rather than indirect measurement by competition. In addition to binding affinities, the upper and lower size limits of nucleic acid fragments that will incorporate could be determined with the use of a large range of ODN lengths.

Table 3: The Effects of ODN Length on Incorporation into $\alpha_2$M*  

<table>
<thead>
<tr>
<th>Molar Ratio Unlabeled: Labeled</th>
<th>Ratio of Bound Ligand in Presence of Competing Ligands (10-mer:20-mer)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T</td>
</tr>
<tr>
<td>1.56</td>
<td>2.16</td>
</tr>
<tr>
<td>3.13</td>
<td>2.84</td>
</tr>
<tr>
<td>6.25</td>
<td>2.30</td>
</tr>
<tr>
<td>Avg</td>
<td>2.50 (± 0.47)</td>
</tr>
</tbody>
</table>

4.4.3 Incorporation of Double-Stranded ODN

The next form of nucleic acids examined for their ability to incorporate into $\alpha_2$M* were double-stranded (ds) ODN. Immunostimulatory CpG ODN must be single-stranded to exhibit their biological activity, and as a result, all studies on $\alpha_2$M*-ODN complexes have employed single-stranded (ss) ODN to maintain continuity. To assess the ability of dsODN to incorporate, competition binding studies similar to those described above were performed. Labeled ssODN #1826 was mixed with $\alpha_2$M and increasing concentrations of dsODN #1826. Following activation with HNE, samples were analyzed by PAGE under native conditions, and the amount of ssODN incorporation was measured by fluorescent imaging. As shown in Figure 23, the amount of ssODN bound is reduced by increasing
concentrations of dsODN; at fivefold molar excess of dsODN, the amount of bound ssODN is reduced by 80%. These results demonstrate that both single- and double-stranded ODN share a binding site and that the binding affinities are comparable.

These results are somewhat unexpected in light of the data collected on the influence of base composition. As previously discussed, poly-G, -C, and -T demonstrate the ability to incorporate while poly-A does not. If base composition affects incorporation, it is reasonable to speculate that the nitrogen base portions of the nucleotides are directly involved in the binding mechanism, such as the proposed mechanism of nucleophilic substitution through the carbonyl oxygen of G, C, and T. However, the nitrogen bases are largely inaccessible to such a chemical reaction in a dsODN, yet dsODN still exhibit the ability to incorporate. This discrepancy cannot be resolved at this time, but further experimentation could elucidate this issue. For ease and cost issues, a single ligand (ODN #1826) was labeled and measured in these studies, and the behavior of other ligands is inferred by their ability to inhibit binding. It is possible that the unlabeled ligands interact with ODN #1826 in some capacity that is independent of α2M and that this interaction prevents incorporation. If this were the case, a ligand would appear to have the ability to incorporate into α2M*, when, in fact, it could not. Future studies can address these issues by direct measurement of the incorporation of these ligands as well as analysis by PAGE and SDS-PAGE to examine the mechanisms of binding.
Figure 23: Double-stranded and single-stranded ODN exhibit similar binding affinities and share a binding site on $\alpha_2$M*. Single-stranded ODN was labeled and mixed with $\alpha_2$M and increasing concentrations of double-stranded ODN of the same sequence. Following activation with HNE, the samples were analyzed by PAGE under native conditions. A) $\alpha_2$M* was detected by Coomassie brilliant blue staining, and B) bound ODN was detected by fluorescent imaging. The amount bound in the absence of competing ligand was assigned a value of 100. Numbers 0-100 indicate the molar ratio of unlabeled to labeled ODN. Data shown here are means of the experiment performed in triplicate ± s.d.

4.4.4 Incorporation of Ribonucleic Acids

The ability of RNA to incorporate into $\alpha_2$M* was determined by similar procedures. ODN #1826 and $\alpha_2$M were mixed in a 5:1 molar ratio with increasing concentrations of unlabeled RNA strands with the same sequence as ODN #1826. Following activation by HNE, samples were analyzed by PAGE under native conditions, and the amounts of bound ODN #1826 were measured by fluorescent imaging. As
shown in Figure 24, the amount of ODN #1826 that binds is relatively unaffected at the lower concentrations of RNA. As the molar ratio of RNA to ODN exceeds 25:1, ODN incorporation decreases, eventually reaching a level that is half the amount of ODN bound in the absence of RNA. These data indicate that RNA does have the ability to incorporate into $\alpha_2$M*, but RNA cannot entirely inhibit ODN from binding, which is evidence that the mechanism for RNA binding partially overlaps the mechanisms by which ODN bind. As previously discussed in this thesis, ODN exhibit the ability to incorporate by different mechanisms: covalently through Cys949 and G1x952, and non-covalently. It is possible that RNA shares one of these mechanisms, and at higher concentrations, completely inhibits ODN from binding by this shared mechanism. If true, this shared mechanism would account for approximately half of the ODN incorporation. To determine the mechanisms by which RNA bind requires additional testing. Analysis of the products from this experiment by native and denaturing PAGE could lend insight into how the presence of RNA affects each individual mechanism of ODN binding. Future studies will employ direct measurement of incorporated RNA, and samples will be analyzed by PAGE and SDS-PAGE to quantify the covalent and non-covalent fractions.
Figure 24: RNA partially competes with DNA for binding to $\alpha_2\text{M}^*$. Labeled ODN and $\alpha_2\text{M}$ were mixed with increasing concentrations RNA oligomers. Following activation with HNE, samples were analyzed by PAGE under native conditions. Bound ODN were quantified by fluorescent imaging; the amount bound in the absence of competing ligand was assigned a value of 100. Data shown here are means of the experiment performed in triplicate ± s.d.
4.5 Simultaneous Incorporation of ODN and Protein Ligands

The use of \( \alpha_2M^* \) to delivery ODN to immune competent cells is not the first attempt to utilize \( \alpha_2M^* \) to strengthen immune responses. \( \alpha_2M^* \) has been used previously to package and deliver peptide antigens to enhance the adaptive immune response to these proteins. As discussed earlier, mice required a peptide dose that was 100-fold lower to produce a response when the peptide was packaged in \( \alpha_2M^* \) than when the peptide was mixed with CFA or IFA [111]. Furthermore, \( \alpha_2M^* \)-Ag complexes elicited antibody titers that were four to five orders of magnitude greater than those elicited by a similar amount of free antigen alone [112]. These results were largely the basis for this project. Provided that the target cells are the same, it was reasoned that \( \alpha_2M^* \) could enhance the immunological response to CpG ODN in precisely the same manner, by facilitating delivery to immune competent cells.

There is another level of complexity to the development of \( \alpha_2M^* \) complexes for immune response modulation; we hypothesized that if both an antigen and adjuvant molecule, such as CpG ODN, could be incorporated within the same \( \alpha_2M^* \) molecule, then the immunological response could be strengthened even further. This reasoning was founded on experiments examining the effects of chemically conjugating protein antigens to CpG ODN. The immune responses elicited by the CpG ODN-Ag conjugates were much stronger than those elicited by equal amounts of CpG ODN and antigen mixed together [137-140]. It was reasoned that this enhancement was the result of more rapid
uptake as well as obligate uptake of both molecules by the same subset of cells. Every cell that took up the antigen also received a dose of the adjuvant, and both were localized to the same subcellular compartments.

Simultaneous packaging of both antigen and adjuvant into $\alpha_2M^*$ would likely yield the same benefits, and we theorize that $\alpha_2M^*$ packaging offers additional advantages over current strategies that could lead to significantly enhanced immune responses. In addition to ensuring that cells would invariably be exposed to both antigen and adjuvant simultaneously and that they would localize to the same subcellular compartments, $\alpha_2M^*$ packaging offers specific and more rapid delivery. Antigens are often taken up by non-specific mechanisms, which are slow and inefficient, but chemical conjugates are taken up faster than free antigens because they are endocytosed via ODN-dependent mechanisms [138, 139]. Data in this thesis, however, demonstrate that $\alpha_2M^*$ packaging increases the rate of CpG ODN uptake by sixfold, demonstrating significantly greater uptake than what occurs through ODN-dependent mechanisms. In addition to more rapid and efficient uptake by responsive cells, both antigen and CpG ODN are protected from enzymatic digestion prior to endocytosis; P=O CpG ODN protection has been demonstrated in this thesis, and antigen protection from serum proteinases has been shown elsewhere [126, 141, 142]. Given the rapid uptake and protection that $\alpha_2M^*$ packaging provides, we hypothesize that the immune responses to $\alpha_2M^*$-Ag-ODN ternary complexes will be enhanced beyond the increases that have been measured from the packaging of each independently. This is based on the synergistic effects that are observed by the simultaneous uptake of the ODN-Ag conjugates. If the activity of each is enhanced by $\alpha_2M^*$-aided delivery, this enhancement may be increased many fold
through the simultaneous delivery in the same manner as the enhancement of ODN-Ag conjugates.

To generate these ternary complexes, both the antigen and CpG ODN must be able to incorporate into $\alpha_2$M* simultaneously, but it is possible that they will compete for binding, thus precluding their mutual incorporation. To determine whether these ligands will co-incorporate, competition reactions similar to those described above were performed. ODN #1826 and $\alpha_2$M were mixed in a 5:1 molar ratio with increasing concentrations of competing protein ligands. The proteins used were hen egg lysozyme (HEL) and $\alpha$-lactalbumin, which were chosen because each had been previously shown to incorporate into $\alpha_2$M* [109]. Both have similar molecular masses (14.3 kDa) and significant sequence identity but differ greatly in the charges they carry at a physiologic pH. With an isoelectric point of 10.5, HEL is positively charged, while $\alpha$-lactalbumin has an isoelectric point of 4.3 and carries a negative charge. We hypothesized that charge-charge interactions could influence interactions between the proteins and ODN thereby affecting co-incorporation. Following activation with HNE, samples were analyzed by PAGE under native conditions, and amounts of bound ODN were measured by fluorescent imaging.

The results, shown in Figure 25, confirm that it is possible to co-incorporate a protein ligand and CpG ODN simultaneously into $\alpha_2$M* and that the protein ligands can exert an influence on the incorporation of ODN. ODN binding is not affected by the presence of lower concentrations of $\alpha$-lactalbumin, but at ratios of $\alpha$-lactalbumin to ODN above 100:1, ODN binding is reduced. At maximal inhibition, ODN binding is reduced by 55%, which was observed in the presence 200-fold molar excess $\alpha$-lactalbumin, with
respect to ODN. Interestingly, the presence of HEL shows the opposite effect; HEL actually enhances the incorporation of CpG ODN into $\alpha_2$M*. Even at the lower concentrations of HEL, there is a dramatic increase in the level of ODN binding, which reaches a plateau that is 180% greater than in the absence of HEL. The $\alpha_2$M*-ODN complexes formed in the presence of protein ligands were also analyzed by SDS-PAGE to determine the mechanism of ODN binding. Recall that 75% of bound P=O CpG ODN is covalently linked. In the presence of 200-fold excess HEL, total binding increases by 180%, but the amount of covalently bound ODN is undetectable (data not shown). Likewise, in the presence of 12.5-fold molar excess $\alpha$-lactalbumin, the portion covalently bound is reduced from 75% to 12.6% (data not shown).

![Graph showing the influence of competing protein ligands on ODN binding to $\alpha_2$M*](image)

**Figure 25:** The influence that competing protein ligands have on ODN binding to $\alpha_2$M* is dependent on their charge. Labeled ODN and $\alpha_2$M were mixed with increasing concentrations of HEL or $\alpha$-Lactalbumin. Following activation by HNE, samples were analyzed by PAGE under native conditions. Bound ODN was quantified by fluorescent imaging; the amount bound in the absence of competing ligands was assigned a value of 100. ■ = HEL; □ = $\alpha$-lactalbumin. Data shown here are means of the experiment performed in triplicate ± s.d.
These findings reveal that CpG ODN and protein ligands share a common binding site on \(\alpha_2M^*\), but this does not prevent their co-incorporation. At the lower concentrations tested, \(\alpha\)-lactalbumin inhibits covalent binding of CpG ODN but does not affect the total amount bound. Results with HEL were even more promising for the development of \(\alpha_2M^*-Ag-ODN\) ternary complexes. In the absence of HEL, the molar incorporation ratio of P=O ODN is 0.5:1, but in the presence of HEL, this amount nearly triples. Based on the ability of ODN to interact with positively charged proteins [143, 144], we hypothesized that ODN and lysozyme are associating via charge-charge interactions and that this is responsible for the increase in ODN incorporation ratio. HEL incorporates in a molar ratio of 8:1, and may draw ODN into the \(\alpha_2M\) binding pocket as HEL itself incorporates.

To investigate the nature of this enhanced ODN incorporation, we sought to establish whether CpG ODN and HEL interact in solution. With each dissolved in PBS, HEL and CpG ODN were mixed in a molar ratio of 100:1, respectively. Saturated ammonium sulfate was added in a volume ratio of one part ammonium sulfate to two parts PBS solution to induce precipitation of HEL. Solutions were incubated at 25\(^\circ\) C for 1 h and then centrifuged at 12k g for 15 min. Supernatants were collected, and the amounts of ODN that remained in solution were measured. As controls, the same procedures were followed for separate solutions of HEL and ODN. In the presence of HEL, 93.4% of the ODN precipitates out of solution with the HEL, compared to a loss of only 12.1% in the absence of HEL (data not shown). The same experiment was repeated with \(\alpha\)-lactalbumin substituted for HEL, but there is no detectable loss of ODN from solution when \(\alpha\)-lactalbumin precipitates (data not shown). These results support the
existence of an interaction between CpG ODN and HEL in solution and that this interaction is based on charge-charge interactions between the two molecules.

Taken together, we have shown that $\alpha_2$M*-Ag-ODN ternary complexes can be generated. In addition, the protein ligand can actually facilitate the incorporation of the CpG ODN, which could further enhance the ability of these complexes to elicit an immune response. This information can be utilized in the selection of test antigens to optimize CpG ODN incorporation, thereby maximizing the therapeutic potential of these compounds.
Chapter 5: Epilogue
5.1 Summary of Biological Studies

This thesis is the first to document the interaction between α₂M* and CpG ODN, determine the biological effects of this interaction, and characterize the chemical nature and factors that influence incorporation. A previous report identified α₂M* as one of two proteins to which intravenously injected ODN would possibly bind; following injection of labeled ODN, blood samples were analyzed by HPLC, and ODN were shown to bind two proteins that displayed column retention rates corresponding to albumin and α₂M* [145]. Beyond this identification of similar retention rates, no further data were collected on the potential interaction between intravascular ODN and α₂M*. Another study details a process by which ODN can be bound to α₂M through a synthetic linker molecule but does not examine the biological implications of the conjugates formed [113]. Here, we demonstrate that ODN can incorporate into α₂M* without the need for synthetic linkers but rather through natural interaction of the two molecules, under the appropriate conditions.

When ODN and α₂M are mixed in a 5:1 molar ratio, proteolytically activated α₂M* binds 90% of the ODN, which yields an incorporation ratio of 4.5 moles of ODN/mole α₂M*. The ODN are bound through both covalent and non-covalent interactions, and ODN bound by both mechanisms remain intact when the complexes are subjected to an electrophoretic field. When stored at 0°C, the amount of dissociation after 18 months is only 5%. At 37°C, complexes with P=O and P=S ODN exhibit half-lives of four and five days, respectively. Since these complexes are being developed as therapeutics, it is important to determine whether their stability will limit clinical use
since a large portion of time invariably elapses between the manufacturing of these compounds and their administration to patients. Given their prolonged stability at 0°C, this will not limit the clinical utility of these complexes.

When incubated at temperatures above 0°C, there is dissociation of bound ODN from α2M*. It is presumed that the non-covalent portion of ODN dissociates and that the covalently bound portion of ODN remains intact, but complexes incubated at elevated temperatures have not been analyzed to determine whether this is the case. If covalently bound ODN do not dissociate at elevated temperatures, then the degree of dissociation would be limited to the non-covalent fraction even when the complexes were subjected to elevated temperatures for prolonged periods. This concept yields another question about potential differences between the covalent and non-covalent fractions: biological activity. In all biological assays described here, complexes were stored at 0°C prior to the experiments; both covalent and non-covalent fractions were bound, so the biological activities of each fraction could not be discerned. It is conceivable that covalently bound CpG ODN would not demonstrate activity because the bound α2M* portion may cause steric hindrance between the CpG dinucleotide and TLR9. In this case, only the non-covalent fraction would exhibit biological activity upon uptake and dissociation from α2M* within the acidic endosome. However, chemically linked antigen-ODN conjugates have demonstrated enhanced immune cell activation; this could be a product of the intrinsic activity of the antigens used, or the bound protein fragment could serve to stabilize the interaction between the CpG ODN and TLR9. If the latter is true, then covalently bound CpG ODN might exhibit enhanced activity. The specific activities of each bound fraction, covalent and non-covalent, could be determined by incubating
complexes at elevated temperatures to dissociate the non-covalent fraction followed by measuring the biological activity of the ODN that remain bound, the covalent fraction. The results of this experiment could yield valuable information about the nature of the interaction between the CpG dinucleotide and TLR9 as well as expand out knowledge of the properties of α₂M*-ODN complexes.

After generating and purifying complexes, we have demonstrated their enhanced biological activity in a murine in vitro model. Using murine MΦs, we have shown that α₂M*-aided delivery enhances both potency and efficacy in the ability of CpG ODN to elicit a cytokine response. In addition to increasing the response strength, α₂M*-aided delivery alters the kinetics of the cytokine production; cells stimulated with α₂M*-ODN respond more quickly and the response peak occurs earlier. This enhanced response is, in part, the result of more rapid uptake; ODN bound to α₂M* are taken up by cells sixfold faster than free ODN. This drastically shortens the duration of exposure required to elicit an immune response; cells treated with α₂M*-ODN complexes began responding with as little as 15 min of exposure to the complexes, while free CpG ODN did not begin to elicit a response until after 2 h of exposure.

The ability of α₂M*-complexes to stimulate human cells was measured. PBMC were treated with α₂M*-ODN complexes, and the activity enhancement was even greater in this system than observed in the murine MΦ model. Complexes exhibited greater potency, and the strength of cytokine responses increased by as much as 15-fold. The greater enhancement observed here may be a result of the complexity of the system. The presence of multiple cell types facilitates the immune response by allowing communication between cells, which allows activated cells to act on each other in a
positive feedback mechanism. The presence of cells incapable of responding also creates a sink by which CpG ODN can be removed from the system and prevented from stimulating a response since cells that do not express TLR9 reduce the pool of active CpG ODN. \( \alpha_2 \text{M}^- \)-bound ODN, however, are targeted for uptake by cells that are responsive: DCs and B cells in humans.

We predict that the \textit{in vivo} immunostimulatory activity of CpG ODN bound to \( \alpha_2 \text{M}^- \) will be enhanced even more than what we have observed \textit{in vitro}. The \textit{in vitro} model is optimized to detect a response to CpG ODN; tissue culture is a closed, static system with CpG ODN in constant contact with cells, and a relative paucity of nucleases. The \textit{in vivo} model, however, offers many more obstacles to CpG ODN-induced immunostimulation. CpG ODN can be degraded by nucleases, bind serum proteins non-specifically, or be taken up by unresponsive cells, and a significant portion is removed from vascular circulation by renal clearance. However, CpG ODN incorporated within \( \alpha_2 \text{M}^- \) adopt the pharmacokinetics of \( \alpha_2 \text{M}^- \) and so are targeted for receptor-mediated uptake by M\( \Phi \)s, DCs, and B cells. This targeted delivery offers a distinct advantage over the use of liposomes, which are taken up by cells non-specifically. CpG-independent immune stimulation and a high level of toxicity also significantly reduce the attractiveness of liposomes as a delivery vehicle for CpG ODN. With the combination of targeting CpG ODN to immune competent cells for receptor-mediated uptake, protection from nuclease digestion, and prevention of non-specific binding to plasma proteins, \( \alpha_2 \text{M}^- \)-aided delivery may greatly enhance the biological activity of CpG ODN.

Preliminary \textit{in vivo} studies have shown that \( \alpha_2 \text{M}^- \)-bound CpG ODN exhibit a 25-fold enhanced potency compared to free CpG ODN in the ability to elicit the production of
TNF (Figure 26). Compared to the four- to sixfold increase observed in vitro, this significant increase in enhancement likely results from $\alpha_2$M*-aided delivery as well as the protection from the numerous in vivo barriers to CpG ODN immunostimulation.

Figure 26: $\alpha_2$M*-aided delivery enhances the in vivo potency of CpG ODN by 25-fold. Mice were treated with equal amounts of free or $\alpha_2$M*-bound CpG ODN by i.v. injection, and TNF was measured in the liver 1 h after treatment. ■ = $\alpha_2$M*-ODN complexes; □ = free CpG ODN. Data represent means of the experiment performed in triplicate ± s.d.

Thus far, comparison of free and $\alpha_2$M*-bound CpG ODN biological activity has been through the measurement of cytokine production. To obtain a more detailed understanding of how $\alpha_2$M*-aided delivery affects the biology surrounding CpG ODN, numerous studies can be performed. Murine immune responses can be further characterized by studying the lytic activity of NK cells and cytotoxic T cells following $\alpha_2$M*-ODN treatment. B cell maturation, proliferation, and antibody production can be measured. To characterize murine responses in vivo, mice can be immunized with an
antigen and α₂M*-bound CpG ODN. However, long term studies, in which mice are treated multiple times would require the use of murine α₂M* instead of the human protein. For *in vitro* purposes, human and murine α₂M* exhibit similar behaviors; both proteins bind human and murine LRP with similar affinities and are taken up at similar rates. However, the human protein has minor differences that will induce an antigenic immune response in mice over time. As a result, immunization studies would require the incorporation of CpG ODN into murine α₂M*. We have established methods by which murine α₂M is purified from murine plasma, and we are in the process of developing procedures by which ODN may be incorporated into murine α₂M for the purpose of immunization studies.

Despite immune response indicators like cytokine production, cellular activity, and antibody production, predicting the protective immunity that these molecules will provide remains difficult. The final murine testing of these complexes will require *in vivo* administration followed by a challenge, such as a tumor load or infectious agent. Demonstration that treatment with α₂M*-ODN complexes is more effective at treating or preventing cancer or infections is the next goal in our evaluation of these complexes as potential therapeutics. Similar experiments have already been conducted by many investigators in the evaluation of free CpG [146-150]; our lab is currently in the process of establishing the execution of these *in vivo* models for the evaluation of α₂M*-ODN complexes.

In addition to their evaluation as therapeutics, α₂M*-ODN complexes may aid in our understanding of the biology surrounding the CpG-induced immune response. In the process of characterizing the uptake of α₂M*-ODN complexes, we found that cellular
uptake was not affected by the ODN backbone. The uptake of free ODN, however, is
affected by the backbone composition, sequence, and length [74, 151, 152]. Furthermore,
other investigators have found that ODN of different backbone compositions, sequences,
and lengths demonstrate different abilities to elicit immune responses [69-72, 153]. ODN
differ in their potency, the cells they act on, and the strength and nature of the immune
responses they stimulate. Since uptake and trafficking to the endosome are essential
steps in the activity of CpG ODN, it has been difficult to determine whether the
differences in activities are the result of altered uptake and trafficking or the result of
distinct interactions between the CpG ODN and TLR9. $\alpha_2M^*$-aided delivery will bypass
differences in uptake and trafficking to allow for the examination of how ODN
differences affect their interaction with TLR9. A greater understanding of this interaction
will likely facilitate the discovery and design of CpG ODN with greater clinical efficacy.

Among these CpG ODN differences include the existence of three distinct classes
of ODN that differentially activate human immune cells. Classes A, B, and C are defined
by their differences in sequences and backbone composition. These differences result in
their uptake by different cell types and qualitatively different immune responses. In this
thesis, $\alpha_2M^*$-ODN activity was measured by cytokine production from PBMC. Class A,
B, and C ODN act on different cell types as a result of differences in uptake, but $\alpha_2M^*$-
bound CpG ODN will bypass this uptake selectivity demonstrated by immune competent
cells. It will be interesting to identify the cell types responding to $\alpha_2M^*$-ODN
complexes, which may be accomplished by FACS analysis. Following treatment of
PBMCs with labeled $\alpha_2M^*$-ODN complexes, cells can be stained using antibodies against
cell-specific markers. FACS analysis will simultaneously detect $\alpha_2M^*$-ODN complexes
and identify the cell types that have taken up the complexes. Cell stimulation can also be measured by staining for surface markers of activation. Antibodies against CD19, CD11c, CD14, and CD16 have been obtained to identify B cells, DC, monocytes, and NK cells, respectively. In addition, antibodies against CD25, CD69, CD86, and HLA-DR will be used to detect cellular activation. These studies will expand both our understanding of how \( \alpha_2 \text{M}^* \)-aided delivery affects the immune response as well as the underlying mechanisms of the cellular response to CpG ODN.

In the process of studying the uptake of \( \alpha_2 \text{M}^* \)-ODN complexes, we discovered that the uptake of free CpG ODN is positively influenced by the presence of \( \text{Ni}^{2+} \). The uptake mechanisms of CpG ODN continue to receive attention; numerous proteins expressed by a large number of cell types have been identified as being involved in the uptake of CpG ODN. This finding could lend further assistance to the identification of cellular uptake mechanisms of CpG ODN, and possibly improved vaccine adjuvant formulations.

We have shown that ODN will incorporate into \( \alpha_2 \text{M}^* \) that has been converted to its receptor recognized form, \( \alpha_2 \text{M}^* \), but not into in the native conformation. This has interesting implications for potential physiological relevance of this interaction. Data collected have led to the hypothesis that \( \alpha_2 \text{M} \) serves as a sensor of proteolysis. \( \alpha_2 \text{M} \) has the ability to bind and inhibit proteinases from all four major mechanistic classes, and binding leads to proteolytic activation and conversion to its receptor-recognized form, leading to rapid uptake by LRP-expressing cells, including DCs, M\( \Phi \)s, and B cells. In addition to binding proteinases, \( \alpha_2 \text{M}^* \) can concurrently bind other macromolecules, such as growth factors, cytokines, protein antigens, and nucleic acids, and this binding also
demonstrates a lack of specificity. Taken together, this is an elegant mechanism by which immune competent cells may rapidly sample their external environment in the presence of extracellular proteinases, which reach very high levels in the settings of inflammation and infection. This theory is strengthened by the observations that \( \alpha_2 \text{M}^* \) enhances the immune response to antigens. If immune competent cells could quickly sample the extracellular environment, they could formulate a response more quickly and tailor that response to combat the specific pathogens present. There is a paucity of evidence at this time to support the existence of \( \alpha_2 \text{M}^* \)-nucleic acid complexes, but the reagents and conditions used to generate complexes in this thesis do mimic physiological conditions. \( \alpha_2 \text{M} \) is constitutively present in plasma, bacterial DNA fragments are present at sites of infection, and neutrophil elastase is one of the most abundant proteases at sites of inflammation. Furthermore, the reaction that generates complexes occurs at physiological pH levels and temperatures.

The \( \alpha_2 \text{M}^* \)-LRP uptake mechanism could have evolved as a means by which LRP-expressing immune cells (DC, MΦs, and B cells) could rapidly endocytose fragments of pathogen DNA to enhance their ability to respond. Of these three cell types, DC and B cells express TLR9 and are responsible for the immune response to CpG ODN, a mimic of bacterial DNA. Supporting this theory is the selectivity that \( \alpha_2 \text{M}^* \) demonstrates for unmethylated DNA. This could promote the ability of immune competent cells to sample foreign DNA and prevent endogenous DNA from interfering with the response by competing for \( \alpha_2 \text{M}^* \) binding; it could also be a mechanism by which autoimmune pathogenesis could be avoided. Further studies are needed to determine whether these complexes are formed in nature, but if they are, these studies will strengthen our
understanding of how the immune system distinguishes foreign invaders from self and how it is able to rapidly formulate specific responses against an extraordinarily diverse world of pathogens.

5.2 Summary of Chemical Characterization

A systematic characterization of the chemical properties of $\alpha_2$M*-ODN complexes was performed. P=O and P=S ODN were incorporated into $\alpha_2$M*, and the stoichiometries and mechanisms of incorporation were determined. Using ODN #1826, P=O ODN incorporate in a ratio of 0.5 moles/mole $\alpha_2$M*, and P=S incorporate in a ratio of 2 moles/mole $\alpha_2$M*. By altering the sequence, however, this ratio varies; ODN #2395 incorporated with a ratio of 4.5 moles/mole $\alpha_2$M*, which was the highest observed. Both P=O and P=S ODN incorporate non-covalently and covalently, through Cys$_{949}$ and Glx$_{952}$. The relative stabilities of complexes were measured over a range of temperatures and pH levels.

Numerous variables that affect incorporation were also identified. Competition for binding was demonstrated between labeled and unlabeled ODN, which indicates that binding is specific and saturable and that binding does not occur through the fluorescent label. Competition studies demonstrate that P=O and P=S ODN compete for binding, and P=S ODN demonstrate a higher affinity for binding. Even at 100-fold molar excess, P=O ODN was unable to reduce the binding of P=S ODN. Methylation reduces the amount of incorporation, which as implications in the selectivity of $\alpha_2$M* for foreign versus endogenous DNA. If $\alpha_2$M* does serve to bind extracellular DNA for rapid uptake
by immune competent cells, the increased affinity for unmethylated DNA would lead to the preferential binding and uptake of bacterial DNA rather than DNA from endogenous origins. This selectivity would increase the sensitivity of the system by preventing endogenous DNA from interfering with the uptake of bacterial DNA, and it could also be a means by which autoimmune reactivity is minimized.

Next, the influence of ODN base composition was examined. By competing with labeled ODN #1826, the relative affinities of G20, C20, T20, and A20 ODN were measured; all exhibited the ability to reduce the binding of ODN #1826 except for A20, which showed no competition even at 100-fold molar excess. Based on these findings, binding likely occurs through guanine, cytosine, and thymine but not through adenine bases. This leads us to hypothesize that binding occurs through the carbonyl oxygen the nitrogen bases. These studies should be repeated with the use of labeled N20 ODN make direct measurements of their binding rather than relying solely on competition studies. Measuring the covalent and non-covalent fractions of bound N20 ODN could also lend further insight into the nature of this reaction.

The binding affinities of ODN of different lengths were measured by comparison of N10 and N20 incorporation. We found that 20-mer ODN bound with an affinity that is twice that of the 10-mer counterparts. These studies should be repeated with direct measurement of incorporation rather than indirect measurement by competition with another ligand. A broader range of ODN lengths should be tested to better define a trend of binding affinities and to identify size limits for incorporation. In this thesis, the largest ODN used was a 22-mer, and other members of our laboratory have successfully incorporated ODN as large as 40-mers. With a molecular weight of approximately 12
kDa, these 40-mer ODN are still dwarfed by the largest proteins that have been incorporated into α₂M* (HIV gp120, with a molecular mass of 120 kDa). However, with the linear nature of nucleic acids, the molecular mass limitations for incorporation may be drastically different from those of globular proteins.

The binding affinities of both double-stranded DNA and RNA oligonucleotides were also assessed. Double-stranded ODN exhibited a similar binding affinity to that of single-stranded ODN of the same sequence. This finding is interesting in light of the influence that base composition has on incorporation. If binding occurs through the nitrogen bases, they should be sterically inaccessible in a double-stranded ODN, thus reducing binding affinities. These potential contradictions could be resolved by additional PAGE analyses of these complexes to measure the fractions that are bound covalently and non-covalently. When sequence-matched RNA and DNA oligonucleotides competed for α₂M* binding, we found that RNA was only partially able to inhibit DNA incorporation. The shape of this competition curve does not indicate differences in affinities but rather the inability of RNA to bind α₂M* by one of the mechanisms that its DNA counterpart employs. To verify this, the α₂M*-ODN complexes that are formed in the presence of 100-fold molar excess of RNA oligonucleotides should be analyzed by native and denaturing PAGE to measure the fractions of ODN that are bound covalently and non-covalently. In addition to this analysis, RNA binding should be measured directly to verify that the observed competition is not an artifact of direct interaction between RNA and DNA strands.

The final studies performed in this thesis involved the co-incorporation of ODN and protein ligands into α₂M*. While lysozyme increased the total amount of ODN that
incorporated and α-lactalbumin decreased the total amount at high molar excess ratios, both reduced the amount of covalently bound ODN. It is evident that ODN and these protein ligands share a common covalent binding site, the thiolester bond. The opposite effects on the total amount of binding lend further insight into this interaction. 

Lysozyme, which incorporates into α2M* with a molar ratio of 8 moles/mole α2M*, facilitates the incorporation of ODN, while α-lactalbumin, has no effect until it is present in a molar ratio of 100-fold excess, when it reduces ODN incorporation. This difference appears to stem from charge-charge interactions between the proteins and ODN. The positively charged lysozyme binds ODN by charge-charge interactions and increases the amount of incorporated ODN because of its own high incorporation ratio. Like ODN, α-lactalbumin, carries a negative charge, eliminating any interaction between these molecules, which leads to no effect on the incorporation of ODN at low α-lactalbumin concentrations until the level of α-lactalbumin is so high that it prevents ODN from incorporating.

5.3 α2M*-Antigen-ODN Ternary Complexes

The ultimate goal of this project has always been to improve the clinical effectiveness of CpG ODN through α2M*-aided delivery. The potential for α2M* to enhance the activity of CpG ODN was based on the evidence that protein antigens bound to α2M* produced much stronger immune responses than free antigen alone or in combination with the strongest experimental adjuvants. Evidence reported here demonstrates the enhanced biological activity of CpG ODN bound to α2M*; they exhibit
greater potency and efficacy, and the immune responses elicited begin and peak earlier. This has been shown using both murine and human in vitro systems, and the preliminary in vivo murine data show even greater effects from α2M*-aided delivery. Based on these data, we may observe the greatest results by packaging both a protein antigen and CpG ODN into α2M* together; this α2M*-Ag-ODN complex would contain both the target for the adaptive immune response as well as the adjuvant to ensure that the response is strong enough to induce protective immunity.

Feasibility of this technology stems from the lack of specificity that α2M* displays for binding molecules but the high specificity it exhibits in cellular binding and uptake. α2M* has the ability to bind all four major classes of proteases, as well as growth factors, hormones, protein antigens, and nucleic acids. Once α2M has been converted to its receptor-recognized form, α2M* is taken up very rapidly by LRP-expressing cells. In the plasma, α2M* is taken up predominantly by hepatocytes and Kupffer cells. In peripheral tissue, DCs, B cells, and MΦs are responsible for α2M* uptake [154]. With respect to vaccine design, these cells are the targets for both the antigen and CpG ODN adjuvant. The incorporation of each separately into α2M* increases the activity of each. α2M* protects them from inactivation through enzymatic digestion or non-specific binding to proteins or cell surfaces, and it targets them for uptake by APC. The increased responses might translate into protective immunity with efficacy that begins earlier and lasts longer, and requires fewer doses. The increased potency might reduce the dosage requirements, thus alleviating side effects.

In addition to the data showing that α2M* can enhance the activity of both antigens and CpG ODN, there is evidence that co-delivery of these molecules also
increases the immune response. Chemical conjugation of protein antigens and CpG ODN elicits immune responses with greater production of cytokines and antibodies as well as increased cytotoxic T cell activity [137, 139, 140]. Investigators attribute the enhancement, in part, to the increased uptake of antigens. CpG ODN are taken up faster than antigens, and their uptake dictates the uptake of the conjugates, so antigens are taken up with the efficiency of CpG ODN uptake. They also attribute the enhanced immune responses to the concomitant cellular exposure to both antigen and adjuvant. Every cell that is exposed to the antigen also receives an activation signal in the form of CpG ODN.

\(\alpha_2\text{M}\)-Ag-ODN complexes would benefit from these same mechanisms. While CpG ODN uptake facilitates antigen delivery, data in this thesis demonstrate that \(\alpha_2\text{M}\)-aided delivery of CpG ODN increases their uptake sixfold. In addition to the rate increase, \(\alpha_2\text{M}\) provides specific targeting to responsive cells. Like the antigen-CpG ODN conjugates, \(\alpha_2\text{M}\)-aided delivery will ensure the concomitant uptake of both antigen and CpG ODN.

Characterizing the chemical nature of \(\alpha_2\text{M}\)-ODN complexes was performed for the purpose of therapeutic development. It was necessary to confirm that stability would not limit their clinical utility. In addition, their ultimate efficacy may rest on the ability to incorporate a large number of CpG ODN into each \(\alpha_2\text{M}\). The maximum solubility of \(\alpha_2\text{M}\) is approximately 8 µM, which represents the upper limit of these complexes in vaccine formulations; by increasing the molar ratio of ODN incorporation, however, the concentration of CpG ODN may greatly exceed this value. To this end, we investigated the ODN variables affecting incorporation and identified several that might increase this ratio. Based on the data collected, the optimal incorporation ratio might be achieved with
the use of P=S-modified ODN with a high content of guanines and a low content of adenines. The effects of ODN length require further study, but data collected so far indicate that longer ODN incorporate with greater affinity.

The co-incorporation of ODN and protein ligands was likewise an important finding. It was possible that these two ligands would compete for binding to the extent of completely preventing their simultaneous incorporation. While both proteins tested competed with ODN for covalent binding, the total amount of ODN was not reduced until the amount of α-lactalbumin reached 100-fold molar excess. The presence of lysozyme actually increased the total amount of ODN incorporation, leading to the conclusion that charge-charge interactions between protein and ODN ligands can promote ODN incorporation. Based on these findings, we may increase the amount of ODN incorporated within α₂M* with the co-incorporation of protein ligands that carry a high positive charge and themselves incorporate in a high molar ratio.

5.4 Conclusion

Vaccines are among the most significant developments in the history of medical science. Their impact on global health cannot be overstated; they have lead to the eradication or near elimination of numerous diseases that previously caused immeasurable morbidity and mortality. Vaccines are inexpensive, easily distributed and administered, and can offer long-lasting protection; they are the pinnacle of cost-effective medicine. However, we have yet to succeed in the prevention of some of the most devastating diseases: HIV and AIDS, tuberculosis, malaria, and hepatitis C. As our
understanding of the immune system has expanded, vaccines and immune system modulators are becoming common treatments for increasingly diverse diseases, now including cancer and immune system pathologies. Previously, vaccine development had relied heavily on empirical investigation, but as we learn more about the how the immune system functions, we may develop new vaccines through rational design; the development of CpG ODN and $\alpha_2$M* as therapies are two such strategies. Based on our knowledge of requirements for strong innate immune responses to promote effective adaptive responses and protective immunity, $\alpha_2$M*-aided delivery of CpG ODN and antigens is a rationally designed technology that may yield significant contributions to our continued efforts in combatting disease.
Chapter 6: Reference Material
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**Biography**

Ryan Berger Anderson was born in Mankato, MN on November 10th, 1976. His parents, Arlyce Mae and Berger Alvin Anderson also have an older son, Erik Thomas Anderson. Ryan attended Frederick High School in Frederick, MD and graduated in June of 1995. He attended St. Mary’s College of Maryland and earned a B.A. in Chemistry in May of 1999. His Honors Project was titled “Extraction, isolation, and identification of medicinally active compounds from a West African plant species”. In August 2000, he entered the Duke University School of Medicine in Durham, NC and is currently pursuing dual M.D. and Ph.D. degrees in the Medical Scientist Training Program. His doctoral thesis is titled “Incorporation of CpG oligodeoxynucleotides into α2-Macroglobulin: Development of a novel vaccine adjuvant delivery mechanism”. Upon completion of his dissertation, he plans to complete medical school, pursue clinical training in Anesthesiology, and remain in academic medicine.