June 2015

Control of Inflammation Using Drug Delivery Strategies in in vitro Models

Patricia R. Wardwell
Syracuse University

Abstract

Regulation of inflammation is a crucial component of the immune system in response to injury and infection. In otherwise healthy individuals, an initial acute inflammatory response will subside once the injury or infection is eradicated. However, in certain disease states including autoimmune disease and persistent infection, miscommunication between cells of the immune system leads to a chronic inflammatory response, contributing to disease pathology and exacerbating symptoms. A major regulator of inflammation communication at the cellular level is transcription factor (TF) NF-κB. Under normal conditions, NF-κB is bound to an inhibitor in the cytoplasm. In a chronic disease state, NF-κB is overactive and found in the unbound form, resulting in increased production of inflammatory signals.

Transcription factor decoys (TFD) are small nucleic acid sequences (~20 base pairs) that mimic the binding site for the TF on the native DNA, but do not encode for any proteins. By binding to the TF in the cytoplasm, TFD have potential to limit excessive immune signaling and inflammatory protein production. Unfortunately, clinical success of TFD has been hampered by a lack of an effective delivery method. Lack of stability and ease of degradation of the TFD require a protective carrier for delivery; however many synthetic carrier systems induce toxicity or an enhanced inflammatory response. In disease states characterized by excessive inflammation, treatment-induced toxicity or immune response is highly undesirable.

The Bader lab has previously reported a nanoparticle carrier system based on natural polysaccharides, designed specifically for the treatment of rheumatoid arthritis. The materials used in this system have properties that can be exploited for the additional application of DNA delivery. This thesis will detail the adaptation of polysialic acid-N-trimethyl chitosan

nanoparticles to be used as delivery vehicles for an NF-κB TFD treatment in in vitro models of rheumatoid arthritis and cystic fibrosis.

Control of Inflammation Using Drug Delivery Strategies in *in vitro* Models

By

Patricia R. Wardwell

B.S. Syracuse University, 2011

Dissertation

Submitted in partial fulfillment of the requirements of the degree for Doctor of Philosophy in Bioengineering in the Graduate School of Syracuse University

June 2015

Acknowledgements

My deepest thanks and gratitude to my advisor and friend, Dr. Becky Bader. Becky, thanks for always believing in me, for allowing me to start working in your lab six (!) years ago, and introducing me to research. Every day you would push me to become a better scientist, researcher, writer, and general thinker, and I am immensely grateful for your instruction and friendship over the years.

Thanks to my amazing family for incredible amounts of support. Mom and Dad, your unwavering encouragement and support means the world. Robert, thanks for being there for me as a friend and/or drinking buddy the last four years. Lauren, thanks for always providing a place to stay in Boston, and comic relief with your snapchat selfies.

To my thesis committee members, thank you for your wisdom and input. Dr. Martin Fostner, thank you for serving as our resident microscopy expert, and for being available to help with experiments countless times.

Thank you to the Syracuse Biomaterials Institute (SBI) and the Biomedical and Chemical Engineering (BMCE) department at Syracuse University, and NSF for all playing a role in funding at some point. Thank you to Karen Low and Lynore de la Rosa for all they do to keep SBI running smoothly. To SBI students and faculty, thanks for embracing the collaborative environment, and offering advice in areas from statistical analysis, to electrophoresis experimental technique.

Thank you to the many undergraduate researchers I worked with and trained over the years. Especially Liz Luke, Phil Chioniere and Wes Ryan, thank you all for helping with feeding cells and taking nanoparticle measurements, and being generally awesome people.

Table of Contents

Section Page

List of Figures and Tables

Figures Page

Tables Page

List of abbreviations and acronyms

Disease states and in vitro model related terms
RA-Rheumatoid arthritis
CF-Cystic fibrosis
DMARD-Disease modifying anti-rheumatic drug
MTX-Methotrexate
IB3-1 CF airway cell line
SW982-RA synovial fibroblast cell line
CFTR-Cystic fibrosis transmembrane conductance regulator protein
TLR-Toll-like receptor
ELISA-Enzyme linked immunosorbent assay

Transcription factor decoy terminology
ODN-oligodeoxynucleotide
TF-transcription factor
TFD-transcription factor decoy
IκB-Inhibitor of NF-κB
IκK- Inhibitor of NF-κB kinase
NF-κB-Nuclear factor kappa-light chain enhancer of activated B cells

Inflammatory proteins
IL-1β-Interleukin-1β
IL-6-Interleukin 6
IL-8-Interleukin 8
GM-CSF-Granulocyte Macrophage colony stimulating factor
TNF-α-Tissue necrosis factor α
LPS-Lipopolysaccharides

Anti-inflammtory proteins
IL-4-Interleukin 4
IL-10-Interleukin 10
IL-13- Interleukin 13

Drug delivery terminology
PSA-Polysialic acid
TMC-Trimethyl Chitosan
PEG-Polyethylene glycol
NP-Nanoparticle, PSA-TMC
NPODN-Nanoparticle coated with ODN
NPSCO-Nanoparticle coated with scrambled sequence ODN
NP-MTX-Nanoparticle loaded with MTX
NP-ODN-MTX-Nanoparticle loaded with MTX and coated with ODN
LipoODN-Lipofectamine mediated delivery of ODN

1. Introduction

Nucleic acid based drug constructs are promising therapeutic candidates and have garnered a great deal of interest in recent years. These constructs, including DNA plasmids, DNA oligonucleotides, and RNA oligonucleotides, have the potential to target a variety of diseases through the control of genetic material. Plasmid therapy, for example, is based on the replacement of a defective gene with a new intact one via plasmid integration. RNA oligonucleotides (siRNA, miRNA) are naturally occurring, but can also be exogenously supplemented, and are essential for controlling certain aspects of gene expression. Finally, DNA oligomers have emerged as antisense oligonucleotides and transcription factor decoys, which bind to specific DNA sequences or transcription factors, respectively. The following work will describe the use of a transcription factor decoy against NF-kB.

Despite the interest in and potential of nucleic acid drugs, the primary barrier to clinical use of these materials is a safe and effective method of delivery. Nucleic acids are very unstable *in vitro*, as well as *in vivo*, and are subject to degradation by nucleases and other enzymes. In addition, charge interactions between the negatively charged nucleic acid backbones inhibit entry into negatively charged cell membranes. *In vivo*, nucleic acids undergo rapid clearance via glomerular filtration, or recognition and intracellular degradation. In sum, the two major barriers to nucleic acids being effective therapeutics are the negative charge which prevents cellular uptake and the short nucleic acid molecules lack of stability and susceptibility to nuclease activity [1]. To overcome stability problems, chemical modifications such as phosphorothioate or methyl phosphonate are often applied to the nucleotide backbones [2]. While these modifications enhance stability, they do not necessarily lead to increased delivery efficiency, resulting in the need for a high dose and frequently repeated delivery. This is not a sustainable method for

delivery, as phosphorothioate nucleic acids have been shown to have a concentration dependent toxicity [3, 4].

Methods that do not involve directly modifying the nucleic acid therapeutic itself, such as viral vectors, cationic lipid formulations and more recently, cationic polymer formulations, exist to overcome the barriers to nucleic acid delivery. The advantages and disadvantages of these different methods will be discussed in chapters two and three. In general, drug delivery systems for nucleic acids must have the following attributes: biocompatibility and biodegradability, reticuloendothelial system avoidance, non-immunogenicity, cellular uptake capability, and cell or tissue specificity [5]. Recently, polysaccharides have attracted interest as materials for drug delivery systems due to an inherent lack of toxicity, a propensity for degradation with existing enzymes, and in some instances, innate bioactivity[6]. This dissertation describes the use of a polysaccharide based polysialic acid-N-trimethyl chitosan (PSA-TMC) nanoparticle system as a non-immunogenic, non-toxic, biodegradable delivery system for small nucleic acids. Although chitosan-nucleic acid polyplex systems have been reported previously, this system is distinguished by the addition of polysialic acid, a polymer that has been shown to impart stealth properties on nanosystems [7]. In addition, when compared to other chitosan based NA delivery systems, PSA-TMC nanoparticles have smaller sizes and lower polydispersities, making them more suitable to drug delivery applications [8].

A transcription factor decoy ODN is a short nucleic acid sequence that mimics the nuclear binding site associated with specific transcription factors, but does not contain any genetic coding sequences, effectively preventing transcription factor activity beyond initial binding. To demonstrate anti-inflammatory activity, the NF-κB decoy ODN-coated PSA-TMC nanoparticles were administered to several *in vitro* models, including CF via interleukin-1β (IL-1β) or

Pseudomonas aeruginosa lipopolysaccharide (LPS) stimulation of IB3-1 bronchial epithelial cells, CF via co-culture with *P. aeruginosa* bacteria, and RA via SW982 synovial sarcoma cells stimulated with interleukin-1β, as well as synoviocytes isolated from RA joint tissue. Using these models, we show that free ODN and PSA-TMC nanoparticles coated with scrambled ODNs do not have substantial impacts on the inflammatory response; however, decoy ODN-coated PSA-TMC nanoparticles were able to reduce the secretion of interleukin-6 and interleukin-8, as well as expression of granulocyte-macrophage colony stimulating factor.

In sum, PSA-TMC is a non-toxic, non-immunogenic, biodegradable nanoparticle system expected to effectively incorporate and safely deliver small nucleic acids. This claim has been investigated using an NF-κB inhibiting oligonucleotide in several *in vitro* models, as described in the following sections.

The thesis is constructed as follows: general background information regarding drug delivery system design challenges (chapter two), followed by specific background information regarding inflammation in RA and CF, concluding with the goals to be achieved and hypotheses tested by this work (chapter three). Chapter four will be devoted to adaptation of PSA-TMC nanoparticles to be DNA carriers, while chapters five-seven will focus on detailed description of development and testing on in vitro models, and chapter eight will summarize the work described and discuss potential future directions of this project.

2. Challenges of drug delivery

This chapter was adapted from a textbook chapter written by P.R. Wardwell and R.A. Bader published in Engineering Polymer Systems for Improved Drug Delivery, and provides an overview of the challenges provided by the environment of the human body and the chemical and physical characteristics of therapeutics themselves for drug delivery applications. Although not everything discussed in this chapter is relevant to the specific work subsequently described in the thesis, the information presented here is vital to understand the complexity of drug delivery, and the degree of thought, planning, and experimentation that must go into designing new drug delivery methods.

Abstract

Recent advances in drug development have led to the discovery and production of a variety of therapeutic molecules, with the potential to target and treat many diseases [9]. Their use is somewhat limited, however, by the drug delivery methods available today, and the obstacles put in place by the human body. The body is essentially a complex network of compartments within which the desired sites of action lie. In order to reach these targets, the drug molecules have to cross a variety of boundaries, usually in the form of epithelial membranes or mucosal barriers, as well as face exposure to a harsh environment of degrading enzymes and varying pH levels [10]. Therapeutic characteristics including hydrodynamic radius, charge, hydrophilicity, and permeability can affect movement [11]. The impact of these obstacles on bioavailability differs among users, resulting in variable therapeutic efficacy [12].

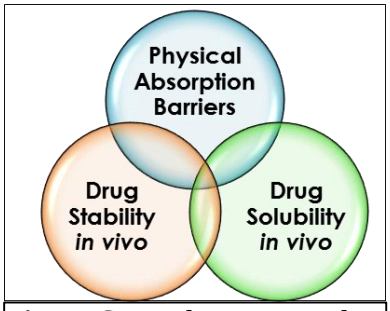

Fig. 1: Several intertwined factors affect the design of a drug delivery system.

In general, the ability of the drug molecule to reach its target organ and have the desired effect is hindered by obstacles that can be broken into three interrelated categories as follows: (1) *in vivo* drug solubility, (2) *in vivo* drug stability and (3) physical barriers to absorption (Fig. 2-1) [13, 14]. In order to travel to the site of action and have maximum efficacy, the drug must be soluble and stable within the aqueous environment of the body. Furthermore, soluble and stable therapeutic agents are associated with increased ability to permeate the physical barriers that hinder absorption. Based on the obstacles introduced above, development and enhancement of methods for improving delivery of therapeutic molecules that lack stability and solubility is imperative. Many methods for improved delivery rely on polymer based compounds in the form of (1) implantable networks for controlled release; (2) carrier systems including nanoparticles, liposomes, and micelles for therapeutic encapsulation; and (3) polymer-drug conjugates.

This chapter will provide an overview of the various physical and chemical challenges encountered in the physiological environment that prevent therapeutics from reaching the site of action. Additionally, the concept of dosing maintenance and the therapeutic window will be explored. Finally, a brief introduction will be given on how polymer based carrier systems and polymer conjugates can be used to overcome these barriers

2.1. History of challenges in drug delivery

As science has advanced, the discovery of potential drug molecules has increased. However, due to complexity of the human body and the drug molecules themselves, application of new therapeutics is somewhat limited. Thus, as new drug discoveries were made, advances in delivery mechanisms became increasingly necessary [15]. To optimize efficacy and minimize negative effects, a high concentration of non-metabolised drug must reach the site of action preferentially over non target tissues [16].

In the mid 1900s, progess in the developent of new drug delivery systems was initiated. Until this time, the majority of drugs were delivered through conventional methods such as injections (parenteral, intramuscular, subcutaneous.), oral delivery (solutions, tablets) or transdermally, in the form of cream or ointment [17]. These methods, although effective at the time, each come with disadvantages. The injection route is painful, invasive, and often times requires administration by a trained clinician; thus patient compliance is low, resulting in a reduced therapeutic efficacy [18]. Additionally, since the drug is often times injected directly into the blood stream, the effect is somewhat short lived, and then the potential for sustained effect is diminished. Oral delivery methods are associcated with high patient compliance, however many drug molecules can not survive the harsh environment of the gut or be absorbed through the intestinlal epithelial barrier [19, 20]. Topical delivery again generally improves patient compliance, however this method is limited to local delivery, as many therapeutics cannot diffuse thorugh the protective layers of the epidermis [21]. Drug delivery systems aim to mitigate the limitations of these conventional delivery methods.

In the 1950's a break through in oral drug delivery systems was made with an encapsulation methods known as the Wurster process [22]. This in turn led to the development of other microencapsulation methods. In the late 1960's, Alejandro Zaffaroni, a pioneer in drug delivery research, designed the first controlled release drug delivery system in the form of a transdermal patch [23]. His research is conisdered by many to form the foundation of all subsequent drug delivery research.

Liposomal systems were also developed in the 1960's for controlled release. A liposome is an artificial vesicle made from two lipid bilayers, resulting in a hydrophilic outer shell and inner core, with a hydrophobic layer between the two. The dual natured attitude toward water

allows for encapsulation of both hydrophobic and hydrophilic drug molecules within the carrier system. Current liposomal systems incoroporate poly(ethylene glycol) to reduce undesired uptake by the reticuloendothelial systems. Liposomes are beyond the scope of this project, but are covered in depth in many references[24-28].

Polymer-drug conjugates, also referred to as polymeric pro-drugs, have been explored as drug delivery systems. Through conjugation, the drug molecule can be held in an inactive form until release at the site of action, thereby reducing non-specific toxicity and enhancing therapeutic efficacy. Conjugation can increase therapeutic stability and solubility. Drug delivery systems, particularly those based upon polymers, have allowed scientists to surpass the barriers frequently encountered *in vivo.*

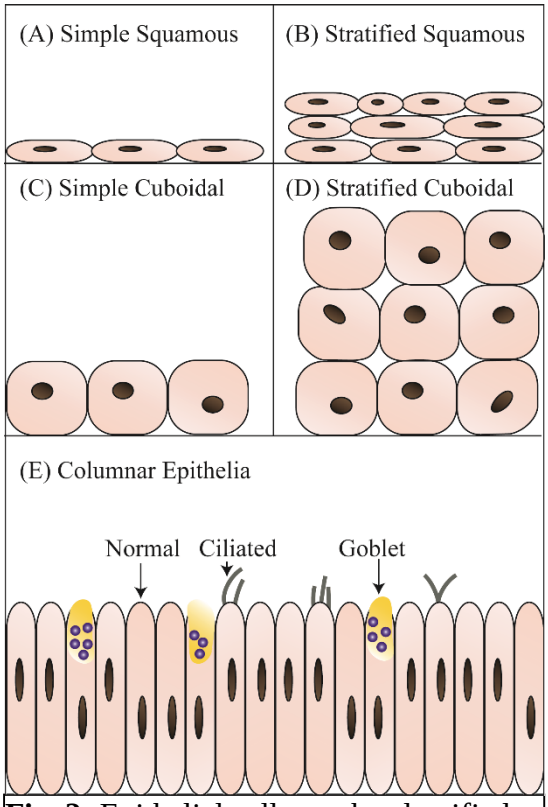

Fig. 2: Epithelial cells can be classified by morphology and alignment.

2.2. Physical barriers

In order to reach the systemic circulation and/or the site of action, all molecules must cross a series of physiological barriers, particularly epithelial, mucosal, and endothelial membranes. These membranes exist throughout the body, with varying complexity, thickness, and permeability [29]. Membrane properties allow some molecules to cross easily, while others are not able to cross at all. The barriers to drug delivery, and their associated properties will be discussed in detail in this section.

2.2.1. Epithelial membranes

Epithelial membranes line the interior and exterior of numerous organs. As individual organs serve distinct purposes and functions, epithelial membranes differing in cellular morphology and arrangement are utilized. The epithelium functions mainly in protection and transport, but also assists in the regulation of secretion, absorption, excretion, filtration, and diffusion of molecules, such as nutrients, waste, and drugs [30, 31]. As shown in Fig. 2, epithelial cell shape can be categorized as squamous, cuboidal, or columnar [32]. These cells can be arranged into layers by a process known as stratification. Squamous cells are generally flat and wide, as illustrated in Fig. 2 (A,B). Consequently, these cells are typically found in areas with a high amount of material exchange. The lungs, for example, utilize a simple squamous epithelium [Fig. 2 (A)] to allow for rapid exhange of gases, as thinner membranes are easier to cross. Cuboidal cells, as shown in Fig. 2 (C,D), are most commonly found in the epidermis and mainly function as structural maintenance cells. Like the simple squamous cells of the lungs, columnar epithelial cells [Fig. 2 (E)] are also found in areas that require a large surface area for material exchange; however, this morphology facilitates greater regulation of the exchange. As columnar cells are more elongated than squamous cells, they allow for an increased number of lateral cell junctions that regulate the connectivity of the membrane. For example, a simple columnar epithelium is found in the small intestine to allow for the absorption of ingested nutrients. Columnar epithelia cells can be specialized with cilia that sweep unwanted particles

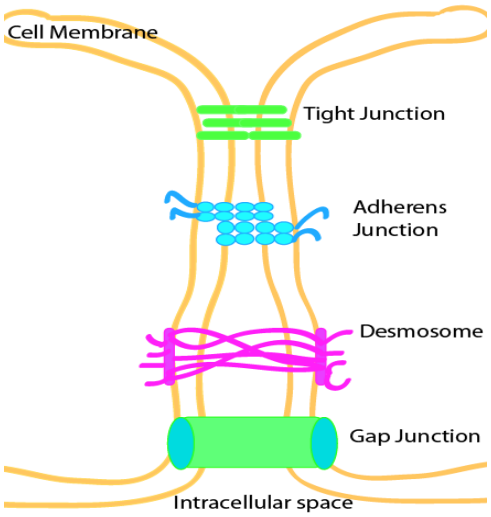

Fig. 3: Tight junctions, adherens junctions, desmosomes, and gap junctions serve as connections between epithelial cells and act to reduce the membrane permeability of molecules.

away from the area, enhancing the protective nature of the the membrane. Ciliated columnar cells are found in other areas aside from the small intestine, including the respiratory passages. Additionally, specialized cells known as goblet cells are often scattered among other columanr epithelial cells. As will be discussed further below, goblet cells secrete mucus that protects the body from potential pathogens and provides a medium for transport. As a general rule, as the complexity of an epithelial membrane increases, the permeability of molecules, including drugs, decreases.

Contact between neighboring epithelial cells is provided by several types of cell junctions, which can reduce permeability of the membrane to select molecules. These junctions include tight junctions, adherens junctions, desmosomes, and gap junctions (Fig. 3). Tight junctions are paracellular connections often found towards the apical surface, that seal off the pathway between cells, preventing harmful substances as well as therapeutic molecules from traveling through the membrane [33]. Tight junctions are composed of transmembrane proteins with adhesive properties including occludins, claudins, and recently discovered tricellulin [33]. Adherens junctions are tyically located below the tight junctions. The primary compenents of these cells are cadherins, which are adhering proteins requiring the presence of calcium to maintain adhesiveness. These junctions include actin and myosin filaments on the intracellular side of the membrane, resulting in an ability to generate contractile force, which in turn acts to control and maintain the cells proper shape and tension. The combination of tight and adherens junctions is referred to as the junctional complex [33]. Desmosomes are the next type of junction found moving away from the apical surface. Desmosomes are also composed of calcium dependent adhesive molecules; however, they are specifically called desmosomal cadherins. Desmosomes primary job is to connect epithelial cells to each other on the lateral surface, giving

the membrane strength and durability. They differ from tight junctions in their level of permeability. As desmosomes primary function is not to create a seal between two compartments, the level of permeability is much higher. Unlike the other junctions discussed in this section, gap junctions allow for transport between cells and do not have a significant role in strengthening cell-cell structural connections. They can be thought of as small, water-filled channels spanning the intracellular space between two adjacent cells. The channels themselves are composed of proteins called connexins, which assemble to form a ring structure and are able to span the intracellular space.

The major limiting factor to absorption of orally administered drugs is the low permeability of the gastrointestinal epithelial membrane [34]. Within the gastrointestinal tract, drug absorption occurs primarily in the lower portions of the small intestine where the tight junctions, and consequently the epithelium, are most permeable [35]. This leakiness of tight junctions is associated with a decreased transepithelial electrical resistance (TEER). The realization of the importance of tight junctions and TEER in trans-epithelial movement has led to the development of several methods to enhance permeability. For example, several investigators have found methods to target occludins and claudins, the primary proteins involved in tight junctions [36, 37]. While useful for drug delivery applications, the disruption of tight junctions also reduces the protective function of the epithelial membrane; so application of tight junction modulation must be highly specific and easily reversible.

Rather than targeting intercellular delivery through the tight junctions, other investigators in the realm of drug delivery have focused on enhancing absorption through the primary columnar epithelial cells of the intensitine, the enterocytes. Enterocytes posess villi and microvilli on the apical surface that increase the surface area for absorption [38]. Among

enterocytes are specialized cells known as M cells, or membranous cells. They typically are found covering sections of lymphoid tissue known as Peyers Patches. M cells present antigens that can be targeted to enhance the efficiency of the transcytosis of macromolecules and non-bioactive molecules relative to enterocytes [39]. Enterocytes produce the glycoprotein enzymes necessary for transporting materials across the epithelia. Recently, enterocytes were found to express many of the same drug metabolising enzymes originally thought to only be found in the liver [40]. Actions such as co-administration of enzyme competitor molecules to decrease the catalytic activity of enzymes (belonging to the Cytochrome P450 family, see Section 2.3.1) on the primary administered drug can be taken to enhance absorption and efficacy. Absorption can be further hindered by other columnal epithelial cells, goblet cells and ciliated epithelial cells [41]. Goblet cells secrete mucus that forms the basis of the mucosal membrane, as discussed below. Ciliated cells hinder absorption through the enterocytes by creating drug molecule movement [41].

Transdermal drug delivery is another favored non-invasive route of administration hindered by the relative impermeability of the epithelial membrane. The major limiting factor of transdermal delivery is the outermost layer of skin known as the stratum corneum. This layer is between 10 and 20 um thick and can be thought of as a brick and mortar type of system; where the bricks are the cells, composed mainly of cross linked keratin, while the mortar is a dense mass of extracellular matrix proteins and lipids. This architecture requires drug transport to take a tortuous path of diffusion through the intercellular lipid mass. Thus, only a limited number of molecules, specifically those that are lipophilic, have a low effective dosing requirement, and possess a molecule weight of <500 daltons, can travel this route [42]. Below the stratum corneum is the avascular epidermis, composed primarily of squamus epithelial cells near the

stratum corneum and cuboidal epithelial cells approaching the demis [43]. The epidermis provides another non-vascularized barrier that must be traversed by the drug before reaching the dermis, the desired destination of most transdermally administered drugs. This is the inner most layer of the skin epithelium where blood vessels and nerve endings are contained [42].

2.2.2. Endothelial membranes

Drugs that enter and exit systemic circulation must cross the endothelial barrier provided by the blood vessels. The anatomy of blood vessels varies with the type; however, each artery, vein, and capillary consists of a thin, inner membrane of squamous endothelial cells, which provides the main barrier to drug absorption [44]. In most healthy systemic vessels, this endothelial sheet is continuous with cells, connected by impermeable tight junctions and adherens junctions.

Capillaries, consisting of a single endothelial membrane and a small amount of connective tissue, are the most common type of blood vessel and the site of blood/tissue-material exchange. Therefore, capillaries are of interest in many drug delivery applications. The capillary endothelium can be targeted using ligands specific to receptors expressed by endothelial cells. Angiogenic vessels associated with tumors and inflamed tissue provide several examples of the specific targeting mechanism. For instance, the endothelium of newly formed vessels often overexpress key proteins and molecules, including vascular endothelial growth factor (VEGF), adhesion molecules such as vascular adhesion molecule (VCAM) and e-selectin [45]. Atherosclerosis, a disease characterized by vascular inflammation, stiffening, and plaque buildup, also is characterized by upregulated adhesion molecules (VCAM-1, ICAM-1, and selectins). Potential plaque rupture provides further prospective targets, as proteins such as fibrin are released into the vessel near the plaque [46]. Additionally, endothelial cells contain

vesicles specifically designed to transport materials across the cytoplasm [47]. In highly angiogenic states, as often associated with pathologies such as tumor growth and inflammation, the capillary endothelium becomes increasingly discontinuous. This discontinuity that results from the rapid and somewhat disorganized assembly of vessels can be used to facilitate enhanced permeation and retention (EPR) of drug molecules, as will be discussed in subsequent sections [47].

2.2.3. Mucosal membranes

Many epithelial layers, particularly the gastrointestinal tract membrane, are accompanied by another barrier membrane known as the mucosal membrane. The mucosal membrane contains a viscoelastic, gel-like substance, mucus, comprised of the glycoprotein mucin. The mucosal membrane has several physical and chemical properties that affect the absorption and bioavailability of therapeutics [48]. For example, the diffusion coefficients of various macromolecular compounds in mucus is typically 30-50% of the diffusion coefficient of the same compounds in an aqueous environment, indicating much slower movement [49, 50]. Furthermore, mucosal membranes can serve as physical barriers to absorption. The viscoelastic properties of mucus result in entrapment of compounds and agglomeration of particles. This agglomeration effectively increases the size of compounds and, thus, contributes to the reduction in the diffusion coefficients [50].

2.2.4. Routes of transport

There are several classifications regarding how a molecule moves across biological berriers. Active transport (requiring an energy input) and passive transport (not requiring an energy input) transport are the two main categories of movement, with passive transport having

numerous sub categories. Transport mechanisms can also be divided into paracellular (between cells) and transcellular (through cells) routes. Fig. 4 provides a pictoral representation of the different transport routes.

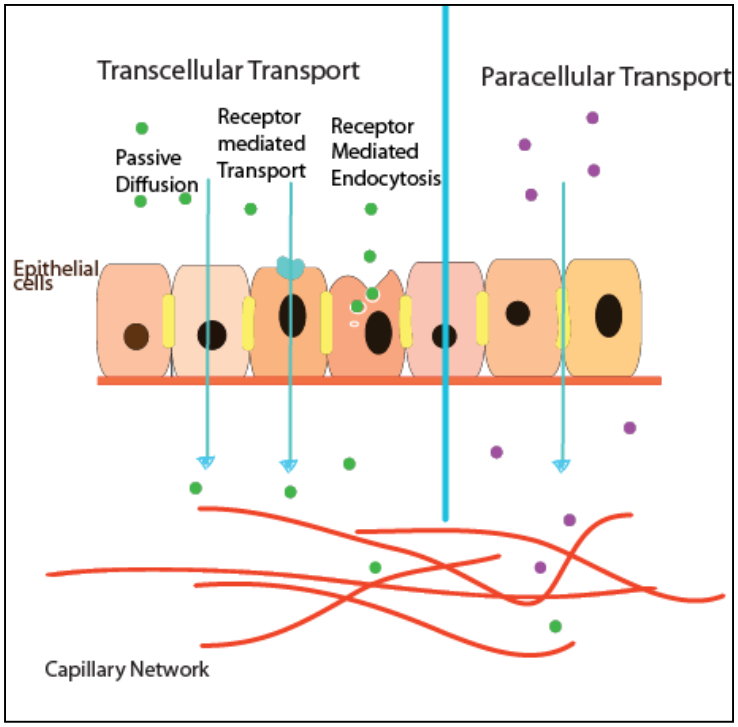

Fig. 4: The general paths a drug molecule can use to cross a membrane. Typically, small, polar molecules (purple dots) can passively diffuse across an epithelial membrane by passing between the cells. This is particularly prevalent in the lower portions of the small intestine,. Lipid soluble molecules have the ability to diffuse accross a membrane, but not between cells (green dots).

The structure of the cell membrane plays a significant role in drug transport. Cell membranes are composed of phospholipid bilayers, with hydrophilic head groups on the edgesand hydrophobic tails composing the core. This structure imposes the following limitations on types of drugs which can cross the membrane by transcellular passive diffusion: (1) the drug must be lipid soluble, (2) the drug must possess a low molecular weight, and (3) the drug must be in a position to travel from an area of high concentration to an area of low concentration. Drugs can also cross a membrane by passive diffusion paracellularly. This type of transport requires the drug molecule be water soluble, of low molecular weight, and be traveling along a concentration gradient. In both paracellular and transcellular passive diffusion, the driving force is the concentration gradient. The concentration gradient is the fundamental idea behind all types of diffusion, and modeled by Ficks Law.

Depending on the cellular architecture of the barrier, the drug molecule may have to diffuse through or between more than one layer of cells. Under these circumstances, each different layer must be accounted for, resulting in an effective barrier to drug absorption. For a given drug molecule undergoing passive diffusion, the rate of absorption will be linear with respect to the concentration [51].

A variation on passive diffusion is a process known as facilitated diffusion. This process still relies on the concetration gradient as the driving force and requires no energy input, however membrane-embedded proteins are required for entry to the cell. Facilitated diffusion can be thought of as two different categories as well: carrier mediated transport and channel mediated transport. Carrier mediated transport requires transport proteins, which function by binding to the molecule to be transported, moving across the membrane, and releasing it on the other side. No direct change is made to the transport protein in this process. These proteins are structurally selective for the drugs that they transport and are saturable. Thus, the maximum rate of absorption will dependent on the concentration of receptor, not the concentration of drug [52]. Channel mediated diffusion requires continuous, aqueous pores spanning a lipid bilayer membrane. Charged or polar drugs are typically able to travel through these channels faster than passive diffusion through the membrane; therefore, their rate of absorption is increased [52].

The transport mechanisms discussed above have relied on the concentration gradient for the driving force and therefore have not required any outside input of energy. In constrast, active transport most often refers to the the movement of solute against a concentration gradient and does require an energy input. In drug delivery applications, active transport typically refers to transmembrane pumps that use ATP to transport drug molecules from areas of low concentration to high concentration. Protein pumps can move molecules either into or out of a cell and are also

crucial in maintaining ion balances across membranes. Some examples of transmembrane protein pumps include the sodium potassium pump (Na+/K+), the sodium hydrogen pump in the gastrointestinal tract, and the calcium ion pump [52, 53].

2.2.5. The blood brain barrier

The blood brain barrier (BBB) is a general term for the system of membranes acting in a protective manner to keep the central nervous system (CNS) impermeable to molecules from the systemic circulation. The CNS is the most convenient route of delivery for therapeutics capable of treating nervous system disorders, including medications for the treatment of stroke, many types of cancer, Alzheimer's disease, and human immunodeficiency virus (HIV) [54]. Unfortunately, the physiology of the BBB makes transport into the CNS from systemic circulation difficult. The BBB is comprised mainly of cerebral microvascular endothelial cells, which give rise to structural differences in brain capillaries compared to other systemic capillaries. As shown in Fig. 5, endothelial cells lining the brain capillaries contain tight junctions, creating a less permeable barrier between the blood and the CNS [55]. Non brain capillaries contain endothelial cells as well, but the spaces between them generally contain more, as well as larger, openings than brain capillaries. As a result, molecules <500 Da can passively diffuse through the openings and pass into the tissues. In contrast, the tight junction regulated brain capillaries do not allow for diffusion between cells, leaving only membrane diffusion as a

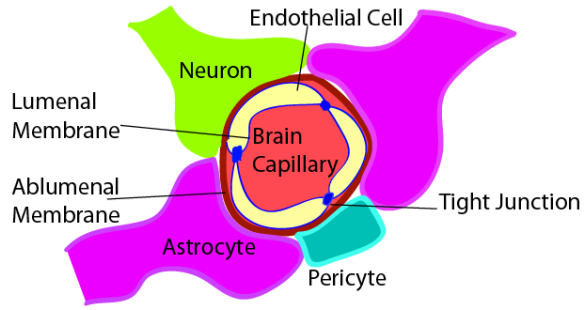

Fig.5: A representative cross sectional view of the blood brain barrier. The BBB is comprised of the endothelial cells of brain capillaries connected by nearly impermeable tight junctions. Additionally, the astrocytes, pericytes, and neurons lining the capillaries further hinder transport of drugs into the CNS.

means of transport into the brain [56]. Small (<500 Da), lipophilic drug molecules have the best chance of achieving transport with this process[56]. This requires solutes to diffuse through two membranes; the lumenal and ablumenal membranes of the endothelium. Furthermore, surrounding the ablumenal membrane of the capillary are astrocytes, pericytes, and neurons, which provide additional membrane barriers between the systemic and brain blood flows [57].

A number of transmembrane transport systems are present in the endothelial cells of the BBB. These transporters are generally facilitative, with the main function being to allow for the uptake of nutrient materials [58]. These transport systems can be exploited in applications to improve delivery to the BBB. In addition to facilitative transport systems, transmembrane transport systems exist within the endothelial cell layer that function to keep molecules out. These transmembrane transport systems, known as efflux pumps, are also present in the intestinal track and liver. Many efflux transport pumps employ ATP hydrolysis as energy to power the pump, and thus are termed ATP Binding Cassette family proteins, (ABC) transporters. This class of proteins is very large and structurally diverse, giving rise to individual ABC pump subfamilies. The subfamilies are denoted with letters A-G, and are distinguished from each other by the types of drugs they transport. In general, ABC substrates are typically hydrophobic or amphipathic, and the differences that distinguish the substrates for each subfamily are subtle. For example, lipids, bile salts, and peptides are all transported by subfamilies A, B, and G. Additionally, organic anions, as well as conjugates with anionic residues such as glutathione, sulfate, or glucoronyl have receptor specificity for ABCC [59, 60].

P-glycoprotein (P-gp) is a well characterized efflux pump present in the BBB. This protein associated with the expression of the multi-drug resistant gene family (MDR family). A variety of lipophilic drugs have been identified as substrates for P-gp. For example, cyclosporine

A (CysA), an immunosuppressive agent, has been shown to be hindered by P-gp when crossing the BBB. However, in the presence of agents shown to suppress the MDR genes, including chlorpromazine and various steroid hormones, uptake of CysA can be significantly improved [61]. P-gp is a transporter present in many other tissues, including the liver, intestines, and kidneys, and will be further discussed in Section 2.4.1.

2.3. Metabolic and chemical concerns

The mechanism of transport of drug in the body depends on several chemical and physical properties, including molecular weight, hydrodynamic radius, lipid solubility, partition coefficient, and polarity [62]. To affect the target tissue, a drug must enter the cells in an active form. In general, drugs are metabolized, resulting in either a decrease in the usable concentration of drug molecules or an increase in the amount of metabolite concentration that will have an undesirable effect on the target tissue. Knownledge of physiochemical properties of drug molecules *in vivo* is essential for predicting therapeutic efficacy. This section will discuss the effects of drug metabolism on drug absorption, including the use of transport proteins and enzymes to hinder or aid absorption.

2.3.1. The first pass effect

First pass metabolism (first pass effect) occurs mostly in oral delivery applications when the concentration of administered therapeutic is reduced by metabolic efforts of the body before reaching systemic circulation. The principle organs involved in first pass metabolism are the small intestine and the liver [63].

2.3.1.1. Enzymatic hepatic metabolism

Despite the many barriers to absorption in the intestine, many drugs are able to cross the epithelium and enter systemic circulation. However, they are not yet in the clear to travel to the

site of action and effect a response. All drugs absorbed through the intestinal epithelium are carried to the liver via the hepatic portal vein. The liver is responsible for breakdown and metabolism of a variety of substances, including steroids, sterols, bile acids, and ecosinoids. Though vital for normal function, the liver provides an undeniable barrier to drug delivery. Drug molecules with structural or chemical properties similar to those of the natural liver substrates will be metabolized in the liver via the same metabolic pathways. Some drugs (for example the glyceryl trinitrate, used to treat angina, and lidocaine, an analgesic) are rendered useless when delivered orally due to extensive metabolism by the liver [64].

The Cytochrome P450 (CYP) enzyme family is the major source of enzyme activity involved in hepatic metabolism. The CYP450 family is a large gene family composed of 57 different members. The majority (70-80%) of phase one drug metabolism is carried out by about 15 of these, belonging to classes designated CYP1 and CYP3. Phase one metabolism refers to modifications of the basic structure of a drug molecule. CYP enzymes can catalyze modifications, including hydroxylations; O, S, and N dealkylation, oxidation, demethylation, and deamination. The result of these modifications is structurally changed metabolites, which are either eliminated immediately, used as substrates for phase two metabolism, or retain the ability to be therapeutically active. Codeine, for example, typically undergoes an O-demethylation catalyzed by CYP2D6, resulting in a structural change to morphine, a drug with an increased activity level [64]. Although the goal of phase one metabolism is generally to detoxify compounds transported in the bloodstream, the opposite effect can sometimes occur, resulting in a secondary metabolite that is more toxic than the original drug molecule. For instance, phase one metabolism of chemotherapeutic Tamoxifin results in a metabolite with genotoxic hepatocarncinogenic properties. In instances such as this, phase two metabolism is extremely

important in preventing the toxic molecule from not only damaging the liver, but also from entering systemic circulation once again [65].

Phase two metabolism is carried out by a class of enzymes known as transferases. As the name might imply, transferases catalyze the transfer of material. In the case of drug metabolism, the transferase catalyzes the transfer of a hydrophilic moiety from a donor molecule to the metabolite molecule, typically reducing the toxicity or allowing for neutralization reactions to occur. Like CYP enzymes, transferases are also typically thought of as gene superfamilies. Some of the major transferase families include glutathione-S transferase (GST), sultphotransferase (SULT), N-acetyltransferase (NAT), and UDP-glucuronosyltransferase (UGT). GST catalyzes reactions with non-polar compounds, SULT results in sulphation of steroid hormones and bile acids, NAT results in acetylation of amine groups, and UGT leads to glucuronidation of molecules, such as the hepatic product bilirubin, pain reliever acetaminophen (toxic in instances of overdose), pain reliever morphine, and other non-steroidal anti-inflammatory drugs (NSAIDs) [64]. Toxicity of a phase one metabolism by product is highly dependent on its rate of production in relation to its rate of reaction with phase two metabolizing transferases.

2.3.1.2. Enzymatic intestinal metabolism

In addition to physical barriers preventing absorption, metabolic enzymes, including lipases, proteases, and glycosidases are present in the gastrointestinal tract to breakdown ingested food and release energy and nutrients [66]. While highly efficient at providing energy and nutrients, these enzymes are also responsible for the degradation of drug products, preventing absorption and/or resulting in a loss of function. Two major proteins responsible for metabolizing drugs in the gastrointestinal tract are CYP3A and the P-glycoprotein efflux pump

[67]. These complexes are found in several locations, including the intestine and liver. In the intestinal tract, they are often found within the membrane of individual enterocyte cells, the primary cells for drug absorption. Methods to circumvent CYP metabolism of drugs in the intestine include co-administration of a CYP inhibitor or inducer. A simple method of inhibition is co-administration of a secondary drug which competes with the primary drug for access to the active site of a CYP enzyme. Drugs with a high affinity for CYP450 that can be used as secondary drugs for competition include cimetidine, (used to treat ulcers), ketoconazole (used to treat fungal infections) and Indinavir, a protease inhibitor used to treat viral infections [64]. Additionally, a metabolite of the primary administered drug may sometimes form an inactive complex with the catalytic site of the CYP enzyme. This method of inhibition is often known as mechanism based P450 inhibition [68]. CYP inhibitors decrease the effect of the enzyme, allowing for increased absorption of drugs including sirolimus, cyclosporine, and tacrolimus [67].

The most prevalent and arguably most important enzyme of the CYP family in intestinal metabolism of drug molecules is CYP3A4. Found in large quantities in the liver, CYP3A4 is also present in the jejunum portion of the small intestine, primarily located on the villi[38]. Studies have shown the enzyme activity and concentration of jejunum CYP3A4 to be equal to that of the microsomes of the liver [69]. CYP3A4 has a broad range of structurally diverse substrates, with an equally broad range of therapeutic function; however, hydrophobicity is a commonality across substrates. Biotransformation reactions of drug molecules by CYP enzymes are typically considered to be phase one, referring to a basic structural change of the molecule [38].

2.3.2. Efflux systems

Efflux systems are energy dependent transport systems whose function is to protect the body by preventing harmful substances from traversing membranes and entering other body compartments [66]. They are present in many different areas of absorption, but play the most significant role in the gastrointestinal tract and the blood brain barrier. The most well-known and well classified efflux pumps are members of the P-gp family, as mentioned in brief above. P-gp was first characterized as the transport system behind tumor resistance to chemotherapeutics; as it was able to transport the therapeutic agents out of the cell. This efflux pump is transcribed as a result of the multi-drug resistant gene, MDR-1 [38]. P-gp has a large variety of substrates, similar to CYP3A, many of which are large and amphipathic [67]. Additionally, in the intestine, expression of P-gp increases longitudinally throughout the tract, in contrast to the levels of CYP3A expression. This results in a constant source of molecular absorption prevention throughout the gastrointestinal tract, as P-gp's main effect is to pump drug molecules back into the lumen [19].

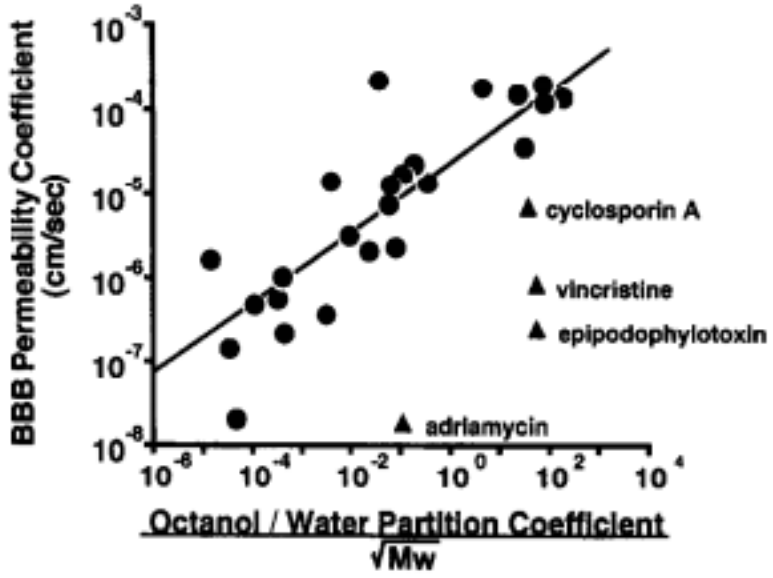

Fig. 6: For drugs with a molecular weight of 500+/- 100 Da, the permeability of the BBB has been shown to increase linearly with the partition coefficient relative to the square root of the molecule weight. P-gp efflux pump has been shown to account for discrepancies, as the outlier drugs in the graph are known to be P-gp substrates.

In sum, the mechanism of membrane crossing for solutes traveling into the brain is usually by receptor mediated transport or diffusion. Properties of the drug molecules can be

tailored to make crossing the BBB more feasible and efficient. If the molecular weight of the molecule in question falls at 500 ±100 Da, the permeability of said molecule increases linearly with the partition coefficient of the molecule. This relationship can be shown graphically by constructing a plot of the log of permeability vs. log of the partition coefficient divided by the square root of the molecular weight, as described by Tsugi and Tamai, and shown in Fig. 6. [61]. An alternative to the molecular weight explanation of decreased permeability is the presence of the P-gp efflux system. This system, also present in the gut, serves to restrict transcellular flux of drug molecules, thus decreasing permeability.

2.4. Physical properties of therapeutics

Several factors are involved in both the amount of and the extent to which a drug molecule will affect the target tissue. In addition to the physiological and chemical properties discussed previously, the route of administration and delivery method, dose and release profile, as well as physiology changes due to pathology play a role in determining the biological effect of a drug molecule.

2.4.1. Bioavailability

Bioavailability is defined as the amount of drug reaching the systemic circulation out of the amount of drug that was administered [70]. Careful dosing and administration is a necessity for efficacious treatment. The amount of drug available at the desired site of action must fall within the therapeutic window [12].

Achieving reproducible oral bioavailability is particularly difficult because there are several factors which must be considered based on the path the drug must travel before reaching systemic circulation. In this case, the overall bioavailability (F) is the product of the portion of

drug absorbed (F_a), i.e. the fraction that passes into the hepatic portal blood unaffected by the enzymes of the gut and intestine, and the fraction of drug that escapes metabolism by the liver (F_h) [38, 70]. This value will vary between drugs due to the differential effects of enzymes, including CYP and P-gp, on individual drugs.

In addition to the effect of the body's metabolism, properties of the drug molecule itself have a large impact. Solubility, for example, is one of the leading factors hindering oral absorption and reducing bioavailability, and a great deal of effort is being put forth to determine methods to increase solubility of therapeutics in aqueous environments [71]. For example, chemical modifications to the drug molecules to generate prodrugs with an increased aqueous solubility are being explored, as are methods of solid dispersion. Solid dispersions typically consist of a hydrophilic matrix material with hydrophobic drug dispersed throughout. The result is an increased dissolution rate and higher bioavailability of hydrophobic drugs. The degree of therapeutic solubility (or dissolution rate) is dependent on the diffusion coefficient of the drug, the concentration of the drug within the dissolution medium, and the solubility of the drug within the dissolution medium. Additionally, physical factors, including the surface area of the formulation available for dissolution and the thickness of the diffusion boundary the drug must travel through, must be accounted for [72].

2.4.2. Release profiles

The amount of drug released into the bloodstream over time is known as the release profile. Release profiles typically depend on the dosage form of the drug, the method of release, and the properties of the carrier system. These release profiles are often categorized as controlled release, pulsatile release, and burst release, as shown in Fig. 7. Controlled release is characterized by a gradual increase in the plasma concentration with time until a maintenance

concentration is reached. This is often the desired treatment method, although somewhat more difficult to attain. Pulsatile release similarly begins with an increase in plasma concentration to a desired level, followed by decreases in concentration, which are counteracted with additional dosing, maintaining a concentration within the therapeutic window. Burst release consists of an initial dose generating a steep rise in plasma concentration. However, the initial drug release is not supplemented with additional doses, resulting in a short time period when the plasma concentration is within the designated therapeutic window [73].

Of particular importance is the carrier system properties, which modulate how the drug molecules are released. Diffusion always plays a role in drug release, however depending on the properties of the source (i.e. the carrier system), the concentration profile will differ. The two primary methods of degradation are bulk and surface erosion (Fig. 8). Surface erosion results in a reduction of the overall volume of the carrier system as degradation occurs at the surface of the material. This also results in a turnover of the surface; a new layer of the material is constantly being exposed to the environment. In contrast, bulk erosion does not result in a volume reduction; rather, the material is lost uniformly throughout the entire volume of the carrier system [74].

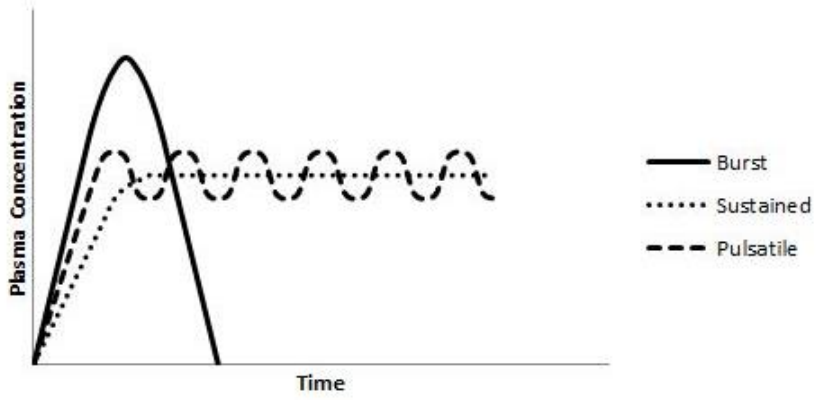

Fig. 7: Three release profiles are commonly observed for drug carrier systems. Sustained/controlled release is characterized by an increase in plasma concentration until a desired maintenance concentration is reached. Pulsatile release is characterized by a cyclic fluctuation in plasma concentration. Burst release is characterized by a large initial increase in plasma concentration, followed by a sharp decline.

Different applications require different release profiles, but, as mentioned above, a sustained plasma concentration is generally most desirable. For example, release of drugs dispersed within polymer constructs, as will be covered in subsequent sections, is dependent on the rate of degradation of the polymer substrate, as well as the diffusion rate of the drug. If the degradation rate is slower than the diffusion rate, the release will follow the pattern of bulk erosion method. If polymer degradation is faster than the diffusion rate, the release will follow the pattern of surface erosion [75].

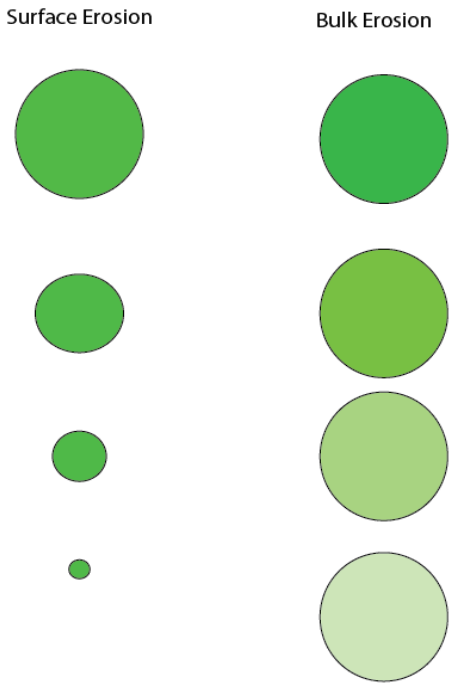

Fig. 8: Surface and bulk erosion are two methods for degradation of carrier systems. Surface erosion results in a decrease in overall volume, shown here by a reduction in size. Bulk erosion results in a decrease in the amount of material within the matrix.

2.5. Polymer carriers as a solution to challenges

The term 'polymer' refers to a long chain molecule composed of many repeating molecules. Polymer encapsulation of and/or modification to drug molecules increases circulation time of the drugs in the body by increasing their size, thereby decreasing the amount of filtration by the kidneys [76]. Additionally, targeting of drugs to specific sites of pathology can be improved by the increase in size due to a phenomenon referred to as enhanced permeation and retention (EPR) [77]. For example, cancer tumors and sites of inflammation show increased angiogenesis and a characteristic "leaky" vessel endothelium (Fig. 9). The leaky vasculature is caused by rapid and disorganized vessel formation, which results in

larger pore spaces between endothelial cells. Polymeric drug delivery systems may be taken into the tissue by transcellular transport, but this is a time dependent process and generally not efficient. In diseased tissue, however, the larger gaps between vascular endothelial cells allows for relatively easy uptake of the drug carriers [77].

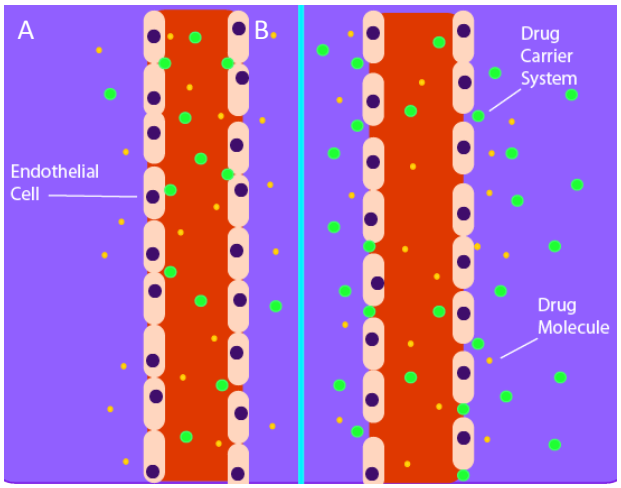

Fig. 1: EPR facilitates for passive targeting of drug delivery systems. (A) Vessels within healthy tissue have narrow gaps between endothelial cells, while (B) vessels within diseased tissue have larger ("leaky") gaps.

In general, polymer carrier systems should have several common characteristics. These characteristics include (1) an ability to be produced easily and on a large scale, (2) applicability to a wide range of drugs, (3) physiological stability, (4) biocompatibility, and (5) acceptability by regulatory committees, such as the FDA [78].

2.5.1. Colloidal polymer carrier systems

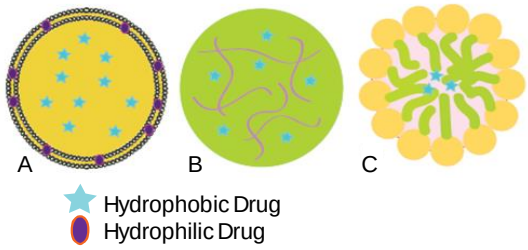

Fig. 10: Colloidal polymer carrier systems include (A) liposomes, (B) nanoparticles, and (C) micelles.

Several kinds of polymer carrier systems can be utilized for drug delivery applications to help avoid some of the previously discussed challenges. In general, colloidal polymer carrier systems are small spherical particles with varying layers and thicknesses [76]. The spherical particle shape of these formulations increases the surface area of the system, allowing for increased absorption. Different particle carrier systems include micelles, liposomes, and nanoparticles [79]. Simplified

versions of these carrier systems are depicted in Fig. 10 and discussed in depth in subsequent section.

Micelles are small, soluble particles that can self-assemble by means of hydrophilic/hydrophobic segregation. In addition to hydrophobic interactions, electrostatic interactions, metal complexation, and hydrogen bonding of the contribute to micelle formation [77]. A block copolymer with separate hydrophobic and hydrophilic segments will have the ability to assemble in an aqueous environment such that the hydrophobic segments compose a core in the center, while the hydrophilic segments compose a shell surrounding the core, thus greatly increasing the aqueous solubility of hydrophobic therapeutics. A popular choice for hydrophilic block copolymer sections is poly (ethylene glycol) (PEG), a widely used polymer in drug delivery applications, and the gold standard for 'stealth cloaking' of carrier systems. Stealth cloaking is a means of avoiding non-specific uptake once administered and functions by creating an aqueous layer around the particle to avoid detection by the immune system [80]. Non-specific uptake by the reticuloendothelial system (RES) is a major obstacle to micelles and other small colloidal carrier systems. The RES is part of the body's defence mechanism and includes cells such as macrophages and monocytes. These cells are phagocytic in nature, and function by engulfing potential threatening substances, eventually accumulating in the liver and spleen for degradation and elimination [81]. This poses a problem for drug carrier systems because uptake by these cells will reduce the amount of therapeutic in the systemic circulation [81].

Poly-L-amino acids, such as poly (D, L-lactic acid) (PDLLA), are frequently used as the hydrophobic portion of micellar block copolymers. For example, Genexol PM, a PEG-PDLLA copolymer micelle loaded with the chemotherapeutic paclitaxel, is currently approved for the

treatment of breast cancer and is undergoing clinical trials in the US for use in pancreatic cancer treatment [82]. Micelle formation is influenced by factors such as the molecular weight of the copolymer, the ratio of copolymer blocks to each other, and the amount of polymer involved. The minimal amount of polymer necessary to form a micelle is referred to as the critical micelle concentration (CMC) [83]. Above this concentration, all additional polymer molecules will be formed into micelles.

Nanoparticle based drug delivery systems consist of sub-micron sized spherical particles, ranging from 10-1000 nm, although the desired size is often around 100 nm [75]. Nanoparticles are typically made of a biocompatible, degradable material and contain drugs either dispersed or dissolved within a core matrix. Two classes of nanoparticles have been identified based on the way the drug is incorporated. Nano*capsules* contain drug molecules confined to the interior of the polymeric shell of the particle, while with nano*spheres* the drug molecules are uniformly dispersed within the polymeric matrix system [84]. Once inside the body, drugs can be released from the particles by means of diffusion, degradation, swelling, or erosion. Efficacy of nanoparticle carrier systems is based on size, particle stability, the amount of drug that can be incorporated, the type of drug that can be incorporate (i.e. hydrophilic or hydrophobic drug molecules, siRNA's or DNA for gene therapy, or proteins), and the potential for different routes of administration (i.e. oral delivery, inhalation, intravenous). Nanoparticles have been shown to be more effective in intravenous delivery than microparticles; which have an increased potential of becoming trapped in the capillaries, some of which are only 5-6 um in diameter. In addition, *in vitro* studies with Caco2 intestinal cells demonstrated that 100 nm particles result in a 2.5 fold increase in uptake compared to 1 um diameter particles and a 6 fold increase in uptake compared to 10 um particles [85]. Furthermore, particles smaller than 200 nm have the ability to escape

processing of the liver and kidney for several circulation cycles, increasing their time in the systemic circulation and hence level of effectiveness[86]. However, a diameter larger than 200 nm will increase the risk for RES uptake, reducing the circulation time and thus the efficacy.

As mentioned in brief above, materials used to generate the nanoparticle systems must be non-toxic, non-immunogenic, non-inflammatory, and non-thrombogenic (which all fall under the general category of biocompatibility). Additionally, the nanoparticles must be stable, be able to avoid detection and uptake by the RES, and be able to be used as carrier systems for a broad array of drug types, including proteins, nucleic acids, and hydrophilic and hydrophobic drug molecules [81]. Some commonly used materials for generation of nanoparticle systems include poly(lactide) (PLA), poly(glycolide) (PGA), poly(lactide-co-glycolide) (PLGA), poly(cyanoacrylates), and poly(caprolactone) [75, 81]. Currently, there are no approved drug encapsulated nanoparticles or on-going human clinical trials in the US, although a great deal of research and development regarding nanoparticle formulations is on-going.

Liposomes are another form of small spherical drug carrier systems. They consist of a lipid bilayer (similar to a cell membrane) surrounding an interior space. This morphology allows for the entrapment and delivery of both hydrophilic and hydrophobic drug molecules. Hydrophobic molecules can be entrapped within the bilayer, while hydrophilic molecules can be carried within the core. The physiochemical properties of the liposomal constituents, including the membrane fluidity, permeability, charge density, and steric hindrance have influence on the types of interactions the liposomes will have with blood and tissue constituents [87].

There are several different types of liposomes. Long circulating liposome's (LCL) can be formed by incorporating hydrophilic polymers into the lipid bilayer to create an aqueous coat on the surface, which prevents marking by immune system opsonins and thereby reduces uptake by

the RES. An example of this modification is called PEG-ylation, where chains of PEG are attached to the particle surface [88]. As discussed above, "stealth" PEG coating generates an aqueous layer. Additionally, LCL liposomes can be tailored with ligands to target specific cell types. For example, long-circulating liposomes prepared with a PEG coating and loaded with Doxorubicin, an anti-cancer chemotherapeutic, were FDA approved in 1995. The efficacy of this system has been further improved via linkage to mAb 2C5, a monoclonal antibody which specifically targets a variety of tumors [89].

Active cationic liposome's have a high affinity for cell membranes and deliver materials to cells by fusing with cell membranes and depositing material into the cell [87]. Nucleic acids are the most common therapeutic form delivered with cationic liposomes. As nucleic acids are negatively charged, stability is increased when complexed with cationic liposomes for delivery. Phase 1 clinical trials were completed for liposomes containing pGT-1, a regulatory gene involved in cystic fibrosis, to the respiratory epithelium. These studies showed promising early results, however the regulatory gene expression was fairly low and relatively short lived [90].

2.5.2. Polymer-drug conjugates

Another method of increasing targeting specificity of drug molecules and avoiding detection and subsequent elimination by the RES is to chemically conjugate polymers to drug molecules. Polymer conjugation serves several purposes. Many chemotherapeutic drugs are very cytotoxic, as their efficacy is generally dependent on their ability to cause death of cancerous cells. These same drugs are also often insoluble in aqueous environments, as found in the human body. Covalent attachment of a water-soluble polymer to an insoluble drug molecule increases the amount of therapeutic in circulation after administration [91]. In many cases, these polymer-drug conjugates undergo phase 1 metabolism to remove the inactive polymer and yield an active

drug. A current example of a drug-polymer conjugate is poly (L-glutamic acid) conjugated to .paclitaxel, yielding PG-TXL. Paclitaxel, a potent anticancer agent, is a molecule with poor aqueous solubility that fights cancer by attacking cellular components controlling processes such as mitosis, transport, and motility, decreasing growth [92]. While the actions of paclitaxel are ideal for attacking tumors, healthy cells are also susceptible to its actions. Conjugation to PG can increase tumor selective uptake and reduce adverse side effects resultant from damage to healthy cells.

Polymer conjugation functions to increase targeting specificity of a particular therapeutic to the desired tissue or region of disease. In addition to polymer alone, receptor specific ligands can be grafted to either the conjugated polymer or the drug itself, which results in site specific accumulation of the therapeutic [93]. Known as active targeting, this is an effective method to reduce non-specific uptake and improve efficacy.

2.5.3. Implantable and transdermal drug delivery systems

Implantable and transdermal drug delivery devices present methods of prolonged administration with relatively stable dosing patterns. These techniques also benefit from the use of polymers, both degradable and non-degradable. Unlike the colloidal carrier systems discussed in section 2.6.1, these drug delivery devices have been in use for longer periods of time and are used more frequently. In general, these devices are used to regulate dosing and increase convenience, rather than improve solubility and stability of the drug molecule itself.

Implantable devices must be embedded in the body, typically subcutaneously. Therefore, there are several important factors that must be met when developing materials for this application. Materials used to construct devices must be chemically inert, hypoallergenic (as to

not invoke an allergic reaction from the immune system), non-carcinogenic, and mechanically stable at the insertion site [94].

2.6 Drug delivery systems for nucleic acids

Recent developments of nucleic acids as drug constructs present new options for treatment of diseases, including cancer, genetic, and inflammatory diseases [95]. However, nucleic acid based drugs represent a unique delivery challenge due to their high susceptibility to enzymatic degradation in a physiological environment, in addition to an intracellular or nuclear site of action.

2.6.1 Viral vectors as nucleic acid carriers

Viral vectors are typically thought of as the most efficient method of gene delivery, in terms of getting the genetic material into a cell. Viruses have naturally evolved throughout history to transfer foreign genetic material into host cells, making them a strong potential vector for nucleic acid delivery [96]. Viral vectors can infect a wide variety of cell types, which is advantageous in terms of number of applications, but a drawback in terms of a lack of control. Despite attempts to target specific tissues with viruses, success has been limited, and continues to present a safety concern. Other problems with viral vectors include potential toxicity, immunogenicity, unpredictability of gene insertion site within the host genome, and potential for gene inactivation by recombination [97].

Several types of viral vectors, including retrovirus, lentivirus, adenovirus, adeno-associated virus, and herpes simplex virus have been reported in studies involving viruses for nucleic acid delivery. Key advantages and disadvantages are summarized in table one [98]. Retroviruses function as gene delivery vehicles through retro-transcribing the viral RNA genome into DNA, and integrating into the host chromatin. These viruses have been shown to have the

ability to attain chronic infection translating to long term gene expression, but are also associated with generation of immunodeficiency, or other malignancies [97]. Lentiviruses are similar to retroviruses in that they both use RNA. The adenovirus consists of a large family of over 50 serotypes, isolated from multiple organs and tissues, resulting in the ability to infect many different cell types [99]. Despite the wide range of potential targets, adenovirus applications remain limited as infection results in an immune response against adenoviral proteins. Adeno associated viruses (AAV) are not directly associated with any human disease, and also normally require co-

Table 1: A summary of various viral vectors currently in use and being researched for DNA delivery applications

Vector	Advantages	Disadvantages
Adenovirus	High transfection efficiency Transfects many cell types Transfects proliferating/non-proliferating cells Easy to produce	Remains episomal, transient transfection Immunogenic with repeated administration Potential for replication competence Lack of targeting
Adeno-associated virus	Ability to establish latent infection, prolonged expression Cell division not required No viral genes in genome	No targeting Potential for mutagenesis Difficult to produce

Herpes simplex virus	Latent expression High in vivo efficiency	Cytotoxic Lack of targeting Requires packaging cell line Does not integrate into genome; transient expression
Lentivirus	Transfects proliferating/non-proliferating cells Prolonged expression	Difficult to manufacture and store Safety concerns Limited clinical data
Retrovirus	Integration into host cell genome Prolonged expression	Inefficient transfection Mutagenesis Requires cell division for transfection Lack of targeting

administration of a helper virus to achieve infection. In addition to requiring an additional virus for infection, AAV stimulate the production of neutralizing antibodies after administration, limiting their potential for repeated administration [97]. As such, clinical trials using AAV have been discontinued due to increased immunogenicity and toxicity [100, 101]. In addition, AAV are difficult to store long term and are problematic for Good Manufacturing Practice (GMP) standards [102].

2.6.2 Cationic lipids for gene delivery

An alternative gene delivery method to viral vectors are cationic lipid based vehicles. Cationic lipids used in gene delivery are amphipathic molecules with polar cationic heads and long hydrophobic tails. In aqueous environments, these lipids will self-assemble into colloidal

structures, or liposomes, with the ability to complex with negatively charged nucleic acids in the form of lipoplexes [102]. Lipoplexes are presumed to enter the cell through endocytosis, making the size of the particle, as well as the charge, important factors in lipoplex design [103]. Ideally, the surface charge of the lipoplex should remain positive to facilitate interaction with the cell membranes. This has an impact on the amount of negatively charged nucleic acids that can be incorporated into the lipoplex [103]. Many commercially available transfection reagents are currently on the market. Of these, Lipofectamine reagents, marketed by Life Technologies, have become the most referenced transfection reagent, with the claim of increased efficiency over other available reagents. However, these compounds do often exhibit cytotoxicity and are prone to accumulation within the liver *in vivo*, leading to significant nucleic acid payload degradation [104, 105].

2.6.3 Synthetic cationic polymers for gene delivery

Recognizing the limitations of immunogenic viral vectors and toxic lipoplexes, recent research has focused on the alternative use of cationic polymers for complexing with and delivering nucleic acids to cells. For example, poly(ethyleneimine) (PEI) is one of the most widely used cationic polymer for nucleic acid delivery [106]. However, PEI exhibits considerable toxicity toward a variety of mammalian cells [107]. The toxicity is twofold, with an initial response to the free PEI remaining in the delivery medium and a delayed response thought to be associated with the cellular processing of the PEI/DNA complexes, likely due to an increase in free PEI. Free PEI is known to interfere with normal cell processes and negatively impact cellular components [106].

Natural polymers present another category of potential materials for gene delivery, as alternatives to viral vectors, lipids, and synthetic polymers. Relevant carrier systems using natural polymers will be discussed in greater detail in chapter three.

2.7 Conclusion

As drug discovery research and development continues to advance, development of compounds with therapeutic potential is evolving. Many of these compounds cannot be fully effective when administered in a traditional manner, invoking the need for the development of novel drug delivery systems to complement the advances in drug development. The trifecta of issues to overcome when administering drugs to the body as summarized in section one include absorption of the drug into various body compartments, stability of the drug and/or carrier system *in vivo*, and finally, solubility of the drug either *in vivo*, or within the carrier system for controlled release applications. Additionally, a well-designed controlled release system with ability to provide a zero order release profile is highly desirable. The body contains many membranous boundaries which function as protective barriers including the epithelium, endothelium, mucosal membranes, and blood brain barrier. These membranes are the targets of several methods to increase permeation and enhance bioavailability of drug compounds. The stability of the drug and/or its carrier system is an important characteristic to be concerned with. If the system does not have adequate stability to be able to reach the site of action intact, its payload will likely be distributed elsewhere, resulting in undesirable effects. Once the drug is delivered to its site of action, adequate solubility is required for the molecule to enter the cell and effect a response.

This chapter was included to demonstrate the intricacies of drug delivery, as the human body contains many barriers and mechanisms to keep substances out. Designing a drug delivery

system requires knowledge of these barriers and mechanisms as a means of exploitation, combined with an understanding of material properties to in order to design carriers with desired traits.

3. Background and Significance

NF-κB decoy oligonucleotides have been proposed as treatment options for several diseases where inflammation plays a major role, including both CF and RA. However, clinical advancement of NF-κB decoy ODNs have been hindered by a lack of efficacy, due to a high degree of instability and a lack of efficient delivery mechanism. This chapter will discuss the specific role of NF-κB in RA and CF, provide an introduction to in vitro models of CF and RA, and finally provide detailed background of existing DNA delivery methods and their shortcomings.

3.1 Nuclear factor kappa-light chain enhancer of activated B cells (NF-κB) signaling pathway

NF-κB is a transcription factor involved in the regulation of a variety of cellular processes, including cellular growth, apoptosis, and inflammatory and immune responses [108].

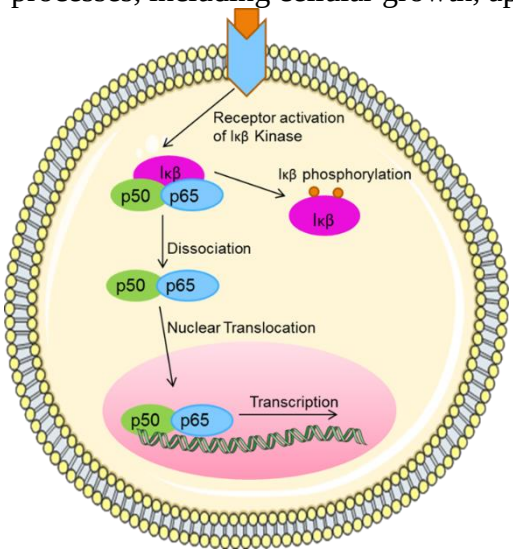

Fig 11. Illustration of NF-kB activation through ligand-receptor interaction

This transcription factor plays a major role in both innate and adaptive immunity and is implicated in a variety of human diseases, including autoimmune diseases, several types of cancer, and some genetic disorders [109].

NF-κB consists of a family of proteins made up of five subunit members: p50, p52, p65, c-Rel, and RelB. The most common, and often referred to as "classic", dimerization of these subunits is p50/p65 [110]. While other dimerizations have been studied, the p50/p65 complex will be the focus of the work described here.

Under normal (unstimulated and healthy) conditions, NF-κB is located in the cytoplasm, bound to an inhibitor known as IκB (inhibitor of κB). When a receptor involved in the NF-κB pathway is triggered by external signals, such as cytokines, growth factors, reactive oxygen species, mitogens, bacterial or viral products, activation of a signaling cascade occurs and initiates the inhibitor of κB Kinase (IκK) [109]. This kinase phosphorylates the inhibitor protein, causing dissociation and leaving the NF-κB dimer free to migrate into the nucleus and initiate transcription [111]. In diseases such as RA and CF, NF-κB over-activation is involved with a state of perpetual inflammation due to autoimmune factors or chronic bacterial infection. These diseases and the role of NF-κB are described in more detail in the following two sections. Fig. 11 contains an illustration of the NF-kB pathway.

3.1.1. Cystic fibrosis and NF-kB

CF is an autosomal recessive genetic disorder caused by a mutation in the CF transmembrane conductance regulator protein (CFTR) [112]. CFTR is a transmembrane ion channel involved mainly in regulating chloride ions. While CFTR mutations manifest in pathology in multiple organ systems, chronic lung infection and inflammation is the greatest cause of morbidity and mortality associated with CF, and will be the focus of the work described here [113]. A lack of a functional protein leads to a decrease in chloride ion secretion in the CF airway. Combined with an increase in sodium absorption due to co-regulation of CFTR the sodium ion channel ENaC, defective CFTR results in dehydration in the lung epithelia, contributing to increased production of extremely viscous, hard to clear mucus [114]. Mucus buildup is associated with chronic bacterial infection, as microorganisms cannot be removed from the airway via normal mucocilliary clearance mechanisms.

Although chronic inflammation is known to be associated with bacterial infection, certain reports suggest that innate inflammation is a component of CF pathology as well. Despite a lack of complete understanding of the onset mechanisms, an inflammatory immune response is certainly present in CF airways. Elevated levels of transcription factor NF-κB have been shown in the CF lung epithelia, and many reports agree that this transcription factor is highly involved in excessive inflammation seen in the CF airway [115]. NF-κB is involved in transcriptional regulation of several pro-inflammatory proteins directly involved in the immune response in CF, including granulocyte-macrophage colony stimulating factor (GM-CSF) interleukin-6 (IL-6) and interleukin-8 (IL-8) [116]. IL-8 is highly involved in neutrophil recruitment, and is present in elevated levels in the CF airway, while IL-6, also present in elevated levels, is involved in immune system activation via B cell stimulation [116, 117].

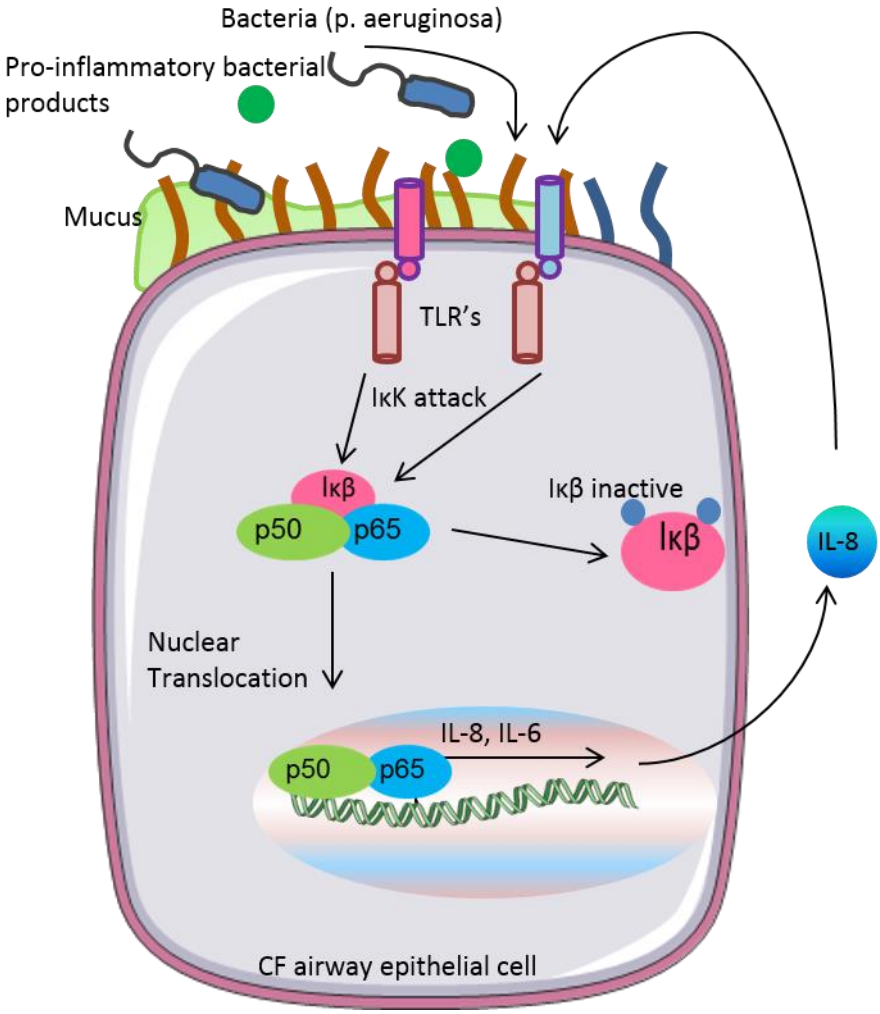

Fig. 12: Illustration of a CF airway epithelial cell, and activation of the NF-κB signaling pathway.

Of the potential bacterial infections associated with CF, one of the most worrisome and difficult to treat is *Pseudomonas aeruginosa*. *P. aeruginosa* has the ability to genetically adapt to acclimate to the environment of the CF airway, allowing survival despite the onslaught of the host immune system [118]. *P. aeruginosa* has been shown to activate the NF-κB signaling pathway via the toll-like receptors (TLR); specifically, through TLR-4 by lipopolysaccharides (LPS) [113]. Pathway activation initiates a protein kinase targeted to the inhibitor of κB (IκB) complex, known as IκK. This kinase phosphorylates IκB, resulting in inhibitor/transcription factor complex dissociation, leaving the p50/p65 NF-κB complex free to undergo nuclear translocation [119]. This process is illustrated in Fig 12, depicting a typical airway epithelial cell response to TLR-4 NF-κB activation.

TLR-4 plays a major role in inter-kingdom signaling within the CF airway, and the Bader Lab has recently been shown to interact and be associated with *Pseudomonas* Quinolone Signal, a quorum sensing molecule secreted from *P. aeruginosa*[120]. PQS, suggested initially to be anti-inflammatory, actually demonstrated increased inflammation in CF airway epithelial cells. An in depth investigation revealed that PQS likely binds to the TLR-4 receptor, indicating yet another role for this diverse membrane receptor.

3.1.2. Rheumatoid arthritis and NF-kB

Rheumatoid arthritis (RA) is an autoimmune disease characterized most notably by chronic inflammation of the synovial membrane of joints. Over time, infiltration of inflammatory cells including lymphocytes, plasma cells, and macrophages to the synovial lining leads to hyperplasia, and formation of a tumor-like tissue known as pannus [121]. An excessive cellular immune response leads to a chronic inflammatory environment, which eventually leads to cartilage destruction and bone degradation [122]. In addition to the influx of immune cells,

native synovial fibroblasts undergo a phenotypic change, characteristic of immune sentinel cells, becoming cells known as activated synovial fibroblasts, or RASF's [123]. The NF-κB pathway is known to be active in RASF's, leading to production of cytokines, chemokines, and matrix metalloproteinase, which contribute to tissue destruction characteristic of RA [123].

While the mechanisms behind RA pathogenesis are not completely understood, it is well accepted that an imbalance of pro-inflammatory and anti-inflammatory cytokines occurs, and plays a major role in joint destruction. Essentially, the levels of anti-inflammatory cytokines (IL-4, IL-10, IL-13) are too low to combat the increased levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, etc.) released by the immune cells and RASFs of the pannus [124]. Pro-inflammatory cytokines IL-6, IL-8, and TNF-α are known to be regulated by the NF-κB pathway, making this signaling pathway a potential target for lowering levels of inflammatory proteins [125].

As the cause of RA remains unknown, so does a cure. Current treatments focus on modulating symptoms and preventing further joint destruction. Although treatments have evolved over the years, researchers and physicians have yet to find a long-term sustainable treatment regimen. Disease modifying anti-rheumatic drugs (DMARDs) are typically used as the first line of treatment. This category of drugs includes immunosuppressants and chemotherapeutics, typically associated with severe side effects. For example, chemotherapeutic methotrexate is widely regarded as the gold standard of treatment, although its use is sometimes discontinued due to hepatic toxicity [126]. In addition to toxicity, this drug has an unpredictable efficacy profile, well tolerated and efficacious for certain patients, while ineffective for others. Recently, biologic agents aimed at interacting with specific cytokines have been introduced. These treatments are effective for some patients, but are associated with a drastically increased

risk of infection, requiring careful monitoring, and are known to have a high risk of infection in severe RA patients [127]. Furthermore, these therapies are extremely expensive, costing upwards of $30,000 annually.

3.2. *In vitro* models for investigating CF and RA

The first step in investigating efficacy of drug carrier systems is very often *in vitro* cell culture models. *In vitro* cell models allow for preliminary testing of a materials safety and efficacy without the extensive resources required for animal testing applications.

Several options for RA *in vitro* models exist, including cell lines and primary cells isolated from tissue obtained from RA patients. Typically, cells used in the models are activated macrophages or fibroblasts, as these are the two cell types highly involved in regulating the pro/anti-inflammatory cytokine imbalance. While *in vitro* models are in general less predictive than *in vivo* models, they provide essential data regarding the effect of the compound in question on a simplified, more direct model of interest. Among *in vitro* models for RA, primary cells are generally considered more predictive than cell lines, and several studies have noted discrepancies in gene expression and protein secretion between cell lines and primary cells[128].

CF *in vitro* models are somewhat complicated, as this disease affects multiple organs and systems. For the work described here, a simple respiratory model was utilized. Specifically, airway epithelial monolayers with the CFTR mutation characteristic of CF airway cells was used throughout the CF *in vitro* experiments. Other airway *in vitro* models include use of sub-mucosal gland (SMG) acini cells, and SMG acini/airway epithelial co-cultures. For the scope of the experiments presented, an airway epithelial model supplemented with a bacterial/mammalian co-culture was sufficient. This model will be discussed in depth in chapters six and seven.

3.3. Drug delivery systems for nucleic acids

Viral vectors are typically thought of as the most efficient nucleic acid delivery agents due to a history of evolution aimed at maximal cell entry [129]. In addition to a high rate of infection, these vectors are able infect a wide variety of cell types. However, viral vectors are associated with major drawbacks, including viral induced immunogenicity, toxicity, mutation of the nucleic acid of interest with the viral DNA, and the potential for inactivation of the gene of interest due to recombination [97].

Several successful colloidal nucleic acid delivery systems, particularly those formed from natural polymers are also being explored as alternative systems [130-132]. For instance, gelatin-based nanoparticles were coated with DNA oligonucleotides with high loading efficiency [133]. Ionically cross-linked chitosan nanoparticles with the ability to entrap plasmid DNA were recently reported by Csaba et al. and were highly effective at transfecting cells *in vitro* and *in vivo* [134]. This supports literature claims stating that chitosan and chitosan-derivatives, such as quaternized chitosan, may be particularly advantageous in the delivery of nucleic acid-based therapeutics by providing biodegradable polymers with low cytotoxicity that reduces enzymatic degradation [8, 131]. Although the cationic nature of chitosan, as well as other non-viral, polymeric vectors, facilitates binding of nucleic acids, the positive charge attracts anionic serum proteins. These proteins tag the carrier system for recognition by the reticuloendothelial system (RES) and lead to elimination from systemic circulation. This is a major problem, and must be addressed when designing drug carrier systems. Hydrophilic polymers such as poly (ethylene glycol) (PEG) are often introduced to avoid RES uptake through the formation a hydration layer that reduces extracellular interactions [135-137].

Despite wide usage in drug delivery applications, an increasing number of reports are suggesting that PEG, although widely regarded as non-immunogenic, is actually quite capable of inducing an immune response in both animal models and human patients particularly when administered repeatedly. For example, Semple et. al observed generation of PEG-reactive plasma immunoglobulin M (IgM) after dosing with PEG-liposomes in mice, as well as rapid clearance of the material from the animals circulation [138]. A recent study by Hamad and coworkers report concentration and molecular weight dependent activation of the complement system by PEG[139]. Complement activation is a cascade of proteins and signaling molecules that function in addition to antibodies to clear pathogenic material from the body. When combined with the antibody response, the complement system has potential substantially increase the immune response, especially in individuals who present PEG sensitivity. A recent study regarding the use of PEGylated liposomes for gene delivery showed although PEG modified carriers resulted in increased circulation time initially, after administration of a second dose, the ABC phenomenon was readily observed [140]. Therefore, it is suggested that the accelerated blood clearance (ABC) mechanism often seen in studies involving PEG and PEGylated materials can be attributed to an increased immune response[141].

In addition to potential immunogenicity, it should be noted that PEG is a synthetic material, and therefore does not always exhibit good biodegradability. To compensate for a lack of biodegradability, low molecule weight polymers are desired for use, as they may exhibit better urinary and/or hepatic clearance. However, low molecular weight PEG (<400 Da) is able to be metabolized by alcohol and aldehyde dehydrogenase, resulting in toxic by-products including diacid and hydroxyacid, which have been shown to lead to acidosis in human and animal studies[142].

Due to the lack of degradability and potential immunogenicity of PEG, in this study we use polysialic acid as an alternative [143, 144]. Preparation, drug loading, and characterization of polysialic acid-N-trimethyl chitosan (PSA-TMC) nanoparticles will be described in detail in chapter four.

3.4. Goals and hypotheses

Based on the positive surface charge of the nanoparticles, nucleic acid oligonucleotides are hypothesized to have the ability to be incorporated with the nanoparticles for *in vitro* delivery. In addition to the ability to load negatively charged nucleic acids, the PSA-TMC delivery is advantageous over other polymeric drug delivery systems for applications in RA and CF treatment. These diseases are both characterized by inflammation and/or infection, as well as an overactive immune system. Therefore, a delivery system with a lack of cytotoxicity and immunogenicity is essential. Polysaccharides, such as PSA and chitosan are non-cytotoxic, non-immunogenic, and can be degraded using mechanisms already in place in the human body, giving them several advantages over synthetic materials [6, 49]. The major hypotheses that have been investigated in this project are as follows:

1. PSA-TMC nanoparticles are a non-immunogenic, biodegradable polysaccharide delivery system and can load oligonucleotides in a more safe and effective manner than existing transfection reagents.

2. Using PSA-TMC to delivery an NF-κB decoy oligonucleotide will modulate the immune response and reduce inflammation in in vitro models of cystic fibrosis and rheumatoid arthritis, as listed below:

i. IB3-1 epithelial cell cystic fibrosis

ii. SW982 rheumatoid arthritis

iii. Primary synoviocyte rheumatoid arthritis

iv. IB3-1/PA01 epithelial cell/bacterial co-culture

These hypotheses were tested via a variety of characterization and *in vitro* cell culture experiments, as described in the following chapters.

4. Synthesis and characterization of PSA-TMC nanoparticles as a delivery system for ODN

The polysaccharide based nanoparticle system PSA-TMC was initially developed in the Bader lab with the intention of loading DMARDs for the treatment of rheumatoid arthritis. In addition to showing DMARD loading capability, the particles proved to be non-cytotoxic, with components known to be biodegradable and non-immunogenic, resulting in a carrier system suitable for drug delivery applications in RA. The Bader lab has previously shown PSA-TMC loaded with DMARDs such as methotrexate and dexamethasone resulted in decreased inflammation in an RA *in vitro* model [145]. A brief collaboration resulted in the realization that aptamers, small nucleic acid sequences, could be effectively loaded into the particle matrix as well. This realization, combined with P.R.Wardwells weird fascination with NF-κB, led to the inception of the idea to use PSA-TMC nanoparticles loaded with transcription factor decoys against NF-κB in an attempt to modulate the immune response in diseases associated with inflammation. This chapter describes merits of PSA and TMC as nanocarrier components, as well as synthesis and characterization methods associated with production of the nanoparticles.

4.1 Introduction

To improve upon viral and cationic lipid transfection vectors, a number of investigators have begun to explore alternative carrier systems, including microspheres and nanoparticles, for delivery of nucleic acids [4, 130, 146-148]. These systems are often viewed as being less efficient in terms of nucleic acid cellular penetration, but this potential concern becomes secondary when considered in conjunction with the ease of production, high loading capacity, and a lack of immune response [133, 134].

Polysaccharides have emerged in recent years as highly desirable materials for incorporation into drug delivery systems. Characteristics including structural diversity, a high potential for modification due to many reactive groups, general aqueous solubility, as well as (in some cases) innate bioactivity contribute to the interest in polysaccharides in drug delivery systems. Polysialic acid (PSA) is a hydrophilic natural polymer which, similar to polyethylene glycol (PEG), facilitates formation of a water layer around colloidal particles. However, as discussed in chapter three, PEG has some serious drawbacks which make it a questionable choice for inclusion in drug carrier systems for diseases characterized by excessive inflammation. Unlike

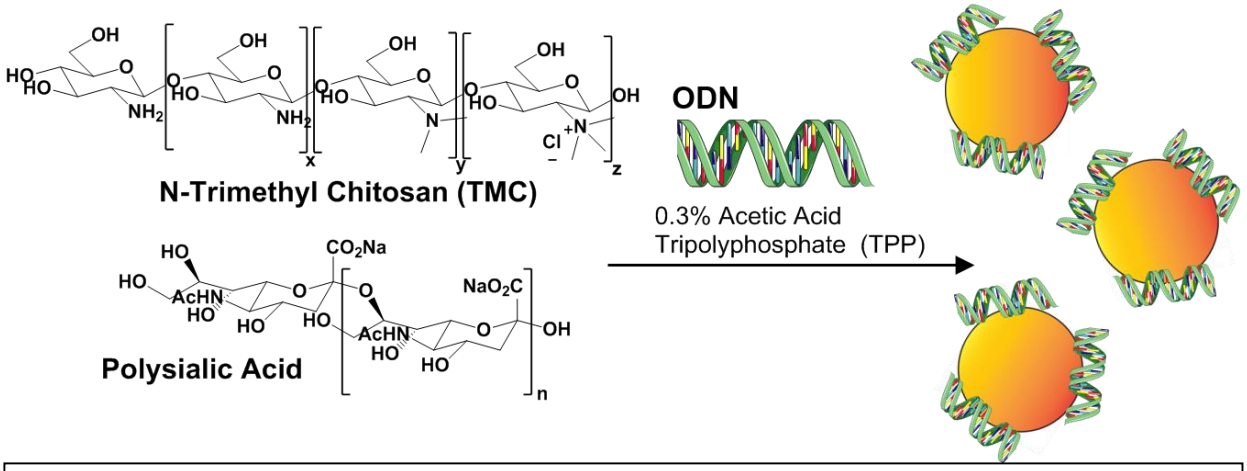

Fig. 13: Synthesis scheme of ionic complexation of PSA-TMC nanoparticles coated with NF-κB Decoy ODN. TMC is positively charged, PSA is negatively charged, and the two polymers interact in the presence of a polyanionic cross-linker via ionic gelation to form nanoparticles.

PEG, however, PSA has no known receptors in the human body, contributing to a lack of immunogenicity and toxicity, two factors of key importance in drug delivery applications aimed at immunomodulation. PSA was first described for drug delivery by Gregoradis as a means of enhancing circulatory stability and half-life of therapeutics through conjugation [137]. The Bader lab recently reported nanoparticles formed by ionic complexation of PSA and N-trimethyl

chitosan (TMC) with a small size (~100 nm), positive surface charge, and a low degree of cytotoxicity[149].

Chitosan has been a material of interest for nucleic acid delivery for some time now. The biocompatibility, lack of toxicity, relatively low cost and general cationic, easily modifiable nature make chitosan an attractive choice for gene delivery applications [150]. However, clinical success of initial chitosan/DNA based polyplexes was limited by low transfection efficiencies, and the high dependence of environmental factors (especially pH) on biological function [151]. These nucleic acid delivery systems are often associated with aggregation, and rapid clearance. Recently, many researchers have reported modifications of chitosan with polymers such as PEG or PEI, generating graft-copolymers for nucleic acid nanocarrier systems with the goal of increasing stability and transfection efficiency [151-154]. While moderate success was seen with these formulations, the use of potentially cytotoxic materials makes these alternatives an undesirable choice for applications aimed at treating excessive inflammation.

An alternative to control the functionality of chitosan across pH ranges is to use TMC, a derivative of chitosan modified via methylation, to improve solubility and potentially transfection efficiency [155]. Incorporation of PSA, a negatively charged, highly hydrophilic material, yields particles with a smaller size and greater stability, improving upon particles made of chitosan or TMC and DNA alone. Zhang et al. recently reported that ionic complexation of PSA with TMC yields a non-cytotoxic nanoparticle carrier system with a size (~100 nm) and positive surface charge. A synthesis scheme depicting formation of particles via ionic complexation of PSA and TMC and subsequent ODN coating is shown in Fig. 13. Although this system was initially developed with the aim of improving RA treatment, the size, stability, and positive surface charge are amenable to ODN delivery [156]. PSA serves as a natural polymer for enhancing the

circulatory stability and facilitating passive accumulation of associated therapeutics within diseased tissue that is characterized by leaky vasculature, as occurs with RA. In the physiological environment, PSA reduces undesirable protein and cell interactions through the formation of a protective envelope of water molecules [135-137]. PSA thus provides a biodegradable, non-immunogenic alternative to synthetic PEG.

Although Gregoriadis et al. pioneered using PSA conjugation as a method of enhancing the circulatory stability of associated therapeutics [135-137], our group is the first to use PSA as the basis of nanocarrier systems [7, 156-158]. Relative to other anionic polysaccharides, electrostatic interaction of the positively charged ammonium groups of TMC with the negatively charged carboxylic acid groups along the highly flexible PSA backbone yields intermolecular complexes of a small size [159-161]. The cationic, quaternized chitosan also facilitates adhesion of negatively charged nucleic acids [162]. The PSA-TMC nanoparticles, therefore, have the potential to serve as ideal, biodegradable, non-immunogenic carrier systems for nucleic acid-based therapeutics.

4.2 Materials and methods

4.2.1 Materials

Polysialic acid (colominic acid, PSA) was obtained from Nacalai USA, Inc. (San Diego, CA, USA). N-trimethyl chitosan (TMC) was produced via quaternization of chitosan (MW 100 Da-300 Da) obtained from Acros Organics (New Jersey, USA), as described previously by Sieval et. Al[163]. Sodium tripolyphosphate (TPP) was purchased from Acros Organics, New Jersey, USA. An NF-κB decoy oligonucleotide (ODN) kit containing NF-κB decoy ODN (5’ CCT TGA AGG GAT TCC CTT CC 3’) and a scrambled ODN (5’ TTG CCG TAC CTG ACT TAG CC 3’) was purchased from CosmoBio (Tokyo, Japan). Methotrexate (MTX) was obtained from Enzo Life Sciences (New York, USA)

4.2.2 PSA-TMC-ODN preparation

ODN coated PSA-TMC nanoparticles were prepared as previously described [145]. Briefly, 6.4 mg of TMC (55% quaternization) were dissolved in 3.0 ml of 0.3% acetic acid in a glass vial. Meanwhile, 3.2 mg of PSA and 1.0 mg of TPP were dissolved in 2.0 ml of DI H_2O. When MTX was incorporated into the particles, 2.4 mg MTX was added to the PSA/TPP solution. The latter solution was sonicated for 10 minutes and then added drop-wise to the TMC solution with stirring. Stirring was continued at room temperature for 20 minutes. At this time, 10 µg ODN were added to the nanoparticle suspension. Stirring was continued for an additional 10 minutes to ensure uniform electrostatic adhesion of ODN to the nanoparticle surface, as well as complete dispersion. Upon completion of stirring, centrifugation at 3000 RPM for 15 minutes yielded a pellet of ODN-coated PSA-TMC nanoparticles.

4.2.3 PSA-TMC-ODN characterization

Nanoparticle size, zeta potential, and polydispersity index were determined using a Malvern Zetasizer NanoZS90 (Malvern Instruments, Malvern UK). Following particle formation and centrifugation, nanoparticles were resuspended at a concentration of 2 mg/ml in DI water and filtered through a 0.45 µM syringe filter. Samples were loaded into cuvettes or capillary cells for measurements of size or zeta potential, respectively, taken at 25 °C.

4.2.4 Determination of ODN and MTX loading

A rhodamine tagged ODN (5’ (TAMRA-X) (C6-NH) CCT TGA AGG GAT TTC CCT CC 3’) was used to assess ODN loading. ODN-coated PSA-TMC nanoparticles were prepared; and, after centrifugation, the supernatant was collected for quantitative analysis of unbound ODN. Supernatant fluorescence values were determined using a synergy 2 multimode plate reader (BioTek Instruments, Winooski VT). Total amount of ODN in the supernatant was

determined from the supernatant fluorescence readings compared to a standard curve of fluorescent ODN, and the following equations were used to determine loading efficiency (LE) and loading capacity (LC):

$$LE = \left(\frac{M_{ODN\ added} - M_{ODN\ supernatant}}{M_{ODN\ added}}\right) x\ 100 \quad (1)$$

$$LC = \frac{M_{ODN\ added} - M_{ODN\ supernatant}}{M_{nanoparticles}} \quad (2)$$

where M_{ODN} added is the mass of ODN added to the initial nanoparticle formulation, $M_{supernatant}$ is the mass of ODN in the supernatant, as determined by fluorescence analysis, and $M_{nanoparticles}$ is the mass of the nanoparticles used for ODN loading.

HPLC was used to determine the amount of MTX loaded into ODN coated nanoparticles. After nanoparticles were pelleted via centrifugation, supernatant samples were saved and analyzed using a Prominence Ultrafast Liquid Chromatography System (UFLC, Shimadzu Instruments, Japan). Samples were run using a 93:7 (v/v) mixture of 50 mM ammonium acetate and acetonitrile mobile phase at a flow rate of 0.75 ml/min with a 100 µL injection volume. The detection wavelength used was 210 nm. To determine the amount of MTX present based on peak area, a calibration curve of 8 known concentrations (50 µg/ml to 0.39 µg/ml) of MTX was constructed. PeakFit 4.2 software was used to analyze peak area.

4.2.5 Analysis of protective effect of PSA-TMC on ODN

PSA-TMC and DNA degradation was assessed using an adapted protocol from Mao et. al. 10 mg PSA-TMC-ODN (equivalent of 1 µg ODN/mg PSA-TMC, designated NPODN) was prepared with ODN coated on the surface, and encapsulated ($NPODN_E$). Bare PSA-TMC (designated NP) was also prepared. After centrifugation, all particles were resuspended in 0.5 ml Millipore H_20 at a concentration of 2 mg PSA/TMC and 2 µg ODN per 100 µl. 3 100 µl aliquots

were prepared from each 500 µl nanoparticle suspensions. ODN, NP, NPODN, and $NPODN_E$ were incubated at 37 degrees C for 15 minutes with 0.0, 0.4 or 4.0 µg DNAse 1 for 15 minutes. The reaction was stopped by adding 100 µl of 10 mM iodoacetic acid for a final iodoacetic acid concentration of 5 mM. Digestion was then carried out with lysozyme and chitosanase. Lysozyme was added at a concentration of 40 U/100 µl, and chitosanase was added at a concentration of 0.1 U/100 µl to all samples digested previously with DNAse1. The control samples were not degraded with lysozyme or chitosanase. Digestion continued for 4 hours at 37 °C. After 4 hours of enzymatic digestion, samples were removed from heat and placed on ice immediately to stop activity. Samples were either frozen or run on a 20% Polyacrylamide gel. All gels were run at 200 mV for 40-50 minutes. The fastest running band on the loading buffer is said to be equivalent of about a 20 bp DNA sequence.

4.3 Results and discussion

4.3.1 Nanoparticle characterization

As expected based on these prior studies, NP possessed a size of close to 100 nm (115 nm) and a positive zeta potential (37 mV), while NP-ODN nanoparticles possessed a significantly larger size with a diameter of 159 ± 15 nm and decrease in surface charge to 23 mV [145, 152]. Furthermore, NP-ODN loaded with MTX led to another slight size increase, insignificantly larger than NP-ODN alone, with a diameter of 184 ± 5.6 nm while maintaining a

Table 2: Characterization data associated with PSA-TMC NP formulations

	Size (nm)	Zeta Potential (mV)	Polydispersity index
NP	115 ± 5.6	37 ± 6.3	0.10
NP-ODN	166 ± 4	23 ± 6.5	0.09
NP-ODN-MTX	184 ± 5	33 ± 6.5	0.09

positive zeta potential of approximately 33 ± 6.5 mV. All nanoparticle formulations possessed size between 100 and 200 nm, favorable for evading the reticulondothelial system (RES) in applications for drug delivery [156]. Size, zeta potential, and polydispersity index of the three different nanoparticle formulations are portrayed in table 4.1.

Systematic analysis of nanoparticle formation via ionic complexation has been investigated previously [164, 165]. Consensus of these studies suggest that initiation of particle formation as well as particle size is highly influenced by charge ratio. A charge ratio of approximately 1:1 (+ :-) is essential for initiation of particle formation, regardless of charge strength. Dragan et. al illustrates this charge dependent phenomenon by varying charge ratios and measuring suspension turbidity. At a ratio of 1:1, a shift in turbidity occurs, indicating particle formation [164]. The PSA-TMC nanoparticle generation described above contains polymer amounts with the exact charge ratios recommended for complex formation. Chitosan to TMC modification results in polymer with approximately 55% modification, or a 55% positive charge. Using a TMC:PSA ratio of 2:1 results in a charge ratio of approximately 1:1. The ideal charge ratio combined with the flexibility of PSA, as described in section 4.1 results in generation of stable, reproducible particles with sizes ranging from ~100 nm (unloaded) to ~200 nm (loaded with MTX and ODN).

4.3.2 ODN and MTX loading

The PSA-TMC nanoparticles were successfully coated with ODN with a loading efficiency and loading capacity of 76.6 ± 8.6% and 0.77 ± 0.09 μg/mg nanoparticles, respectively. HPLC was performed to determine the amount of MTX loaded within the NP-ODN-MTX nanoparticles. Loading capacity and loading efficiency values of .20 mg MTX/mg nanoparticle and 86.7 %, respectively were obtained.

4.3.3 Protective effect of PSA-TMC on ODN

Enzymatic degradation of ODN coated PSA-TMC, ODN encapsulated PSA-TMC, and TMC alone was performed. Based on enzymes present *in vivo*, DNAse1, lysozyme, and chitosanase enzymes were used to test stability. Despite several attempts, and a very high gel percentage, DNA fragments were not detectable. In addition, different post-run stains, including silver stain, methylene blue, and ethidium bromide were used in an effort to detect bands of DNA. A summary of different staining methods can be found in table 4-2.

Table 3: A summary of gel staining attempts to visualize enzymatically digested ODN, and the results

Attempt, 20% Gel	Stain	Result
1	Silver Quest (silver stain)	No band visualization, not even the ladder. This was not an appropriate stain to use.
2	Methylene Blue	Ladder visualization, no bands from any of the samples
3	Ethidium Bromide	No ladder visualization, saw bands from the ODN samples after 15 minute stain incubation, but after 2.5 hour stain incubation, saw nothing.

Potential alternatives to detecting DNA post-degradation include PCR amplification prior to running the gel. However, without knowing the exact degradation pattern, this would require multiple primers, and in general turn into a larger scale study than originally anticipated. This will be a continued investigation, as it provides an excellent introduction to continuing the project for a potential new student.

4.4 Conclusions

Initial preparation of ODN loaded PSA-TMC was performed by incorporating the nucleic acids directly into the polymer network. While this resulted in nanoparticles of desired size and

zeta potentials, the addition of negatively charged materials to the polymer matrix appears to have increased the particle stability beyond what was desired. Therefore, the nucleic acid release from the nanoparticles was extremely slow, and not conducive to the applications being investigated here. To rectify this, nucleic acids were instead coated on the surface of the nanoparticle, as the positively charged particle surface will attract and adhere the ODN's due to electrostatic interactions. As expected, coating the surface with ODN led to acceptable loading levels, with sufficient payload release.

PSA-TMC nanoparticles showed high loading capability of MTX, as expected based on previous studies, and ODN. Upon determination of successful synthesis and loading of PSA-TMC nanoparticles, *in vitro* studies were carried out to determine potential applications of these materials. Previous studies as well as the innate properties of the materials used to develop the nanoparticles have confirmed a lack of toxicity toward rheumatoid arthritis cell line, however toxicity was investigated for other cell lines used in the studies. These cytotoxicity and efficacy *in vitro* studies are described extensively in the following chapters.

5. Modulation of the immune response via NF-kB decoy ODN coated nanoparticles in in vitro models of rheumatoid arthritis

Abstract

The transcription factor nuclear factor-kappa B (NF-κB) is highly involved in regulation of a number of cellular processes, including production of inflammatory mediators. Thus, this transcription factor plays a role in pathology of many diseases, including rheumatoid arthritis, an autoimmune disease hallmarked by an imbalance of pro and anti-inflammatory cytokines. Small nucleic acids with sequences that mimic the native binding site of NF-κB have been proposed as treatment options for RA; however due to low cellular penetration and a high degree of instability, clinical applications of these therapeutics have been limited. Here, we describe the use of N-trimethyl chitosan-polysialic acid (PSA-TMC) nanoparticles coated with NF-κB decoy ODNs (PSA-TMC-ODN) as a method to enhance the stability of the nucleic acids and facilitate increased cellular penetration. In addition to decoy ODN, PSA-TMC nanoparticles were loaded with RA therapeutic methotrexate (MTX), to assess the anti-inflammatory efficacy of a combination therapy approach. Two difference *in vitro* models, a cell line based model as well as a primary RA cell model were used to investigate anti-inflammatory activity. One way ANOVA followed by Holm-Sidak stepdown comparisons was used to determine statistical significance. In general, free ODN did not significantly affect secretion of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-8, (IL-8) while free MTX had variable efficacy. However, PSA-TMC-ODN and PSA-TMC-ODN-MTX resulted in significant decreases in the

inflammatory mediators IL-6 and IL-8 in both cell models. In addition, PSA-TMC exhibited sufficient cellular uptake, as observed through fluorescence microscopy.

These results support our previous findings that PSA-TMC nanoparticles are an effective delivery vehicle for small nucleic acids, and effectively alter the pro-inflammatory state characteristic of RA.

*This chapter has been adapted from an original manuscript by P.R. Wardwell submitted to the journal Arthritis Research and Therapy

5.1 Introduction

Rheumatoid arthritis is an autoimmune disease characterized by inflammation of the synovial tissue of joints. Over time, the infiltration of immune cells to the synovial lining leads to hyperplasia, increased vascular growth, and formation of a tumor like tissue known as the pannus [166]. The physiology of a chronic inflammatory state eventually results in cartilage degradation and bone resorption. An imbalance of pro- and anti-inflammatory cytokines contribute to the state of chronic inflammation. Briefly, the levels of anti-inflammatory cytokines (Interleukin (IL)-4, IL-10, and IL-13) present in the synovium are too low to combat the effects of pro-inflammatory cytokines (tissue necrosis factor-α (TNF-α), IL-1, IL-6, and IL-8) [124]. Of the cells present in the RA synovial lining, ‘macrophage-like’ cells and activated synovial fibroblasts are accepted as the primary mediators of the pro/anti-inflammatory imbalance [167, 168].

NF-κB is a transcription factor involved in the regulation of a variety of cellular processes, including growth, apoptosis, and inflammatory and immune responses [108]. NF-κB dependent gene expression is known to play a critical role in the observed cytokine imbalance, as well as contribute to increased inflammation in rheumatoid arthritis [110, 169]. Under normal conditions, NF-κB is sequestered in the cytoplasm by means of a bound inhibitor known as IkB (inhibitor of κB). External stimulation from inflammatory mediators, including IL-1β, leads to a signaling cascade that results in phosphorylation of the inhibitor, followed by dissociation of the NF-κB/IκB complex and subsequent nuclear translocation of NF-kB. Once inside the nucleus, NF-kB initiates transcription of pro-inflammatory cytokines, including IL-6 and IL-8, two cytokines highly involved in regulating inflammation in RA. IL-6 and IL-8 both possess NF-κB binding sites on their promotor regions, indicating they are highly regulated by NF-κB [170]. Transcription factor decoy oligonucleotides (ODNs) have the

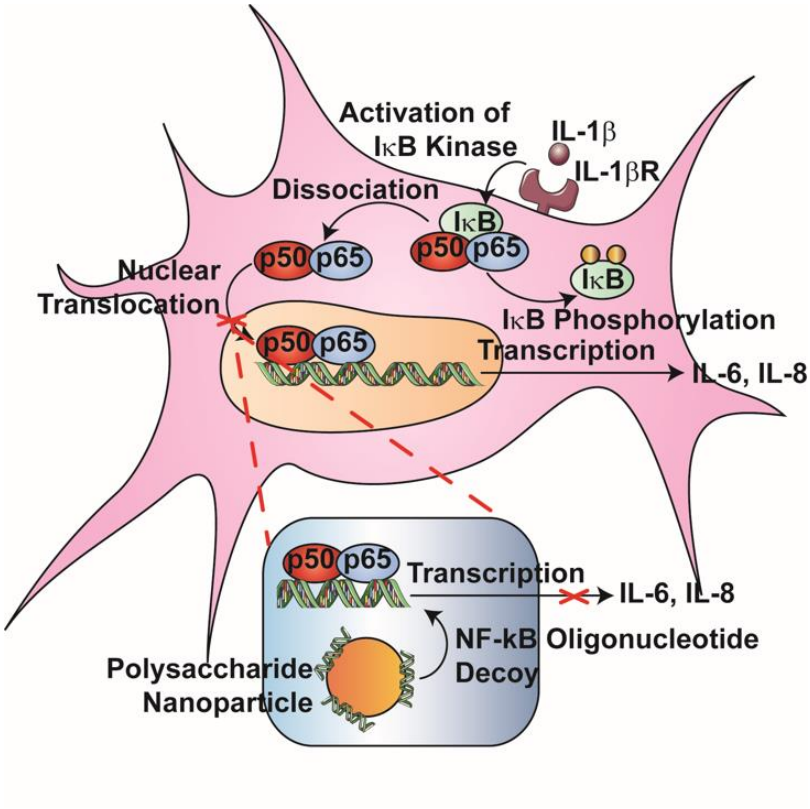

Fig. 14. An illustration of the NF-κβ pathway activation in a RA synovial fibroblast cell. Pro-inflammatory cytokines, including IL-1β activate the cell signaling pathway associated with NF-κβ, including activation of IκK, phosphorylation and inactivation of Iκβ, and translocation of NF-κβ to the nucleus. NF-κβ decoy ODN's can prevent translocation of the transcription factor, as well as subsequent transcription of NF-κβ dependent genes.

potential to reduce inflammation in RA by binding to NF-kB in the cytoplasm, preventing nuclear translocation, and inhibiting NF-κB mediated transcription of pro-inflammatory proteins. The mechanism of decoy ODN is illustrated in Fig. 14.

Transcription factor decoy ODNs mimic the native DNA binding site of the transcription factor, but are only ~ 20 base pairs long, and do not encode any genes. NF-kB decoy ODNs have been proposed as treatment for RA previously [171]. However, despite promising potential for treatment, applications have been limited by low cellular penetration and a lack of stability of the nucleotides, which combined result in low overall bioavailability [172, 173]. As mentioned in the introduction section, methyl phosphonate or phosphorothioate chemical modifications are often applied to the nucleotide backbones as a means to overcome stability problems; however although these modifications enhance stability, they do not necessarily lead to increased delivery efficiency, resulting in the need for a high dose and frequently repeated delivery [2]. Nucleic acids containing these modifications have been shown to have a concentration dependent toxicity toward mammalian cells, therefore this is not a sustainable method for delivery[3, 4].

As discussed in chapter two, methods such as viral vectors, cationic lipid formulations and more recently, cationic polymer formulations, exist to overcome the barriers to nucleic acid delivery. To reiterate nucleic acid drug delivery system requirements as initially stated in chapter one, drug delivery systems for nucleic acids must have attributes including biocompatibility and biodegradability, reticuloendothelial system (RES) avoidance, non-immunogenicity, cellular uptake capability, and cell or tissue specificity [5]. In terms of these attributes, viral vectors are associated with major drawbacks, including viral induced immunogenicity, toxicity, mutation of the nucleic acid of interest with the viral DNA, and the

potential for inactivation of the gene of interest due to recombination [97]. Cationic lipid based Lipofectamine reagents, marketed by Life Technologies®, claim of increased efficiency over other available reagents and have become the most referenced lipid based transfection reagent. However, in addition to marked toxicity exhibited by these reagents in cell lines, these compounds are associated with metabolism by the liver *in vivo*, resulting in degradation of the nucleic acid payload [104, 105].

Due to the drawbacks of viral vectors and cationic lipid delivery methods, cationic polymers have been the focus of recent research as an alternative for nucleic acid delivery. One of the most widely used polymers in nucleic acid delivery is poly (ethyleneimine) (PEI). However, PEI exhibits considerable toxicity toward a variety of mammalian cells [106] [107]. Delivery systems for RA, where the ultimate goal is to reduce inflammation, require materials that do not contribute to inflammation or the immune response and that exhibit low levels of toxicity. Therefore, an alternative delivery method to viral vectors, cationic lipids and synthetic cationic polymers is needed.

A polysaccharide based system containing two natural polymers, N-trimethyl chitosan (TMC) and polysialic acid (PSA) has previously been described. This nanoparticle system is non-cytotoxic, non-immunogenic, and has been shown to effectively deliver encapsulated disease modifying anti-rheumatic drugs (DMARDs) and surface-coated with NF-kB decoy ODNs when applied to *in vitro* models of rheumatoid arthritis and cystic fibrosis, respectively [145, 174]. In this chapter, the use of PSA-TMC nanoparticles as a delivery system to combine treatment of a DMARD, methotrexate, and NF-κB decoy ODN is reported. While combination therapies have been reported before, particularly NF-κB inhibitors, to the best of the author's

knowledge, this is the first report of combining DMARD methotrexate with a transcription factor decoy NF-κB inhibitor.

5.2 Materials and Methods

5.2.1 Materials

Polysialic acid (colominic acid, PSA) was obtained from Nacalai USA, Inc. (San Diego, CA, USA). N-trimethyl chitosan (TMC) was produced via quaternization of chitosan (MW 100 Da-300 Da) obtained from Acros Organics (New Jersey, USA), as described previously by Sieval et. Al[163]. Sodium tripolyphosphate (TPP) was purchased from Acros Organics, New Jersey, USA. An NF-κB decoy oligonucleotide (ODN) kit containing NF-κB decoy ODN (5' CCT TGA AGG GAT TCC CTT CC 3') and a scrambled ODN (5' TTG CCG TAC CTG ACT TAG CC 3') was purchased from CosmoBio (Tokyo, Japan). Recombinant human interleukin-1β was obtained from R and D systems (Minneapolis, MN). Methotrexate (MTX) was purchased from Enzo Life Sciences (Rochester, NY). Alexa Fluor 488 succinimidyl ester was purchased from Invitrogen/Life Technologies (Grand Island, NY).

5.2.2 Cell culture

SW982 cells were obtained from ATCC (Manassas, VA, USA) and grown in Dulbecco's Modified Eagles Medium (DMEM, Fisher Scientific, USA) supplemented with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, Atlanta, GA) until confluent. Primary RA cells were isolated from synovial tissue obtained from two Caucasian RA patients, both women between the ages of 50 and 59. The tissue samples were obtained by Dr. Timothy Damron at Community General Hospital (Syracuse, NY) following written and informed consent by each patient, as required by an Institutional Review Board–approved protocol. Tissue was isolated following a protocol outlined by Zimmerman [175]. Briefly, the synovial tissue was minced finely and

incubated at 37° with 0.1% Trypsin (Invitrogen, Carlsbad, CA) in PBS for 30 minutes. Tissue was then digested for two hours in DMEM with 0.1% Collagenase P. After digestion, tissue was filtered through a 100 µM filter. The resultant solution was centrifuged, and the pelleted cells were resuspended in DMEM with 10% FBS, placed in a T-75 flask, and cultured at 37°C with 5% CO_2. After three passages, the cells were stained with CD44-FITCmAB (Santa Cruz Biotechnology, Santa Cruz CA) to confirm fibroblast cells. To supplement the two sets of primary cells obtained through synovial tissue isolation, Human Fibroblast Like Synoviocytes (HFLS, lot numbers 2884 and 2956, female Caucasian) were obtained from Cell Applications (CA, USA) and cultured in DMEM with 10% FBS at 37°C with 5% CO_2.

5.2.3 Nanoparticle preparation and characterization

ODN coated PSA-TMC nanoparticles were prepared as previously described and detailed in chapter four [145]. Nanoparticle size, zeta potential, and polydispersity index were determined using a Malvern Zetasizer NanoZS90 (Malvern Instruments, Malvern UK). Following centrifugation, nanoparticles were resuspended at a concentration of 2 mg/ml in DI water and filtered through a 0.45 µM syringe filter. Samples were loaded into cuvettes or capillary cells for measurements at 25 °C.

5.2.4 *In vitro* efficacy of ODN-coated PSA-TMC nanoparticles

SW982 cells or primary RASF cells were plated on 24 well plates at a density of 20,000 cells/well. In addition to ODN coated nanoparticles (NP-ODN), bare nanoparticles (NP), MTX loaded nanoparticles (NP-MTX), ODN coated MTX loaded nanoparticles (NP-MTX-ODN) and nanoparticles coated with a scrambled oligonucleotide (NP-SCO) were prepared. As a control, MTX alone was prepared at a concentration of 1.0 mg/ml DMEM media. After centrifugation, all nanoparticles were resuspended in serum free DMEM at a concentration of 1.0 mg/ml. 500 µl

of each treatment group was added to the 24 well plate in duplicate as follows: 1. Media alone (control), 2. ODN alone, 3. NP-ODN, 4. NP, 5. NP-SCO, 6. NP-MTX, 7. NP-ODN-MTX, and 8. MTX alone. The complexes were removed and media supplemented with FBS was replaced after 4 hours to allow for normal growth conditions. 24 hours after initial complex addition, inflammation was induced with the addition of 1.0 ng/ml of interleukin 1β (IL-1β). This concentration has been shown to increase levels of IL-6 and IL-8 when administered to SW982 cells [176].After incubation at 37°C for an additional 24 or 48 hours,, supernatant samples were collected and stored at -80°C for analysis of IL-6 and IL-8.

5.2.5 Quantitative analysis of inflammatory cytokines

ELISA kits for IL-6 and IL-8 were purchased from Peprotech (Rocky Hill, NJ) and run according for manufacturer instructions. Samples were run in duplicate, and each experiment was repeated independently at least 3 times.

5.2.6 *In vitro* cellular uptake

To examine internalization of the nanoparticles, cellular uptake experiments were performed. Prior to nanoparticle synthesis, TMC was tagged with Alexa-Fluor 488® carboxylic acid, succinimidyl ester, mixed isomers in DMSO (1 mg/mL), (Invitrogen, Grand Island, NY). 25 mg of TMC were dissolved in 4 ml of 0.1 M sodium bicarbonate buffer (pH 8.3). 500 µl of AF 488 dye were added, and the solution was stirred for 1 hour at room temperature. Upon completion of stirring, the resultant material was dialyzed for 48 hours against water to ensure removal of un-reacted dye. In addition, the amount of TMC used has an excess of reactive amine groups relative to amount of Alexa Fluor 488, therefore the amount of unreacted dye was expected to be negligible. H^1 NMR confirmed dye conjugation. Cells were plated on lysine coated glass bottom dishes (Mattek Corp, Ashland, MA) at a density of 100,000 cells per dish two days prior to

scheduled imaging to allow for adherence and confluence. On the day of imaging, sterile filtered NP were administered to the plated cells at a concentration of 1 mg/ml. Complexes were incubated with the cells for 45 minutes at 37°C prior to removal. The cells were washed three times with 1X PBS immediately prior to imaging, and imaged using a Nikon Eclipse Ti inverted microscope.

5.2.7 Statistical analysis

IL-6 and IL-8 protein levels were expressed relative to an untreated, stimulated control group, with all data presented as mean ± standard deviation for all groups ($N \geq 3$). One-way ANOVA followed by Holm-Sidak testing for multiple comparisons was performed to compare IL-6 and IL-8 protein secretion following treatment and inflammatory stimulation. All statistical tests were conducted with an alpha value of 0.05.

5.3 Results

5.3.1 Effect of ODN and MTX loaded NP on IL-6 and IL-8 secretion in RA in vitro models

To initially determine efficacy of NF-κB decoy ODN and MTX loaded NP, the SW982 cell line was used as a model of RA. The SW982 cell line has been shown to mimic activated RA synovial fibroblast cells in regards to the expression of inflammatory mediators, particularly when stimulated in 1 ng/ml IL-1β[177]. We have previously conducted cytotoxicity studies of PSA-TMC NP formulations and concluded NP, as well as NP-ODN, NP-MTX, and MTX alone do not impact cellular proliferation at low concentrations and are, therefore, appropriate for this study [145, 174].

To assess the bioactivity of PSA-TMC NP coated with the NF-κB decoy ODN and/or loaded with MTX, the secretion of two potent inflammatory mediators, IL-6 and IL-8, by SW982 cells were investigated. Both of these pro-inflammatory mediators are directly influenced by NF-κB

and play a major role in the inflammatory response in RA. IL-6 is a multifunctional cytokine, with the ability to regulate the immune response, inflammation, and hematopoiesis and plays a crucial role in RA pathogenesis[178]. IL-8 was chosen as a representative chemokine and is responsible for recruiting immune cells to the synovium and contributing to the tumor-like pannus tissue. In addition, IL-8 is involved in up-regulation of inflammation via paracrine signaling mechanisms in the RA synovium [179]. The mechanism of action of MTX in RA treatment and inflammatory activity is currently unresolved; however, the drug is believed to interfere with cell folate metabolism. Furthermore, several reports suggest MTX acts on NF-κB as an inhibitor[180]. We explored co-administration of NF-κB decoy ODN and MTX to observe any potential synergistic activity. IL-6 and IL-8 levels were examined in response to treatment with NP-ODN, NP-MTX, NP-ODN-MTX, ODN alone, and MTX alone using immunoassays.

The IL-6 secretion profile in response to different treatment groups is shown in Fig. 15 In general, we were interested in the IL-6 response of cells subjected to the different treatment

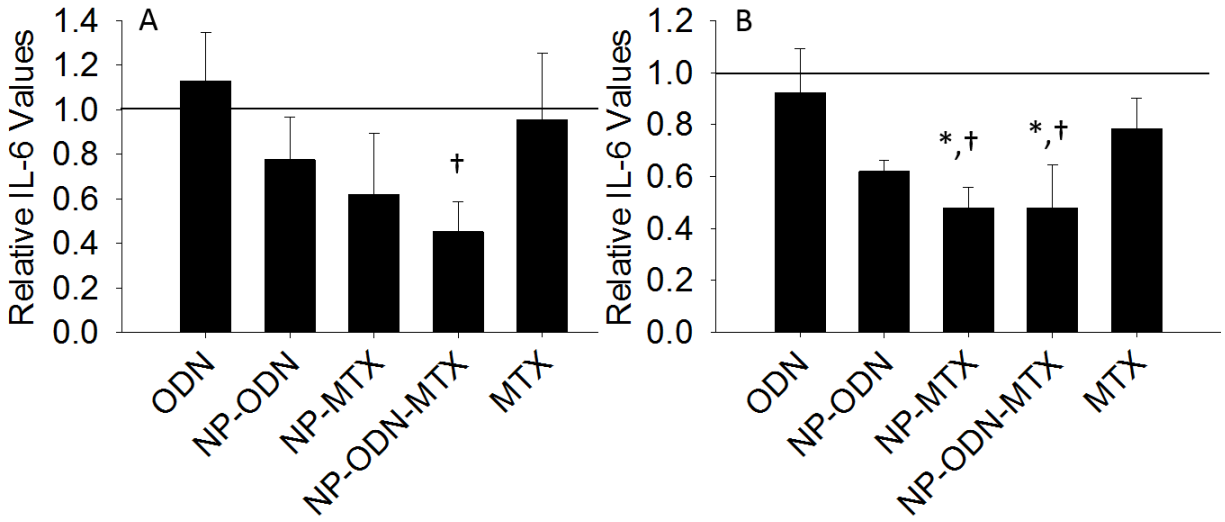

Fig. 15. ELISA was performed to determine levels of IL-6 secretion by SW982 cell line RA model cells, after treatment with ODN alone (ODN), NP coated with ODN (NP-ODN), NP loaded with methotrexate (NP-MTX), NP loaded with methotrexate and ODN (NP-ODN-MTX), and MTX alone (MTX) and stimulation with IL-1B at 24 (A) and 48 (B) hours. Results are expressed as fold changes of IL-6 levels relative to an untreated control, shown as a solid line at 1. One way ANOVA followed by Holm-Sidak multiple comparisons testing was used to determine the impact of treatment on IL-6 secretion. * Represents a significant difference from the control, while † represents significant difference from ODN alone. All data is presented as mean ± SD (N=3).

groups in comparison to untreated control cells and in comparison to cells treated with ODN alone. At 24 hours (Fig. 15A), cells treated with NP-ODN-MTX displayed a significant reduction of IL-6 relative to untreated control cells. A decrease in IL-6 levels in comparison to untreated control cells was also observed following treatment with NP-ODN; however, this reduction was not great enough to be significant. At 48 hours (Fig. 15B), a significant decrease relative to both untreated control cells and cells administered ODN alone was observed following treatment with NP-MTX and NP-ODN-MTX. NP-ODN displayed trends at 48 hours similar to those at 24. This treatment resulted in a decrease in IL-6 levels in comparison to both

untreated control cells and ODN alone; however, the decrease was not great enough to be significantly different.

The IL-8 secretion profile from SW982 cells in response to treatment with different nanoparticle formulations is portrayed in Fig. 16. After 24 hours (Fig. 16A), NP-ODN-MTX resulted in a significant decrease in IL-8 levels compared to ODN administered alone. While the

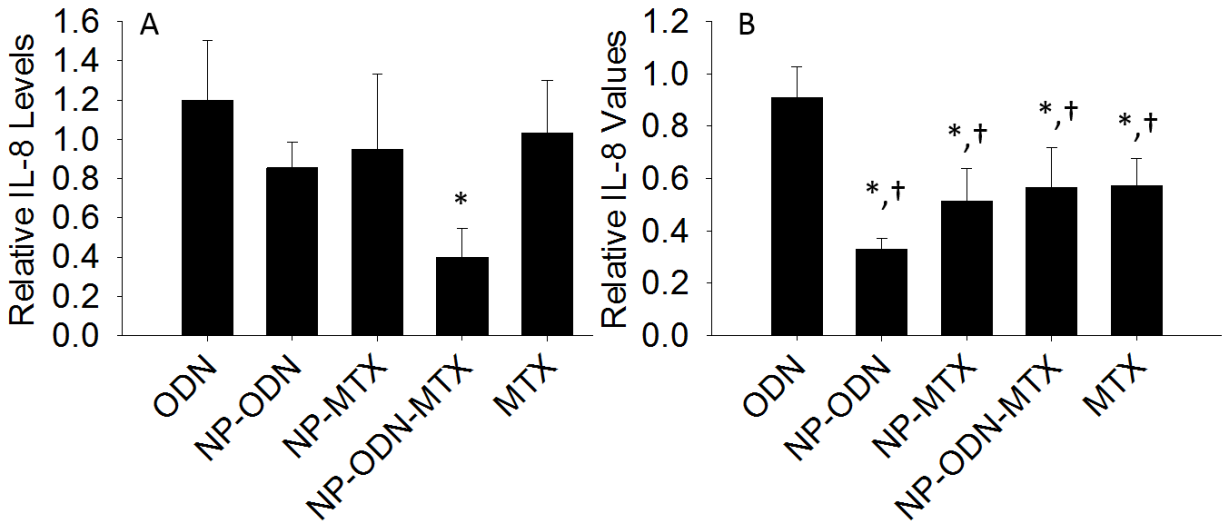

Fig. 16. ELISA was performed to determine levels of IL-8 secretion by SW982 cell line RA model cells, after treatment with ODN alone (ODN), NP coated with ODN (NP-ODN), NP loaded with methotrexate (NP-MTX), NP loaded with methotrexate and ODN (NP-ODN-MTX), and MTX alone (MTX) and stimulation with IL-1B at 24 (A) and 48 (B) hours. Results are expressed as fold changes of IL-8 levels relative to an untreated control, shown as a solid line at 1. One way ANOVA followed by Holm-Sidak multiple comparisons testing was used to determine the impact of treatment on IL-8 secretion. * represents a significant difference from the control, while † represents significant difference from ODN alone. All data is presented as mean ± SD (N=3).

level of IL-8 in response to NP-ODN-MTX treatment was lower than the untreated control, the difference was not significant. At 48 hours, (Fig. 16B) multiple significant decreases in IL-8 levels were observed. Cells treated with NP-ODN, NP-MTX, NP-ODN-MTX, and MTX alone all had IL-8 levels significantly lower than the untreated control cells and cells treated with ODN alone.

These results suggest that PSA-TMC NP can be used to deliver decoy ODN and MTX, alone and simultaneously, to activated RA synovial fibroblasts. To further validate the ability of PSA-TMC nanoparticles to serve as an effective treatment strategy for RA, *in vitro* experiments were also conducted with primary cells. Previous reports have noted discrepancies in cytokine production between cell line models and primary RASF cells [128]. Furthermore, a literature search revealed that immortalized cells, such as SW982, constitutively express the NF-κB pathway, indicating that they may be more susceptible to NF-κB interference than primary RASF cells [181].

Primary RASF cell cytokine secretion was investigated following treatment with ODN, NP-ODN, NP-MTX, NP-ODN-MTX and MTX. At 24 hours (Fig. 17A), a significant reduction in

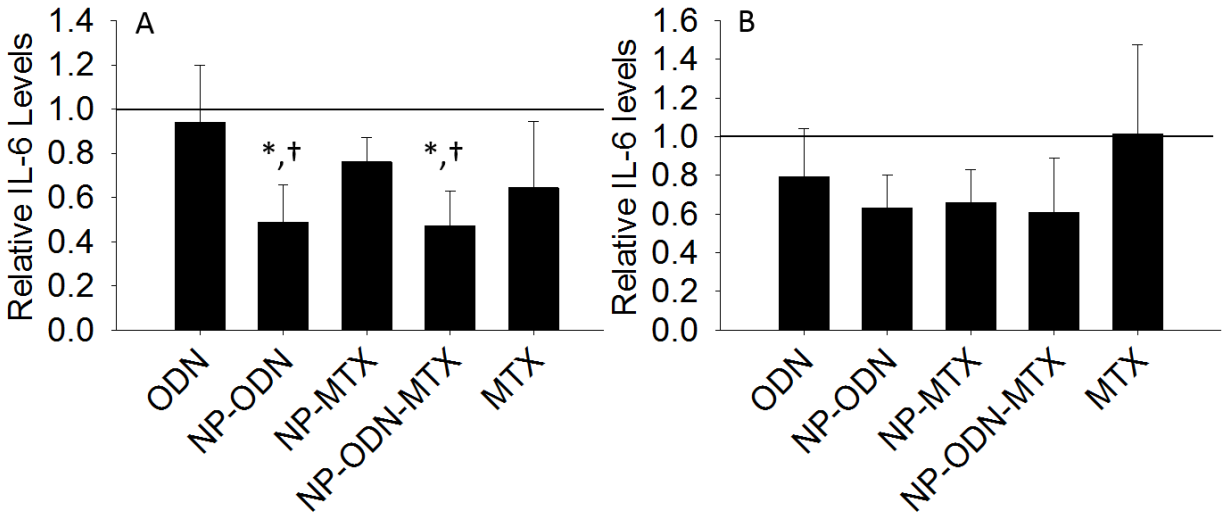

Fig. 17. ELISA was performed to determine levels of IL-6 secretion by primary RA synovial fibroblast cells, after treatment with ODN alone (ODN), NP coated with ODN (NP-ODN), NP loaded with methotrexate (NP-MTX), NP loaded with methotrexate and ODN (NP-ODN-MTX), and MTX alone (MTX) and stimulation with IL-1B at 24 (A) and 48 (B) hours. Results are expressed as fold changes of IL-6 levels relative to an untreated control, shown as a solid line at 1. One way ANOVA followed by Holm-Sidak multiple comparisons testing was used to determine the impact of treatment on IL-6 secretion. * represents a significant difference from the control, while † represents significant difference from ODN alone. All data is presented as mean ± SD (N=4).

IL-6 secretion by the primary cells was observed in response to NP-ODN and NP-ODN-MTX in comparison to untreated control cells, as well as cells administered ODN alone. While cells treated with NP-MTX and MTX alone experienced a decrease in levels of IL-6, the decrease was not great enough to be considered significant. A lack of significant reduction of IL-6 secretion in response the NP-MTX and MTX alone is in accordance with several reports, described in further detail in the discussion, stating that MTX does not have a direct effect on IL-6 levels in primary RASF cells. At 48 hours, (Fig. 17B) although trends similar to 24 hours are seen with reductions in IL-6 levels in response to NP-ODN, NP-MTX and NP-ODN-MTX, significant reductions were not observed in response to any NP treatment.

The IL-8 secretion response of primary RA cells to NP treatments is depicted in Fig. 18. At 24 hours (Fig. 18A), despite similar reduction trends as observed for primary IL-6 secretion and SW982 IL-8 secretion, the differences were not significant. At 48 hours, (Fig. 18B) NP-ODN,

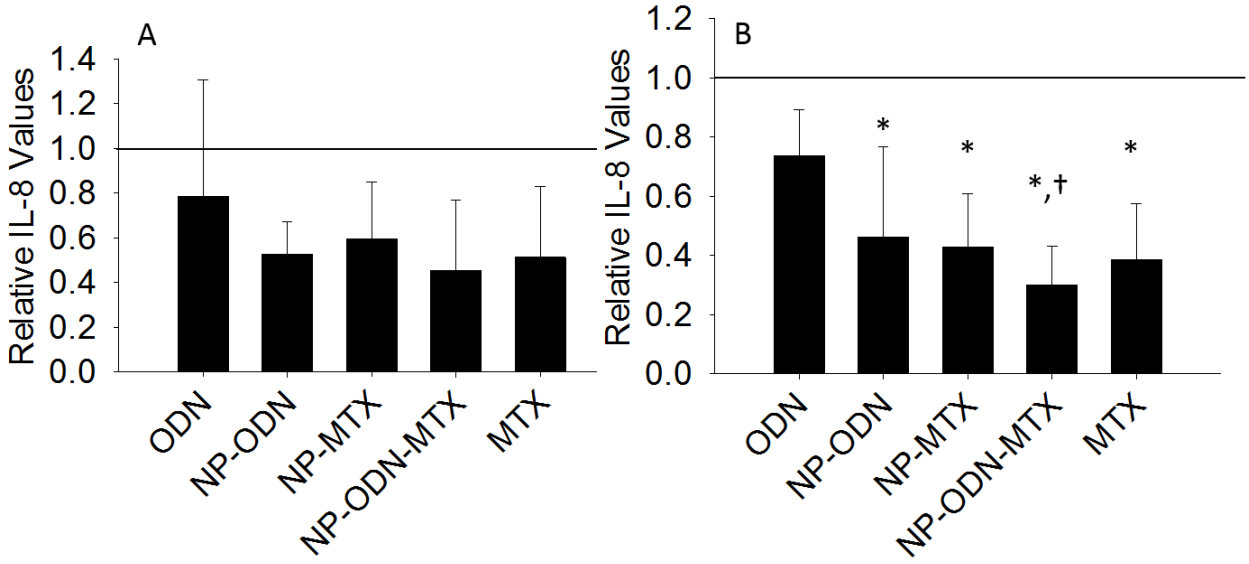

Fig. 18. ELISA was performed to determine levels of IL-8 secretion by primary RA synovial fibroblast cells, after treatment with ODN alone (ODN), NP coated with ODN (NP-ODN), NP loaded with methotrexate (NP-MTX), NP loaded with methotrexate and ODN (NP-ODN-MTX), and MTX alone (MTX) and stimulation with IL-1B at 24 (A) and 48 (B) hours. Results are expressed as fold changes of IL-8 levels relative to an untreated control, shown as a solid line at 1. One way ANOVA followed by Holm-Sidak multiple comparisons testing was used to determine the impact of treatment on IL-8 secretion. * represents a significant difference from the control, while † represents significant difference from ODN alone. All data is presented as mean ± SD (N=4).

NP-MTX, NP-ODN-MTX, and MTX alone all resulted in significant decreases in IL-8 secretion when compared to the untreated control. Furthermore, NP-ODN-MTX treatment resulted in a significant decrease when compared to ODN alone.

5.3.2 Cellular uptake of NP-ODN

To facilitate visualization of carrier uptake and localization of NP *in vitro*, TMC was modified with green (Alexa Fluor 488) fluorescent tag. Tagged NP were incubated with SW982

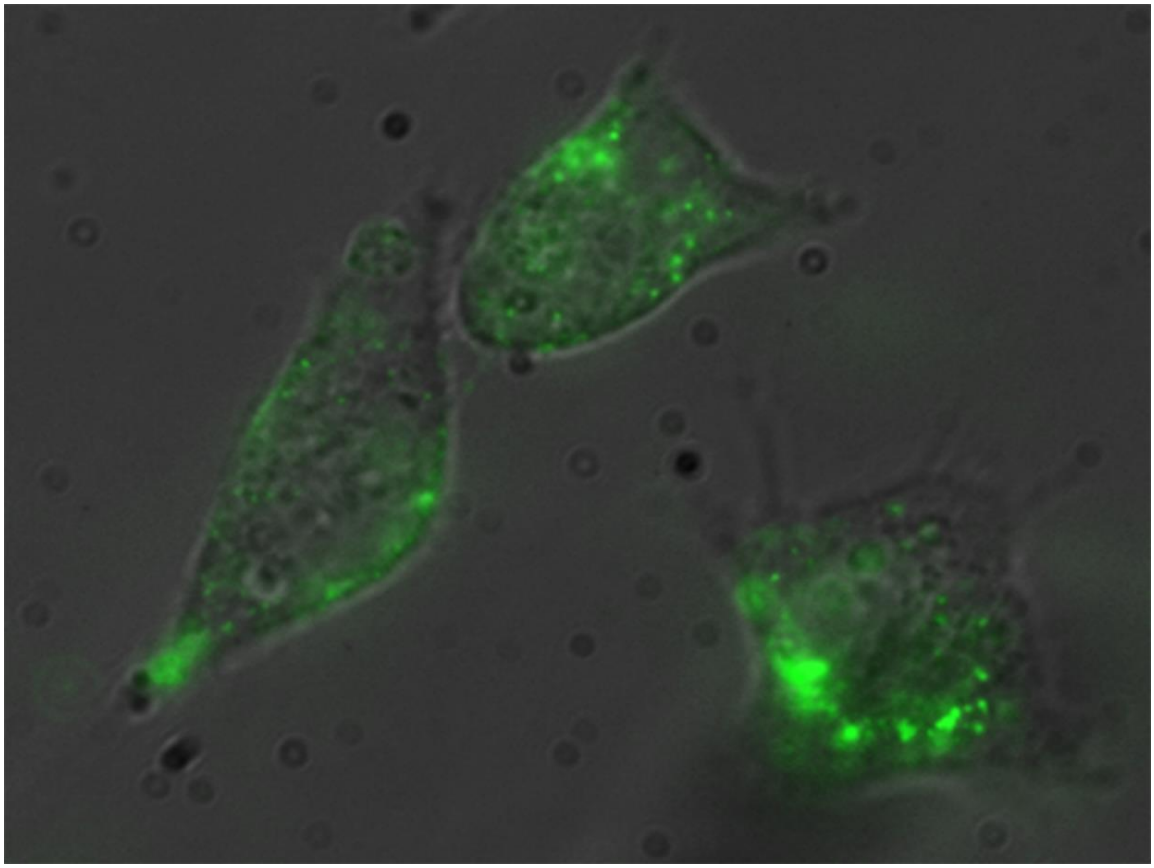

Fig. 19. Cellular uptake visualization of Alexa Fluor 488® tagged PSA-TMC in SW982 synovial sarcoma cells. Cells were incubated with tagged particles for 45 minutes at 37°C, then imaged using an inverted fluorescent microscope.

cells at 37°C for 45 minutes, and visualized. A composite image from the uptake experiments is shown in Fig. 19. The nanoparticles demonstrated sufficient cellular uptake within a short time period.

5.4 Discussion

A major barrier in the advancement of nucleic acid therapies to achieving clinical relevance is a general lack of ability of the negatively charged nucleic acid to enter the negatively charged cell membrane. A number of positively charged carrier systems and transfection reagents have

been explored to overcome this barrier; however, many of these are associated with toxicity, immunogenicity, and/or are highly variable based on cell type [182]. The carrier system presented here, PSA-TMC nanoparticles, has been found to be non-cytotoxic, while maintaining a positive surface charge [145, 156, 174]. The positive surface charge of the particles due to TMC is expected to facilitate interaction with negatively charged cell membranes, increasing ODN cellular uptake. Meanwhile, PSA has no known receptors in the body, making it an optimal choice for a component, as this will likely allow for RES evasion and reduce the likelihood of inducing an immune response. Further, PSA has properties similar to polyethylene glycol (PEG), a polymer commonly used to extend circulation time via incorporation into nanocarrier systems or protein conjugates [7, 145, 149, 158]. In sum, a nanoparticle system based on natural polysaccharides, anticipated to exhibit reduced immunogenicity and enhanced hydrophilicity in comparison to other cationic polymer based nanocarrier systems is reported in this chapter. An NF-kB decoy ODN was chosen as the NA drug of choice for this study due to the known activity of NF-kB in RA pathology.

Under normal conditions, NF-κB is bound to an inhibitor in the cytoplasm. However, in response to an inflammatory stimulus, the inhibitor undergoes phosphorylation, leading to dissociation of the NF-κB/inhibitor complex and subsequent nuclear translocation of the transcription factor. For this study, IL-6 and IL-8 were chosen as the representative cytokine and chemokine, respectively for quantitative analysis. In addition to other roles in the inflammatory response, IL-6 and IL-8 play a role in stimulation of VEGF, a growth factor linked to production of blood vessels [125]. The newly and, hence, typically rapidly formed blood vessels have larger pore sizes between the endothelial barrier than normal blood vessels, which can be exploited for drug delivery via the enhanced permeation and retention (EPR) effect [183]. Colloidal carrier

systems between 100-200 nm can passively accumulate in areas associated with blood vessels with larger, leaky pores in the endothelial barrier, thus, the EPR effect is a means of passive targeting [184]. While RA pathology doesn't exhibit the retention aspect of this phenomenon, the enhanced permeation appears to be great enough to act as a passive targeting mechanism [185, 186].

As NF-κB has a well-established involvement in RA, the transcription factor is an enticing target for drug candidates. However, as demonstrated here and in previous studies, administration of decoy ODN's alone results in low efficacy and delivery with many available reagents results in high degrees of cytotoxicity [174]. In the current study, decoy ODN efficacy was increased when administered via PSA-TMC to primary and cell line *in vitro* models of RA. SW982 cells yielded significantly decreased levels of IL-6 in response to treatment with NPODNMTX at 24 and 48 hours and in response to NPMTX at 48 hours only. As primary cells are isolated from different individuals, it is not unexpected to observe increased variability among cytokine expression and secretion when compared to cell line groups, as seen here [187].

In regards to the effect of MTX on cytokine modulation, conflicting results have been reported. Early research by Loetscher et al. claimed that MTX is ineffective at mediating IL-8 production in RA [188], while Kraan et al. reported decreased IL-8 in synovial fluid after MTX treatment [189]. Similarly, Nishina et al. recently reported MTX effectively reduced IL-6 plasma levels in RA patients [190], while Inoue et al. claimed MTX did not have an inhibitory effect on IL-6 production by RA synovial cells [191]. Previous studies conducted by our group found MTX delivery alone to be inconsistent, providing further evidence that the therapeutic effects of MTX may not be manifested in changes in the cytokine milieu [145].

The cytokine results portrayed in Figs. 15 through 18 in response to MTX alone are reflective of the variable response observed among RA patients. In addition to unpredictable efficacy, MTX is associated with a number of severe, dose dependent side effects, limiting the tolerable dosage level. Several reports advocate for combination therapy of DMARDs, particularly MTX, with biologic therapies. For example, a report by Goekoop-Ruiterman et al claimed increased clinical improvement in early stages of diseases progression with combination therapies [192]. Likewise, claims of increased efficacy of low dose of MTX combined with alternative therapies, such as phosphodiesterase type three inhibitor cilostazol have also been reported [193]. Indeed, primary RASF cells showed a significant response in IL-6 levels to treatment with NP-ODN-MTX and NP-ODN, but not NP-MTX or MTX alone. The primary cell model also resulted in a significant reduction of IL-8 in response to NP-ODN, NP-MTX, NP-ODN-MTX and MTX in comparison to an untreated control, however only NP-ODN-MTX resulted in a significant decrease in comparison to just ODN delivery alone. These results suggest that decoy ODN has the ability to act alone, as well as enhance efficacy of DMARD MTX when delivered in combination. PSA-TMC NP provide a delivery vehicle to safely enhance cellular uptake of ODN, as well as encapsulate and carry MTX to the required site of action.

5.5 Conclusion

In this study, evidence furthering the claim that PSA-TMC nanoparticles can be used to effectively deliver nucleic acid based drugs was obtained. Furthermore, the combination of NF-κB decoy ODN and DMARD MTX was shown to result in an increased reduction in inflammatory cytokines in both a cell line and primary RASF model of RA. To our knowledge, this is the first report of investigating combination therapy of MTX with a decoy ODN. While PSA-TMC nanoparticles have been used to administer both MTX and ODN separately in

previous studies, this is the first time we have attempted to combine these two therapies and report successful modulation of inflammatory proteins in RA in vitro models [145, 174]. Incorporating in vivo testing is necessary to determine both safety and efficacy of PSA-TMC loaded with ODN and MTX, however this preliminary in vitro investigation provides strong evidence to support future studies.

6. Immunomodulation of Cystic Fibrosis Epithelial Cells via NF-κB Decoy Oligonucleotide Coated Polysaccharide Nanoparticles

Activation of the transcription factor nuclear factor-kappa B (NF-κB) signaling pathway is associated with enhanced secretion of proinflammatory mediators and is thought to play a critical role in diseases hallmarked by inflammation, including cystic fibrosis (CF). Small nucleic acids that interfere with gene expression have been proposed as promising therapeutics for a number of diseases. However, applications have been limited by low cellular penetration and a lack of stability. Nano-sized carrier systems have been suggested as a means of improving the effectiveness of nucleic-acid based treatments. Polysialic acid-N-trimethyl chitosan (PSA-TMC) nanoparticles were successfully coated with NF-κB decoy oligonucleotides (ODNs). To demonstrate anti-inflammatory activity, this chapter describes use of a simple *in vitro* model of CF generated via interleukin-1β or *P. aeruginosa* lipopolysaccharides stimulation of IB3-1 bronchial epithelial cells. While free ODN and PSA-TMC nanoparticles coated with scrambled oligonucleotides did not have substantial impacts on the inflammatory response, the decoy ODN-coated PSA-TMC nanoparticles were able to reduce the secretion of interleukin-6 and interleukin-8, proinflammatory mediators of CF, by the epithelial cells, particularly at longer time points. In general, the results suggest that NF-κB decoy ODN-coated TMC-PSA nanoparticles may serve as an effective method of altering the proinflammatory environment associated with CF.

6.1 Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disorder characterized by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator protein (CFTR) that controls chloride ion transport in epithelial surfaces [194]. Disruption of ion transport leads to excessive mucus buildup, affecting many organs; however, chronic lung disease due to persistent infection and inflammation presents the highest morbidity risk [195, 196]. Although the mechanisms behind the onset and perpetuation of inflammation are not fully understood, many reports agree that elevated levels of transcription factor nuclear factor-kappa light chain enhancer of activated B cells (NF-κB) within the lung epithelia are associated with the inflammatory immune response. NF-κB is known to transcriptionally regulate the production of a variety of inflammatory mediators, including interleukin-8 (IL-8) and interleukin-6 (IL-6), cytokines directly involved in the immune response [197]. IL-6 stimulates B cells, leading to increased antibody production, while IL-8 recruits neutrophils to the infected area [198-200]. In contrast to CF bronchial epithelial cells that show significantly elevated levels of IL-6 and IL-8, healthy cells yield levels of these cytokines that are not readily detectable [200].

Pseudomonas aeruginosa, a pathogen commonly found in CF lung infections, is known to activate the NF-κB signaling pathway via interactions with the toll-like receptors (TLR). Notably, *P. aeruginosa* lipopolysaccharides (pLPS) interact with the NF-κB pathway through TLR-4 [201]. As illustrated in Fig. 20, under normal circumstances, NF-κB is sequestered in the cytoplasm by a protein known as inhibitor of κB (IκB). In the event of inflammatory stimuli receptor binding, such as binding of pLPS to TLR 4, IκB kinase (IκK) is activated, which phosphorylates and inactivates IκB. Dissociation of NF-κB then occurs, followed by translocation to the nucleus and initiation of transcription [119]. Blocking NF-κB nuclear

translocation and/or DNA binding with a transcription factor decoy oligonucleotide (ODN) therefore has the potential to reduce inflammation in CF airways (Fig. 20) [202].

NF-κB transcription factor decoy ODNs have previously been explored as anti-inflammatory treatments for several conditions, including CF. A review by De Stefano highlights some of the recent applications of NF-κB transcription factor decoy ODN's in the treatment of inflammatory

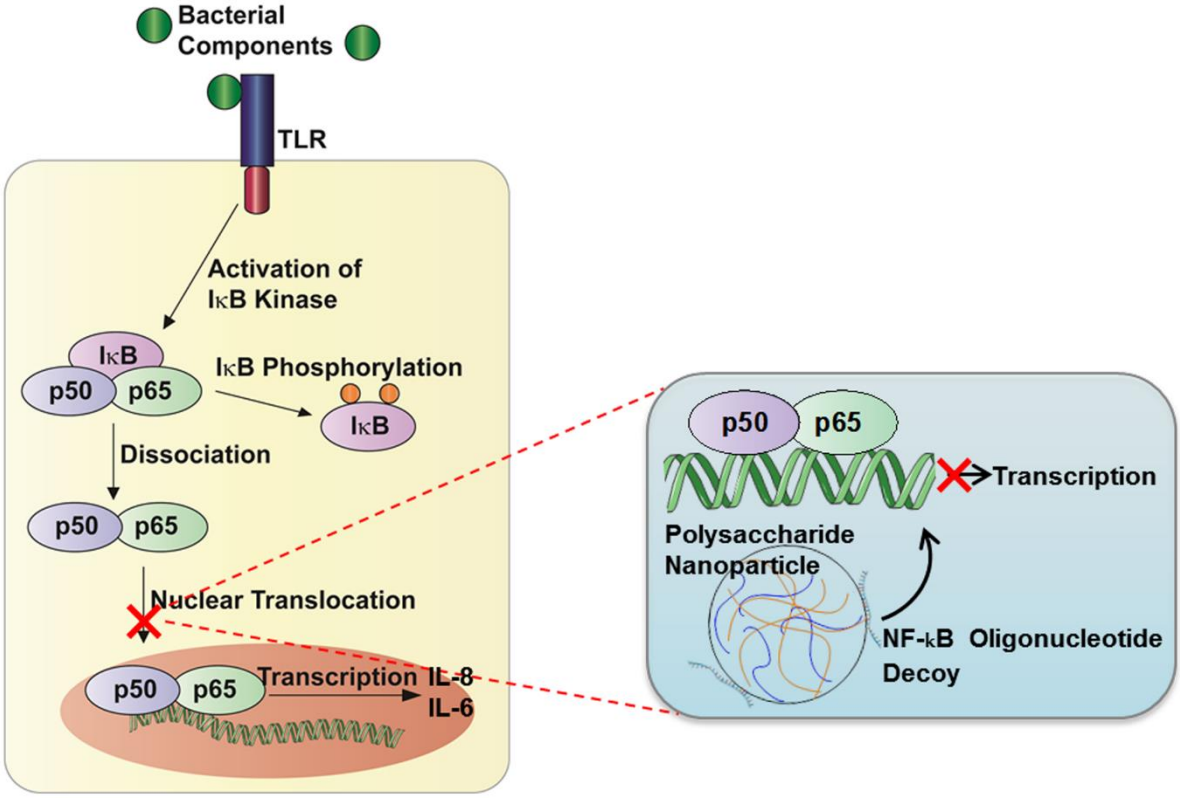

Fig. 20 An illustration of NF-κB pathway activation in a CF airway epithelial cell. P. aeruginosa bacterial components bind to the toll-like receptors, leading to activation of IκK, phosphorylation and inactivation of IκB, and eventual translocation of NF-κB to the nucleus.NF-κB decoy ODNs prevent translocation and transcription of NF-κB dependent genes.

diseases [203], and several groups have reported suppression of inflammatory cytokines in response to an NF-κB decoy ODN in *in vitro* and *in vivo* disease models [4, 204-207]. For example, NF-κB decoy ODNs complexed with cationic liposomes were shown to reduce the expression of IL-8 by IB3-1 bronchial epithelial cells infected with *P. aeruginosa[4]*. However,

use of decoy ODNs as therapeutic agents, particularly for diseases that necessitate systemic administration, has hampered by two major drawbacks: (1) the negative charge prevents cellular uptake and (2) the short nucleic acid molecules are unstable and susceptible to nuclease activity [119]. Reflecting this potential limitation in activity, one clinical trial has been conducted with local administration of NF-κB decoy oligonucleotides using a non-viral vector, but the study was closed due to a lack of demonstrated efficacy, presumably as a result of rapid enzymatic digestion [101]. As detailed in previous chapters, viral vectors and cationic lipid reagents, the current options for nucleic acid delivery are often associated with immunogenicity and cytotoxicity, rendering them inappropriate for use in applications where control of inflammation is the desired outcome [100, 101] [105].

Here, the PSA-TMC nanoparticles, described in detail in chapter four, were coated with NF-κB decoy ODN, and the ability of the ODN-coated nanoparticles to reduce the inflammatory response associated with CF was demonstrated using an *in vitro* model based upon the IB3-1 cell line. Although several immortalized cell lines derived from bronchial epithelial cells exist for therapeutic screening, the IB3-1 CF cell line, which express the mutated CFTR protein, was used for the research presented herein based upon prior use of these cells for gene therapy [4], in conjunction with evidence of a strong pro-inflammatory response to stimuli derived from *P. aeruginosa* [4, 208, 209]. Prior to conducting anti-inflammatory efficacy studies, toxicity of the nanoparticles toward the IB3-1 cell line was assessed. Anti-inflammatory activity was assessed by determining changes in the secretion of IL-6 and IL-8 upon stimulation with either interleukin-1β (IL-1β) or pLPS after pre-treatment of the cells with of the decoy ODN-coated nanoparticles. Similar to other investigators, the direct impact of the decoy ODNs on the NF-κB signaling pathway was evaluated with a luciferase reporter assay using HEK 293 cells.

6.2 Materials and methods

6.2.1 Materials

Polysialic acid (colominic acid, PSA) was obtained from Nacalai USA, Inc. (San Diego, CA, USA). N-trimethyl chitosan (TMC) was produced via quaternization of chitosan (MW 100 Da – 300 kDa) obtained from Acros Organics (New Jersey, USA), as described by Sieval et al [163]. Sodium tripolyphosphate (pure, TPP) was procured from Acros Organics (New Jersey, USA). *P.aeruginosa* LPS (pLPS) was purchased from Sigma Aldrich (St. Louis, MO, USA). An NF-κB decoy oligonucleotide (ODN) kit containing NF-κB decoy ODN (5' CCT TGA AGG GAT TCC CCT CC 3'), as well as scrambled ODN (5' TTG CCG TAC CTG ACT TAG CC 3'), was acquired from CosmoBio (Tokyo, Japan). Recombinant human interleukin-1β was obtained from R and D systems (Minneapolis, MN). A rhodamine tagged ODN (5' (TAMRA-X) (C6-NH) CCT TGA AGG GAT TTC CCT CC 3') was purchased from TriLink Biotechnologies (San Diego, CA). Lipofectamine 2000 transfection reagent and 4', 6-Diamidino-2-Phenylindole, dilactate (DAPI stain) were procured from Invitrogen (Grand Island, NY).

6.2.2 Cell culture

IB3-1 bronchial epithelial cells with the CF CFTR gene mutations were obtained from the Johns Hopkins GCFR cell center and grown on rat tail collagen coated tissue culture flasks. To coat the flasks, rat tail collagen was dissolved in LHC-8 complete growth media (Invitrogen, Grand Island, NY) at a concentration of 30 ng/ml. LHC media has been optimized for the growth of bronchial epithelial cells. 1.5 ml of the coating solution was added to a 25 cm^2 tissue culture flask and incubated at 37^oC, 5% CO_2 for 24-48 hours. Coating media was removed prior to placing cells in the flask, and cells were maintained in LHC-8 with 5% fetal bovine serum (FBS, Atlanta Biologicals, Atlanta GA) at 37^oC, 5% CO_2 until confluent. Human embryonic kidney

(HEK) 293 cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's Modified Eagles Medium (DMEM) with 10% FBS at 37°C, 5% CO_2.

6.2.3 Nanoparticle preparation and characterization

PSA-TMC nanoparticles (NP) were prepared as previously described, and detailed in chapter four [156]. A Malvern Zetasizer NanoZS90 (Malvern Instruments, Malvern UK) was used to determine nanoparticle size, polydispersity (PDI), and zeta potential.

6.2.4 Cytotoxicity of PSA-TMC towards IB3-1 cells

PSA-TMC cytotoxicity was assessed using a CCK-8 cell counting kit (Dojindo Molecular Technologies, Rockville Md.). IB3-1 cells were plated on a 96 well tissue culture plate (5000 cells/well) and allowed 24 hours for complete adherence. PSA-TMC was prepared as described above, resuspended in LHC-8 + 5% FBS media after centrifugation, sterile filtered, and added at concentrations ranging from 10 mg/ml to 0 mg/ml to IB3-1 cells in duplicate. After incubation at 37°C for 24 hours, changes in cellular metabolic activity were determined following the manufacturer's instructions. Briefly, 10 µl of reagent and 100 µl of media were added to each well; and, after incubating at 37°C for 90 minutes, colorimetric absorbance at 450 nm was quantified using a BioTek Synergy 2 multimode plate reader (BioTek Instruments, Winooski, VT).

6.2.5 *In vitro* efficacy of ODN-coated PSA-TMC nanoparticles

IB3-1 cells were plated on collagen coated 24 well plates at a density of 20,000 cells per well. LHC-8 media supplemented with 5% FBS was added to bring the volume per well to 500 µl, and 24 hours were allowed for cellular adherence. Decoy and scrambled ODN-coated PSA-TMC nanoparticles (NP-ODN and NP-SCO, respectively), as well as bare PSA-TMC nanoparticles (NP), were prepared as described in chapter four. Immediately following

centrifugation, NP-ODN, NP-SCO, and NP were resuspended in LHC-8 (without FBS) at a concentration of 1 mg/ml and filtered through a sterile 0.45 micron filter. As a positive control, Lipofectamine 2000-Decoy ODN complexes (Lipo-ODN) were prepared as directed by the manufacturer. In two separate microcentrifuge tubes, 2.5 µl of Lipofectamine 2000 reagent and 500 ng decoy ODN were added to 50 µl of serum free media. After equilibrating for 5 minutes, the reagent media was added to the ODN media, and the resultant solution was incubated for 15 minutes to allow for complex formation. 500 µl of the following treatment groups were added to the prepared 24 well plate in duplicate: 1. media alone, 2. ODN alone, 3. NP-ODN, 4. NP, 5. Lipo-ODN, and 6. NP-SCO. After 4 hours, the treated media was removed and replaced with fresh media containing 5% FBS to allow for normal growth conditions.

24 hours after media replacement, cells were stimulated with IL-1β (2.5 ng/ml) or pLPS (10 µg/ml) to induce inflammation. The concentration of IL-1β used correlates to the concentration that yielded a 50% increase in the secretion of prostaglandin E_2 (PGE_2) upon administration to IB3-1 cells. Similarly, LPS has been reported to increase levels of IL-6 and IL-8 when introduced at a concentration of 10 µg/ml [202]. After incubation at 37°C for 24 or 48 hours, the supernatant was collected and stored at -80°C degrees until analysis. DAPI nuclear staining was conducted immediately after sample collection to determine cell count for normalization. Briefly, DAPI dye (5 mg/ml stock concentration) was diluted to 0.5 µg/ml with 1X PBS and 250 µl were added to each well. After 1 minute, the dye was removed and the cells were rinsed 3 times with PBS. Imaging was performed using an inverted fluorescence microscope (Leica Microsystems, IL, USA). Three representative images were obtained from each well. Each image was counted, and a mean cell number (MCN) was generated for each condition. Total cell number (TCN) was determined using the following equation:

$$TCN = MCN * (\frac{A_{well}}{A_{image}}) \quad (5)$$

where A_{well} is the total area of the well, and A_{image} is the area of the image obtained by microscopy.

6.2.6 Quantitative analysis of inflammatory cytokines

IL-6 and IL-8 ELISA kits were purchased from Peprotech (Rocky Hill, NJ) and run according to manufacturer's instructions to determine the levels of IL-6 and IL-8 in the collected supernatant. Samples were run in duplicate, and each experiment was repeated independently three times.

6.2.7 Cellular response to TMC and PSA

Based upon a recent literature report, TMC can have an anti-inflammatory impact on cells stimulated by LPS [210]. Therefore, 500 µl of TMC and PSA in LHC-media (without FBS) at concentrations of 1 mg/ml were added to IB3-1 cells cultured in a 24 well plate in duplicate. After 4 hours, the treated media was removed and replaced with fresh media containing 5% FBS to allow for normal growth conditions. 24 hours after media replacement, cells were stimulated with pLPS (10 µg/ml) to induce inflammation. After incubation at 37°C for 24 or 48 hours, the supernatant was collected and stored at -80°C degrees until analysis. DAPI nuclear staining was conducted immediately after sample collection to determine cell count for normalization, as described in Section 2.6. IL-6 ELISA (Section 2.7) was used to quantify the amount of IL-6 within the collected supernatant. The experiment was repeated independently three times.

6.2.8 Luciferase reporter assay

HEK 293 cells were transfected with a pMetLuc Reporter Vector, part of a Ready-To-Glow Secreted Luciferase Reporter System acquired from Clontech (Mountain View, CA). For each transfection, 0.5 µg of the NF-κB dependent pMetLuc reporter, which leads to the production

and secretion of luciferase protein in response to NF-ΚB activation, or 0.5 μg of a control vector were prepared in Lipofectamine LTX reagent (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. After incubation with the reporter complexes for 4 hours, cells were treated with ODN-coated nanoparticles and controls, as described in Section 2.6 (media alone, ODN alone, NP-ODN, NP, Lipo-ODN and NP-SCO). Following incubation for an additional 4 hours, the treatment media was replaced with DMEM supplemented with 10% FBS. 24 hours after media replacement, cells were stimulated with IL-1β (2.5 ng/ml) to induce inflammation. Supernatant samples were collected at 24 hours and assayed for luciferase activity using the luminescence assay included in the Clontech kit. Luciferase activity was normalized to the control vector, and each experiment was repeated independently 3 times.

6.2.9 Cellular immunostaining

Immunofluorescent staining of the NF-κB subunit was used to qualitatively verify that the ODN-coated nanoparticles blocked nuclear translocation of NF-κB following stimulation of IB3-1 cells. The staining protocol was optimized based upon prior work by Nadjar et al [211]. 100,000 cells were plated onto collagen-coated confocal dishes (MatTeck Corporation, Ashland, MA) and allowed to adhere overnight. ODN-coated PSA-TMC nanoparticles were prepared as described above and added to a confocal dish at a concentration of 1 mg/ml. As controls, confocal dishes were prepared that contained either media or ODN (500 ng/ml) alone. After 4 hours, ODN and ODN-coated PSA-TMC nanoparticles were removed and replaced with LHC-8 media with 5% FBS. 24 hours later, the cells were stimulated with IL-1β at a concentration of 2.5 ng/ml to induce inflammation. After 48 hours, the cells were fixed in a 4% paraformaldehyde solution for 15 minutes. Subsequently, the cells were permeabilized with a 1.0% Tritonx-100 for 10 minutes after rinsing 2-3 times with 1X PBS. After again rinsing with 1X PBS 2-3 times, a 3% BSA blocking

solution was added to the cells for 30 minutes to prevent nonspecific binding. Following an additional 2-3 rinses with 1X PBS, NF-kB (P65) NLS polyclonal antibody (primary) was added at a concentration of 2 µg/ml, and the cells were incubated overnight at 4°C. The primary antibody was removed, and the cells were rinsed 4 times in 1x PBS. FITC-Goat anti-rabbit IgG (secondary) was added at a concentration of 2 µg/ml and incubated for 2 hours at room temperature. Secondary antibody was removed, and the cells were rinsed 4 times in 1x PBS. TO-PRO®-3 nuclear stain (Invitrogen, Grand Island, NY) was added at a concentration of 5 µl/ml and incubated for 45 minutes. Cells were rinsed and imaged with a Nikon Eclipse Ti inverted microscope.

6.2.10 Statistical analysis

Protein levels were normalized to cell number and expressed relative to the untreated, stimulated control group. Luciferase activity was normalized to the control vector. Data is presented as mean ± standard deviation for all groups (N=3). To compare protein secretion and luciferase activity following different treatments and IL-1β or LPS stimulation, one-way ANOVA, followed by a Holm-Sidak test for multiple comparisons [212, 213], was performed. The Student's t-test was used to compare the sizes, zeta potentials, and PDI of ODN-coated nanoparticles to those of bare nanoparticles. The latter test was also used to compare IL-6 secretion of cells treated with PSA or TMC and stimulated with LPS to untreated, LPS-stimulated cells. All statistical tests were conducted with a significance level (alpha) of 0.05.

6.3 Results

6.3.1 In vitro anti-inflammatory efficacy

Prior to testing of *in vitro* efficacy, the cytotoxicity of the PSA-TMC nanoparticles towards IB3-1 cells was established (Fig. 21). As anticipated based upon prior testing with the MH7A and SW-982 cell lines [145, 156], no changes in cellular proliferation were observed for IB3-1 cells

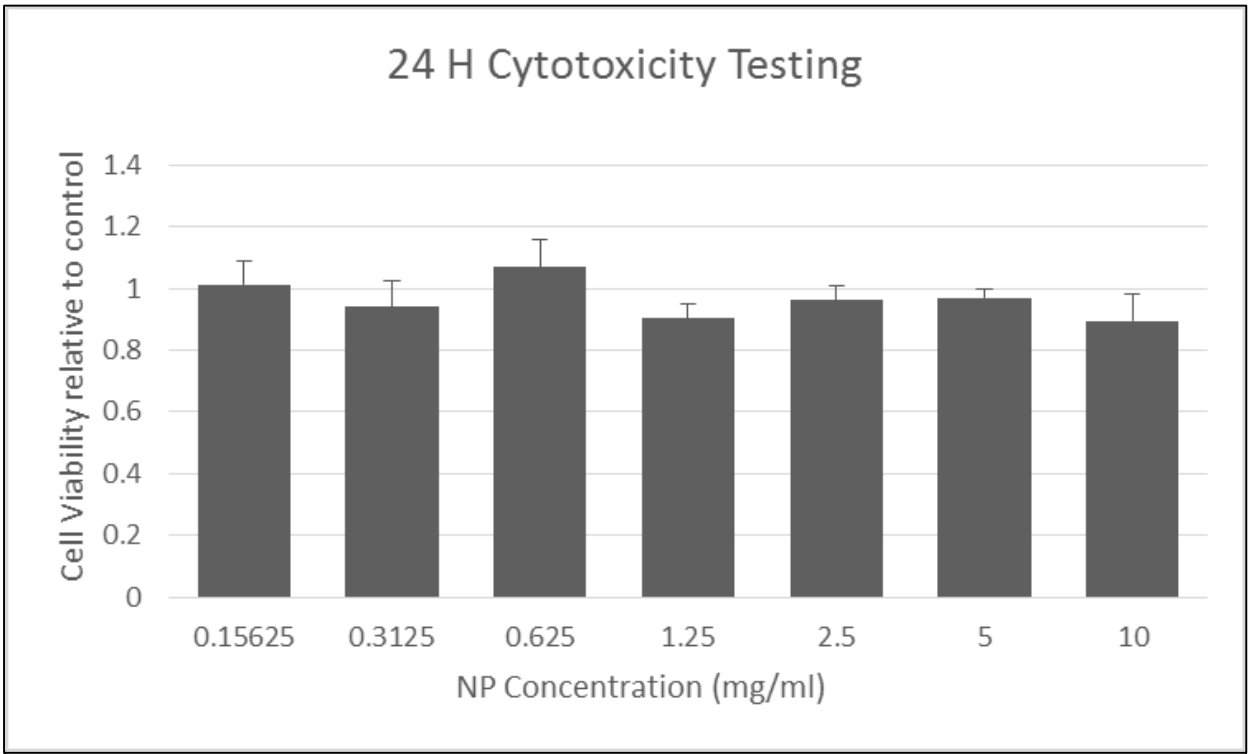

Fig 21: In vitro cytotoxicity of PSA-TMC toward IB3-1 cells. The particles were observed to be non-toxic up to the highest tested concentration, 10 mg.

with addition of PSA-TMC concentrations up to 10 mg/ml [156]. Therefore, bare nanoparticles administered at a concentration of 1 mg/ml for *in vitro* testing (1 mg/ml) were expected to yield a minimal change in cellular inflammatory response.

IL-6 and IL-8 secretion levels were first examined in response to stimulation with IL-1β, a pro-inflammatory mediator secreted by mammalian cells. As shown in Fig. 22B, the ODN-coated nanoparticles evoked a significant decrease in IL-8 secretion at 48 hours relative to stimulated control cells. Although not significant, a similar decrease was observed at 24 hours

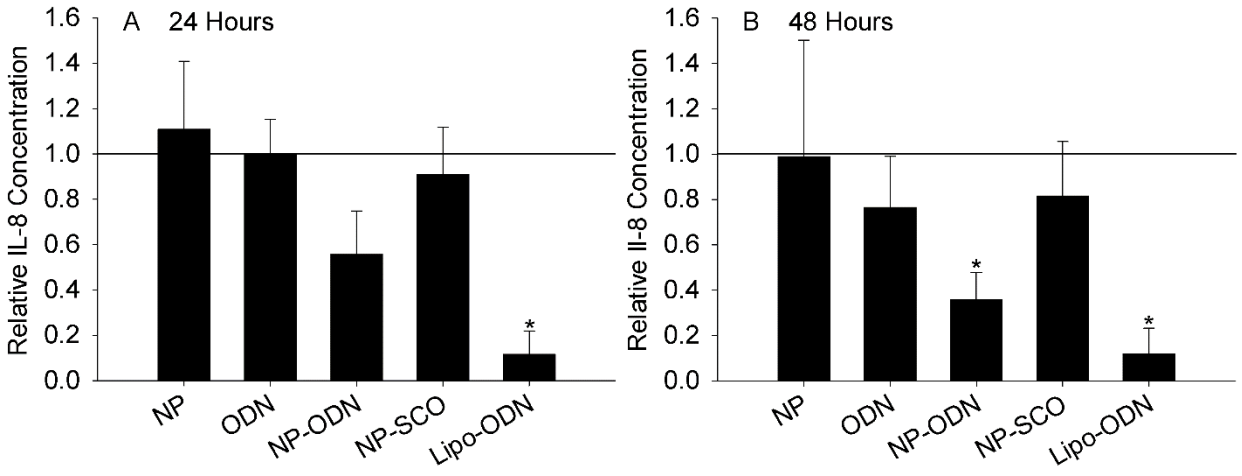

Fig. 22. ELISA was performed to determine levels of IL-8 secretion after treatment with bare nanoparticles (NP), ODN alone (ODN), NF-κB decoy ODN-coated nanoparticles (NP-ODN), scrambled ODN-coated nanoparticles (NP-SCO), or ODN complexed with Lipofectamine 2000 (Lipo-ODN) and stimulation with IL-1β at 24 (A) and 48 (B) hours. Results are normalized to cell number and expressed as fold changes of IL-8 levels relative to a non-treated control, indicated by a solid line at 1. All data is presented as mean + SD (N=3). One way ANOVA followed by a Holm-Sidak test for multiple comparisons was used to assess the impact of treatment on protein secretion. * indicates a significant difference ($p < 0.05$) between the treatment and the non-treated control.

(Fig. 22A). As expected, ODN alone, bare nanoparticles, and nanoparticles coated with SCO did not result in any changes in IL-8 levels. At first glance, ODN delivered using Lipofectamine 2000, a commercially available and commonly used transfection reagent, also appears to effectively lower IL-8 expression at 24 and 48 hours. However, Lipofectamine 2000 was highly cytotoxic to the IB3-1 cells, resulting in significant cell death and consequently lower levels of cytokine production due to low cell number. Additionally, cell counting for normalization was

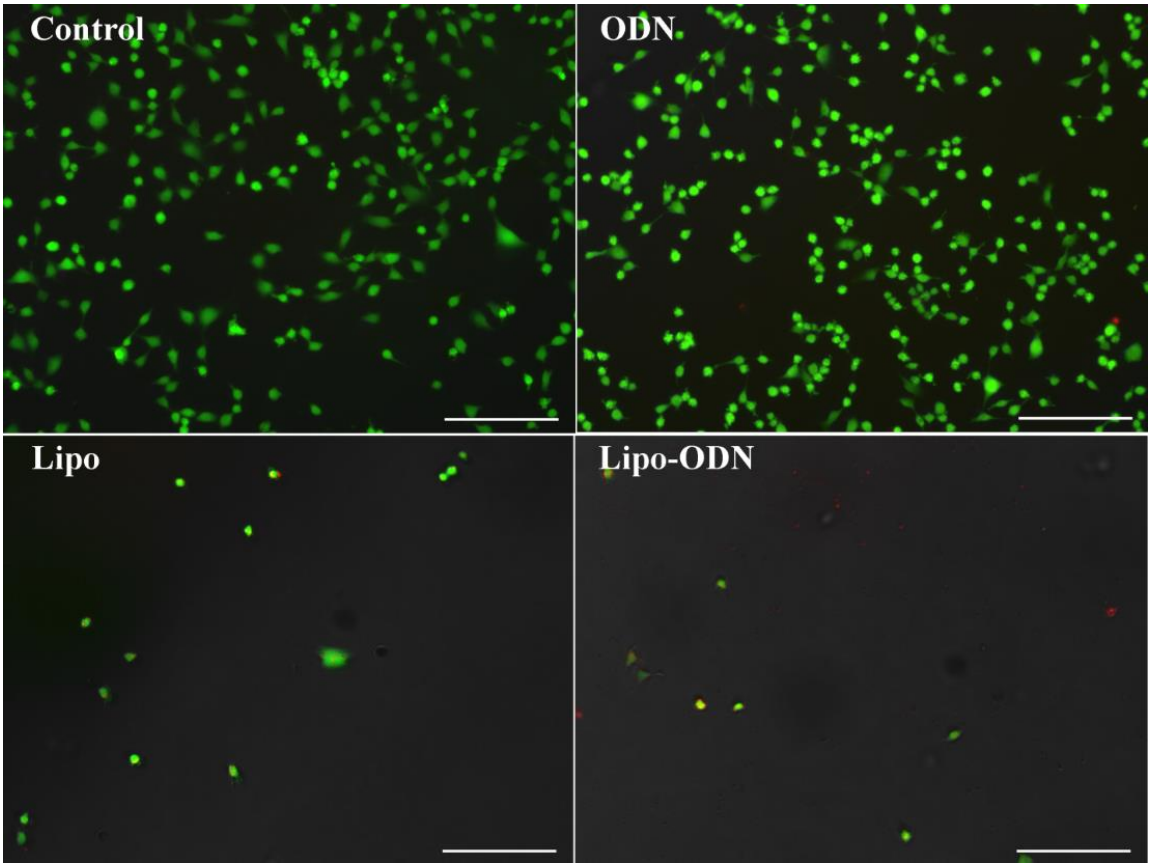

Fig. 23. Live-dead staining was used to verify that Lipofectamine induced cell death in IB3-1 cells. As expected, ODN did not result in a change in cellular viability. (Scale bar = 200 μm)

difficult for this condition due to a general lack of cells. Live-dead staining was used to further demonstrate the cell death that resulted from exposure to Lipofectamine 2000, and shown in Fig. 23.

IL-6 is a potent cytokine involved in general up-regulation of inflammation, whose production, like that of IL-8, is known to be enhanced through an IL-1β mediated increase in NF-κB signaling [214]. Fig. 24 illustrates the response of IB3-1 cells to stimulation with IL-1β

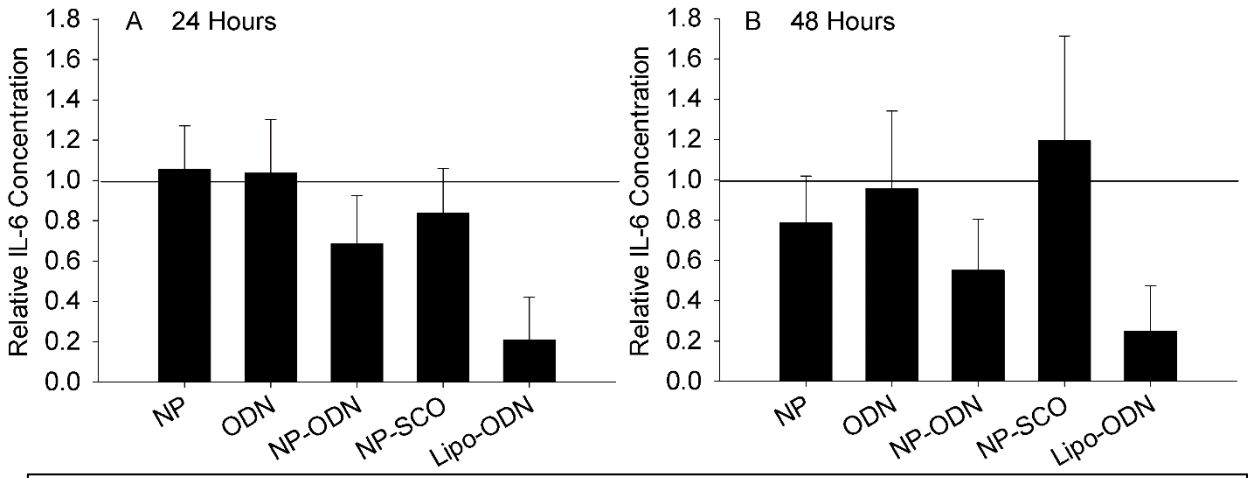

Fig. 24. ELISA was performed to determine levels of IL-6 secretion after treatment with bare nanoparticles (NP), ODN alone (ODN), NF-□B decoy ODN-coated nanoparticles (NP-ODN), scrambled ODN-coated nanoparticles (NP-SCO), or ODN complexed with Lipofectamine 2000 (Lipo-ODN) and stimulation with IL-1β for 24 (A) and 48 (B) hours. Results are normalized to cell number and expressed as fold changes of IL-6 levels relative to a non-treated control, indicated by a solid line at 1. All data is presented as mean ± SD (N=3). One way ANOVA followed by a Holm-Sidak test for multiple comparisons was used to assess the impact of treatment on protein secretion.

following ODN delivery treatments. Consistent with the observations for IL-8, the ODN-coated nanoparticles appeared to lower IL-6 vales compared to the untreated control at 24 (Fig. 24A) and 48 hours (Fig. 24B), with a slightly lower mean value at 48 hours than at 24. Likewise, ODN alone, bare nanoparticles, and SCO-coated nanoparticles did not result in a change in IL-6 secretion.

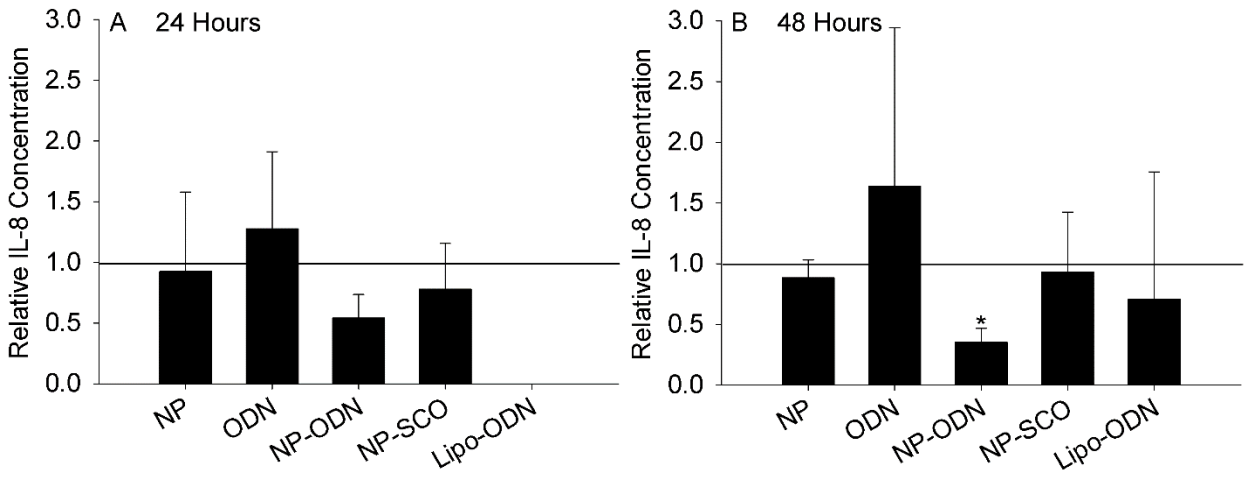

Fig. 25. ELISA was performed to determine the level of IL-8 secretion after treatment with bare nanoparticles (NP), ODN alone (ODN), NF-κB decoy ODN-coated nanoparticles (NP-ODN), scrambled ODN-coated nanoparticles (NP-SCO), or ODN complexed with Lipofectamine 2000 (Lipo-ODN) and stimulation with pLPS for 24 (A) and 48 (B) hours. Results are normalized to cell number and expressed as fold changes relative to a non-treated control, indicated by a solid line at 1. All data is presented as mean ± SD (N=3). One way ANOVA followed by a Holm-Sidak test for multiple comparisons was used to assess the impact of treatment on protein secretion. * indicates a significant difference ($p < 0.05$) between the treatment and the non-treated control.

IL-6 and IL-8 secretion were also investigated in response to stimulation with LPS isolated from *P. aeruginosa*, i.e. a pro-inflammatory mediator released by bacteria cells. LPS is a known activator of the NF-κB pathway via the toll-like receptor-4 (TLR-4) [215]. IL-8 secretion in response to LPS stimulation is shown in Fig 25. Similar to IL-8 levels in response to IL-1β, ODN-coated nanoparticles resulted in significantly lower levels of IL-8 at 48 hours (Fig. 25B). At 24 hours, a decrease in IL-8 when treated with ODN-coated nanoparticles was shown, although the difference was not significant (Fig. 25A). Furthermore, ODN alone, bare nanoparticles, and nanoparticles coated with SCO did not result in any significant changes in IL-8 levels at either time point.

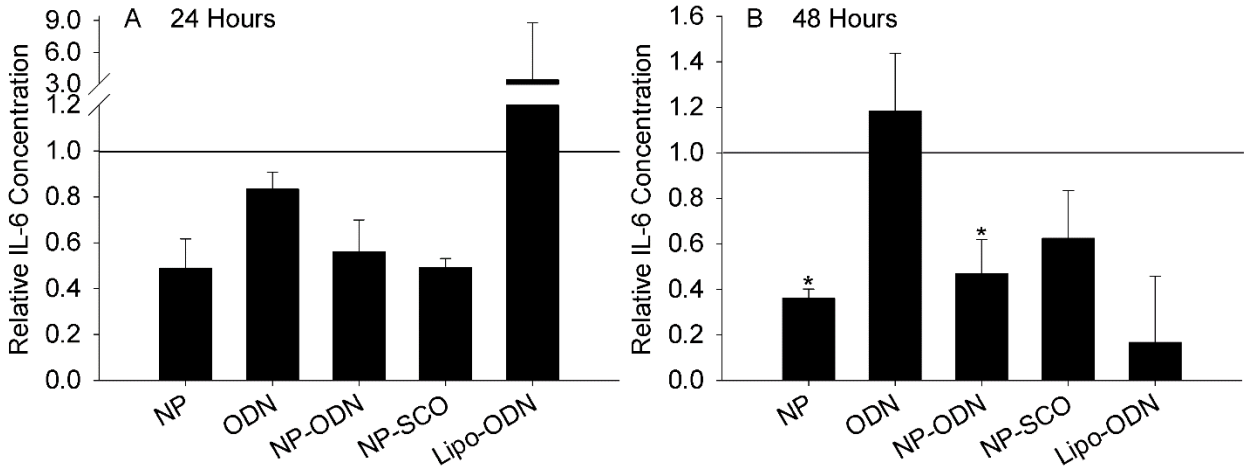

Fig. 26 ELISA was performed to determine the level of IL-6 secretion after treatment with bare nanoparticles (NP), ODN alone (ODN), NF-κB decoy ODN-coated nanoparticles (NP-ODN), scrambled ODN-coated nanoparticles (NP-SCO), or ODN complexed with Lipofectamine 2000 (Lipo-ODN) and stimulation with pLPS for 24 (A) and 48 (B) hours. Results are normalized to cell number and expressed as fold changes relative to a non-treated control, indicated by a solid line at 1. All data is presented as mean ± SD (N=3). One way ANOVA followed by a Holm-Sidak test for multiple comparisons was used to assess the impact of treatment on protein secretion. * indicates a significant difference ($p < 0.05$) between the treatment and the non-treated control.

For LPS induced IL-6 secretion, at 24 hours (Fig. 26A), ODN-coated nanoparticles resulted in a non-significant decrease in IL-6, similar to what was observed for stimulation via IL-1β.

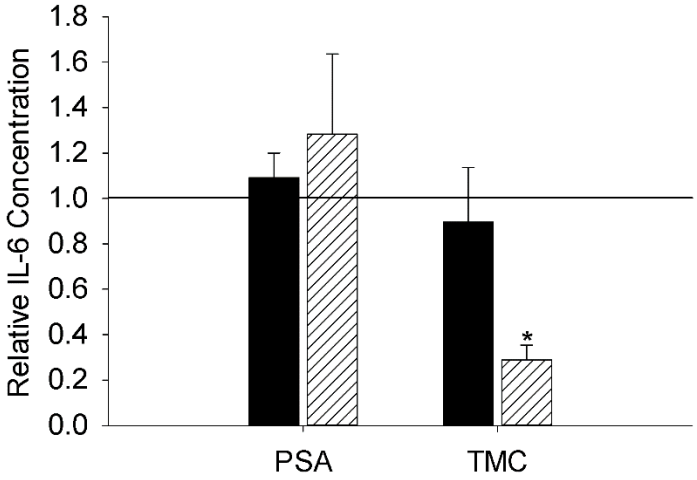

Fig. 27. ELISA was performed to determine the level of IL-6 secretion following treatment with the nanoparticle components, PSA and TMC, and stimulation with pLPS for 24 and 48 hours. Results are normalized to cell number and expressed as fold changes relative to a non-treated control, indicated by the solid line at 1.

However, bare nanoparticles and nanoparticles coated with SCO also resulted in IL-6 decreases,

although the change was not significant. Free ODN alone again did not yield a change in IL-6 secretion. Similarly, at 48 hours (Fig. 26B), ODN-coated nanoparticles, as well as bare nanoparticles, resulted in significantly lower levels of IL-6 secretion than the untreated control. SCO coated nanoparticles resulted in slightly lower IL-6 levels as well, while ODN alone did not effect a change. To further investigate the significant reduction in IL-6 secretion observed at 48 hours for the bare nanoparticles, LPS induced secretion of IL-6 was also assessed following treatment with TMC and PSA alone. As shown in Fig. 27, PSA alone did not result in a significant change in IL-6 secretion at 24 or 48 hours; however, TMC alone resulted in significantly lower IL-6 levels at 48 hours.

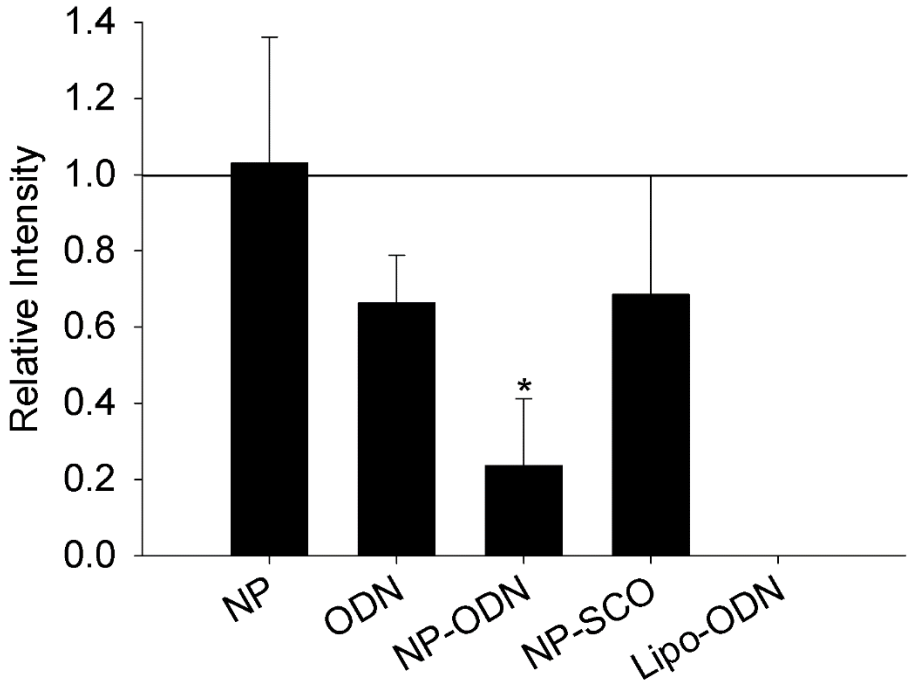

Fig. 28: A luciferase reporter assay was used to examine the effect of the decoy ODN-coated nanoparticles on NF-κB dependent gene expression. HEK 293 cells were transfected with luciferase reporter plasmid, treated with the NF-κB decoy ODN-coated nanoparticles or controls, and stimulated with IL-1β (2.5 ng/ml). All data is presented as mean ± SD (N=3). One way ANOVA followed by a Holm-Sidak test for multiple comparisons was used to assess the impact of treatment on luciferase activity. * indicates a significant difference ($p < 0.05$) between the treatment and the non-treated control.

6.3.2 Luciferase reporter assay

The impact of the ODN-coated nanoparticles on NF-κB dependent gene expression was

further examined using a luciferase reporter assay, whereby induction of NF-κB dependent genes results in the secretion of a reporter molecule into the supernatant. The HEK 293 cell line was used in lieu of the IB3-1 cell line based upon ease of transfection. As shown in Fig. 28, NF-κB decoy ODN-coated nanoparticles resulted in a significant reduction in the expression of NF-κB dependent genes, as indicated by a decrease in luciferase activity. In contrast, ODN alone, bare nanoparticles, and nanoparticles coated with SCO did not result in significant changes in the expression of NF-κB dependent genes. Luciferase activity for cells treated with Lipo-ODN could not be determined due to a lack of viable cells.

6.3.3 Cellular immuno-staining

Fluorescence microscopy was used to add credence to the notion that NF-κB decoy ODN-coated nanoparticles trap NF-kB within the cytoplasm,

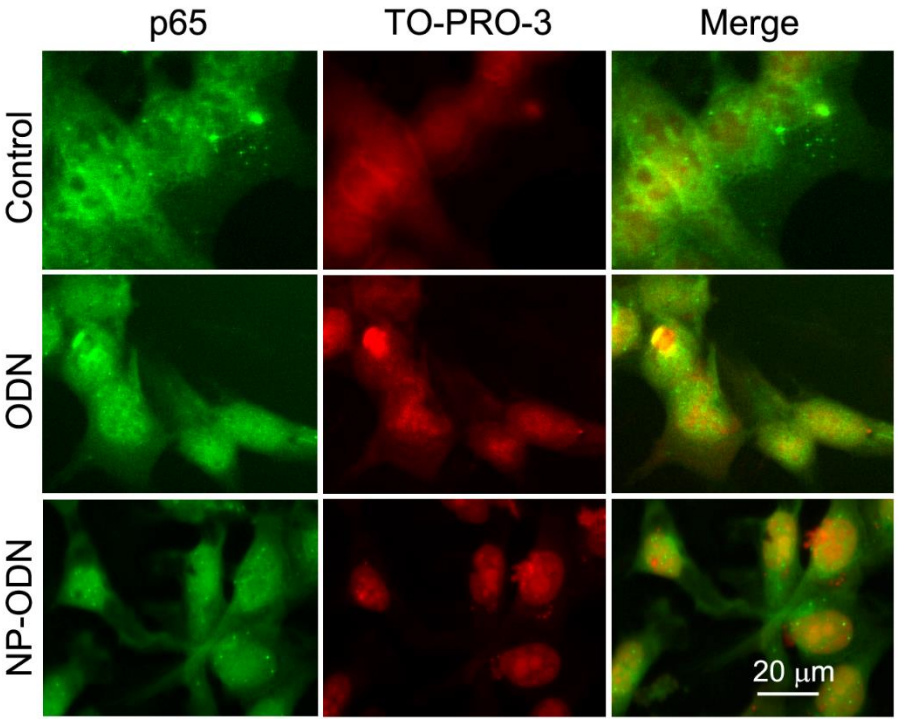

Fig. 29: Intracellular distribution of the NF-κB p65 subunit after treatment of IL-1β stimulated cells with ODN and NP-ODN. Cells were fixed and labelled with anti-NF-kB p65 antibody and nuclei were counterstained with TO-PRO-3. Treatment with ODN alone did not alter distribution of NF-κB relative to the control, while NP-ODN sequestered much of the NF-κB in the cytoplasm.

thereby reducing nuclear translocation and subsequent proinflammatory signaling. IL-1β stimulated cells were fixed and labelled with anti-NF-κB p65 antibody following treatment with

ODN alone or ODN-based nanoparticles (Fig. 29). Counterstaining with TO-PRO-3 nuclear dye allowed for enhanced distinction between cytoplasmic and nuclear NF-kB. As anticipated, ODN-coated nanoparticles trapped NF-kB within the cytoplasm relative to untreated, stimulated cells, while administration of ODN alone did not lead to a distinguishable difference from the control.

6.4 Discussion

A limiting factor in the advancement of oligonucleotide applications in cellular environments is a general inability of the negatively charged nucleic acids to interact with and penetrate the negatively charged lipid bilayer membranes. As a way of circumventing this problem, a number of positively charged transfection reagents are commercially available. Although these reagents have been somewhat successful, many cationic transfection reagents are notorious for being cytotoxic, and their efficacy is highly variable based on cell type. For example, Lipofectamine 2000 yields an overall decrease in cellular protein content at the dose required for optimal transfection efficiency, indicating high cytotoxicity [216]. Additionally, strong uptake of Lipofectamine-based lipoplexes by the RES occurs *in vivo* [217]. As discussed within the Introduction, the positive zeta potential of the PSA-TMC nanoparticles imparted by the quaternized chitosan is expected to facilitate nucleic acid binding, as well as membrane adhesion and penetration, thereby achieving delivery of associated ODNs into the cell. The presence of PSA in the nanoparticles is presumed to reduce protein interactions and limit interactions with the RES [135-137]. Thus, PSA offers a natural, biodegradable, non-immunogenic alternative to PEG for extending circulatory stability.

Bioactivity of the NF-κB decoy ODN-coated PSA-TMC nanoparticles was established by assessing changes in the secretion of two proinflammatory mediators, IL-6 and IL-8, associated with the NF-κB pathway by activated IB3-1 cells. Under normal conditions, NF-κB transcription

factor is located in the cytoplasm, bound to IκB inhibitor. However, stimulation with various pro-inflammatory factors, including IL-1β and LPS, as used in the current study, leads to activation of a kinase responsible for phosphorylating IκB, causing dissociation and inactivation [218, 219]. Without sequestration by IκB in the cytoplasm, NF-κB is free to translocate to the nucleus, and transcription of various proteins, including IL-6 and IL-8, is initiated. A transcription factor decoy ODN introduced to the cytoplasm will intercept NF-κB, thereby preventing translocation to the nucleus and mitigating expression of genes linked to additional proinflammatory mediators (Fig. 21).

Using IB3-1 cells, IL-1β activation of NF-κB has been shown to be a major contributing factor to elevated IL-8 levels in the CF lung epithelium [220]. Nanoparticles coated with ODN effectively lowered IL-8 secretion levels in response to stimulation with IL-1β in this study. Of note, the PSA-TMC nanoparticles did not exhibit cellular toxicity, as indicated by a cellular proliferation assay and consistent with data obtained from other cell types [145, 156]. In contrast, Lipofectamine 2000 was associated with significant cellular toxicity, a highly undesirable characteristic when delivering anti-inflammatory therapeutics. The toxicity observed in this study is not limited to the IB3-1 cell type [216]. While ODN-coated nanoparticles decreased IL-6 secretion in the presence of IL-1β stimulation, the decrease was not great enough to be considered significant. A lack of significance here is likely attributable to IL-6 levels that were near the limit of detection, which resulted in higher variability. ODN delivery with Lipofectamine 2000 appeared to effectively lower IL-6 levels similar to IL-8 secretion. However, once again, the cytotoxicity of the transfection reagent resulted in high amounts of cell death, decreasing the number of cells available to produce and secrete IL-6.

When stimulated with LPS, both IL-8 and IL-6 secretion levels were reduced at 48 hours when treated with ODN coated nanoparticles. Interestingly, bare PSA-TMC nanoparticles appear to have an anti-inflammatory effect on the IB3-1 cells, significantly reducing levels of IL-6 secreted at 48 hours, with a similar trend seen at 24 hours. These collective results suggest that, in the presence of LPS, the nanoparticles have an anti-inflammatory effect on IB3-1 cells that is independent of the NF-κB decoy ODN. Recently, several reports have indicated that chitosan and quaternized chitosan can interact with and modulate the inflammatory effects of LPS in a variety of cell types, however the exact mechanism of this effect is not yet known [221-223]. For example, Ji et al. reported suppression of inflammation by chitosan via modulation of cytokines locally produced in periodontal ligament cells [222]. The potential for quaternized chitosan to modulate inflammation was further validated by the present study.

A luciferase reporter assay was used to demonstrate that the NF-κB decoy ODN nanoparticles directly interfered with the NF-κB signaling pathway. As anticipated, the assay verified that the NF-κB decoy reduced expression of NF-κB dependent genes. The results obtained from the reporter assay are consistent with previous investigators who have used either electrophoretic mobility shift assay or other luciferase reporter assays to demonstrate the bioactivity of the NF-κB decoy when used in conjunction with a carrier system [206, 224-226].

6.5 Conclusion and future work

In general, the results obtained in the current study suggest that NF-κB decoy ODN-coated nanoparticles can effectively mitigate the inflammatory response for sustained periods of time with minimal cytotoxicity. When applied to an *in vitro* model of CF based on IL-1β or LPS activated CF epithelial cells, ODN-coated nanoparticles were able to reduce IL-6 and IL-8 secretion at 48 hours. Although most investigators have only observed decoy ODN effects over a

short time period [4, 204], the results obtained here are consistent with a recent study demonstrating a sustained suppression of inflammation by NF-κB decoy ODN-loaded PLGA nanoparticles [202]. Moving forward, the IB3-1 cells will be exposed to *P. aeruginosa* after treatment with the ODN-coated nanoparticles to obtain a more comprehensive understanding of the anti-inflammatory activity. Furthermore, *in vivo* testing is required to verify that the PSA-TMC nanoparticles are a safe, effective means of delivering nucleic acid based therapeutics to patients afflicted with CF.

This work was supported by NSF grant EFRI-1137186.

7. Modulation of the immune response of *Pseudomonas aeruginosa* infected IB3-1 lung epithelial cells using NF-κB decoy ODN coated polysaccharide based nanoparticles

Abstract

The work reported in chapter six shows encouraging data supporting the efficacy of using PSA-TMC as a carrier system for a decoy ODN aimed at reducing airway inflammation in CF. However, in reality, the CF airway is an extremely complicated environment, with signaling cross-talk between epithelial cells and infectious pathogens, such as *Pseudomonas aeruginosa*, one of the most difficult to treat infections associated with CF. Thus, to increase the accuracy of the *in vitro* model and build a stronger case for the use of PSA-TMC-ODN as a potential CF treatment, a bacterial/mammalian co-culture was successfully developed. Upon method development for co-culture creation, the model was used to conduct similar efficacy experiments as described in the previous chapter. However, unlike the previous chapter, an in depth analysis of inflammatory cytokine gene expression as well as protein secretion is reported and discussed in this chapter, also using slightly different time points than previously investigated. In general, in a more complex, physiologically relevant model and early infection time points of one and four hours, PSA-TMC-ODN significantly reduced IL-6 and IL-8 protein secretion, while ODN administered alone only led to non-significant decreases. This data, combined with previous studies provides a strong foundation for the use of PSA-TMC-ODN as a CF treatment, and provides a transition study from initial *in vitro* efficacy testing toward the next step, *in vivo* safety and efficacy testing.

7.1 Introduction

Cystic fibrosis (CF) is an inherited, autosomal recessive disorder hallmarked by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulatory protein (CFTR). The CFTR protein is involved in regulating chloride ion transport in epithelial membranes[227]. The defective protein affects many organs through production and buildup of heavy mucus; however, chronic lung disease as a response to chronic infection and inflammation presents the greatest risk of morbidity [195, 196]. In CF patients, chronic lung infections of opportunistic pathogens, including *Pseudomonas aeruginosa*, are a major contribution to increased morbidity and mortality. *P. aeruginosa* infections are difficult to treat, as this pathogen is associated with resistance to many antibiotic treatments, attributed in part to the ability of this organism to form biofilms, supplementing the viscous mucus already produced by the host [227]. Currently, conflicting reports exist as to whether infection causes inflammation, or innate inflammation increases the opportunity for infection, as detailed in a recent review by Dhooghe [228]. Regardless of the cause, excessive inflammation in CF is an undisputed aspect of pathology, with chronic bacterial infection being the leading cause of a perpetuated inflammatory response. Typically, an increased immune response would serve to eradicate an infection; however, in the CF lung, the initial immune response is not adequate, resulting in a chronic inflammation response [228]. Persistent inflammation leads to tissue damage, complicating treatment and contributing to increased morbidity and mortality rates.

Transcription factor nuclear factor kappa B (NF-κB) is known to play a major role in a variety of cellular processes, including regulation of the immune response. NF-κB is known to transcriptionally regulate the production of a variety of inflammatory mediators, including interleukin-8 (IL-8), interleukin-6 (IL-6), and granulocyte-macrophage colony stimulating factor

(GMCSF), cytokines directly involved in the immune response in CF [197, 229]. IL-6 stimulates B cells, leading to increased antibody production, while IL-8 recruits neutrophils to the infected area [198-200]. GMCSF is a pleiotropic cytokine involved in activating granulocytes and monocytes, increasing the immune cell presence and enhancing cytokine production [230].

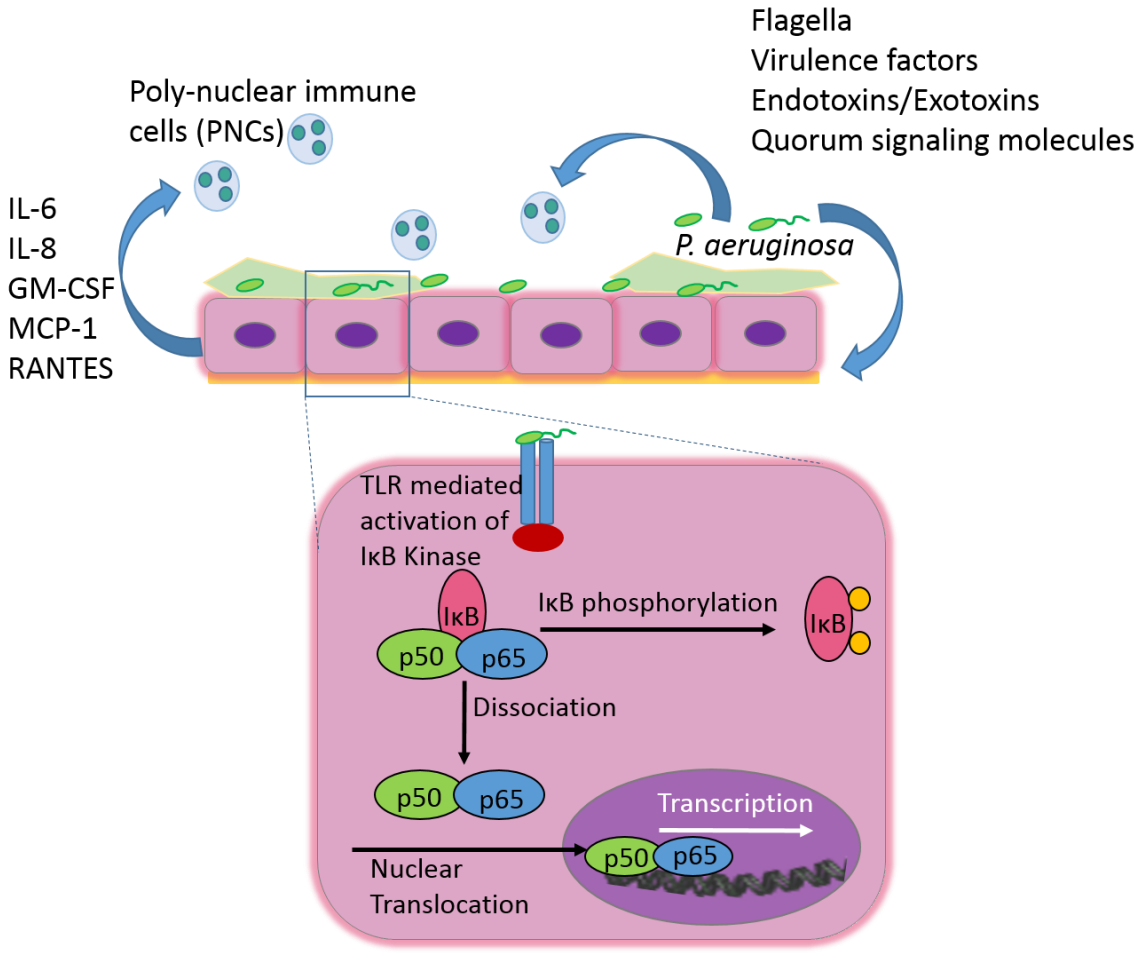

Fig. 30 -Image portraying the complexities of signaling between bacterial and mammalian cells. Specifically, we are interested in the p50/p65 complex of NF-κB.

Interaction of *P. aeruginosa* components with host cells, particularly through binding of toll like receptor four (TLR-4), leads to activation of the NF-kB pathway and subsequent up-regulation of pro-inflammatory proteins [231]. Fig. 30 illustrates the complex signaling and physical interactions of *P. aeruginosa* and the CF lung epithelium.

NF-κB transcription factor decoys (ODNs) have been proposed previously as a means to limit NF-κB activation in CF lung epithelia [203]. While several reports have shown promising *in vitro* and *in vivo* studies, limited clinical success has been seen when translating ODN therapeutics to clinical applications [4, 204-207]. ODN based therapeutics have two major drawbacks: (1) a negative charge that limits cellular penetrability and (2) an extreme susceptibility to enzymatic degradation in physiological environments [119]. To improve delivery and increase stability, a variety of methods have been proposed and tested. Viral vectors have been considered the gold standard for DNA delivery, however, these delivery vehicles are often associated with toxicity when administered repeatedly [100, 101]. Non-viral delivery systems, including lipoplexes, polyplexes, and hybrid lipid-polymer systems have been developed as alternatives [232]. However, these systems all have difficulty evading the RES system, and lipid based reagents especially have tendency to accumulate in the liver, leading to payload degradation and reduced efficacy [104, 232].

Zhang et. al reported a nanoparticle system based on complexation of polysialic acid (PSA) with N-trimethyl chitosan (TMC) as a means of drug delivery for treating rheumatoid arthritis [145, 149]. PSA and TMC are polysaccharides, attractive choices for drug delivery applications due to inherent biodegradability, non-toxicity, and ease of modification [6]. PSA acts similarly to PEG and was pioneered as a drug delivery material by Gregoradis et. al, who developed drug-PSA conjugates with extended circulation times. However, unlike PEG, PSA has no known receptors in the body and does not induce an immune response.

Chitosan is a material commonly used in DNA-polymer polyplex formation, and has been the subject of several comprehensive review articles [140, 233]. However, the positive surface charge of these polyplexes leads to rapid detection and elimination. Incorporating PSA

with a quaternized derivative of chitosan is expected to help evade RES detection, while maintaining the positive surface charge required for electrostatic association with ODN.

We have adapted the PSA-TMC nanoparticle system to be used as a carrier for small oligonucleotides. Previously, we demonstrated the anti-inflammatory efficacy of decoy ODN's delivered via PSA-TMC in a CF *in vitro* model in which soluble inflammatory mediators were used to induce inflammation [174]. In this study, we develop a mammalian-bacterial co-culture model, using human CF airway epithelial cells, and *P. aeruginosa* bacteria to model the pathology of the CF lung. In this complex CF *in vitro* model, we further demonstrate the ability of NF-κB decoy ODN coated PSA-TMC nanoparticles to reduce inflammatory gene expression and inflammatory protein secretion.

7.2 Materials and Methods

7.2.1 Materials

Polysialic acid (Colominic acid, PSA) was obtained from Nacalai, USA, Inc. (San Diego, CA, USA). N-trimethyl chitosan TMC was produced via quaternization of chitosan (MW 100 kDa- 300 kDa) purchased from Acros Organics (New Jersey, USA), as described previously by Sieval et al [163]. Sodium tripolyphosphate (TPP) was also obtained from Acros Organics (New Jersey, USA). *Pseudomonas aeruginosa* bacteria (PA01 strain) was provided by the lab of Dr. Christopher Nomura (SUNY ESF, Syracuse, NY). A live/dead cell viability staining kit was purchased from Life Technologies (Grand Island, NY). An NF-κB decoy oligonucleotide (ODN) kit containing NF-κB decoy ODN (5' CCT TGA AGG GAT TCC CTT CC 3') and a scrambled ODN (5' TTG CCG TAC CTG ACT TAG CC 3') was purchased from CosmoBio (Tokyo, Japan). A QuantiGene mRNA expression level kit was purchased from Affymetrix (Santa Clara, CA) and ELISA kits were obtained from Peprotech (Rocky Hill, NJ).

7.2.3 Mammalian cell culture

The IB3-1 cell line consists cystic fibrosis lung epithelial cells, containing the CFTR mutation. IB3-1 cells were obtained from the GCFR at Johns Hopkins University. Cells were maintained in tissue culture treated flasks coated with collagen in LHC-8 media containing 5% FBS until confluent. Cells were grown in an incubator maintained at 37 °C and 5% CO_2.

7.2.4 Bacterial cell culture

Pseudomonas aeruginosa (PA01) strain was grown on LB media agar plates at 37°C for 24 hours. At this time, 2-3 colonies were picked from the plate and grown for 24 hours in LHC-8 media without gentamicin at 37°C with shaking at 200 RPM. Serial dilution plating was performed to determine bacterial concentration in CFU/mL after 24 hours of growth.

7.2.5 Bacterial/mammalian co-culture development

IB3-1 cells were grown on collagen coated tissue culture plates in LHC-8 media with 5% FBS until confluent. To initiate a co-culture, IB3-1 cells were inoculated with PA01 at a concentration of approximately 10^5 CFU/ml in LHC-8 media without gentamicin or FBS. The co-culture was incubated for 1 hour at 37°C with 5% CO_2. After 1 hour, the supernatant was removed and replaced with LHC-8 media without gentamicin, or LHC-8 media without gentamicin with 0.4% arginine and incubation continued for one or four hours. Arginine has been shown to facilitate bacterial adhesion while maintaining mammalian cell health [234]. At the indicated time points, live/dead staining was performed to assess mammalian cell health.

7.2.6 Nanoparticle preparation and characterization

PSA-TMC nanoparticles (NP) coated with ODN (NP-ODN) were prepared as previously reported, detailed in chapter four. [145, 149, 174]. The supernatant was removed, and all

nanoparticles were resuspended in LHC-8 media to a concentration of 1 mg/ml prior to efficacy testing.

A Malvern Zetasizer NanoZS90 (Malvern Instruments, Malvern UK) was used to determine size, zeta potential, and polydispersity index of the nanoparticles. Samples were resuspended in 2.0 ml DI water, filtered through at 0.45 micron filter, and loaded into disposable cuvettes or capillary cells. All measurements were performed at 25°C.

7.2.7 *In vitro* efficacy

IB3-1 cells were plated on collagen coated 24 well plates at a density of 50,000 cells per well. LHC-8 media containing 5% FBS was used to bring the well volume to 500 µl, and the cells were incubated for two days to allow generation of a confluent monolayer. Bare PSA-TMC nanoparticles (NP), ODN, and SCO coated nanoparticles (NPODN and NPSCO, respectively) were prepared as described in section 2.5. NP formulations were resuspended in LHC-8 media without FBS at a concentration of 1 mg/ml after centrifugation, and filtered through a 0.45 µm pore size filter. Lipofectamine 2000-ODN complexes were prepared according to manufacturer instructions. Briefly, 2.5µL Lipofectamine 2000 reagent and 500 ng decoy ODN were added to 50 µL serum free LHC-8 media. After equilibration for 5 minutes, the components were then added together and incubated 15 minutes to allow for complex formation (Lipo-ODN). 500 µL of each of the following complexes were added to the prepared 24 well plates in duplicate: 1. Media alone, 2. ODN alone, 3. NPODN, 4. NP alone, 5. Lipo-ODN, 6. NPSCO, and 7. Media alone (unstimulated). Complexes were incubated with the cells for four hours/ After incubation, all complexes were removed, and replaced LHC-8 with media containing 5% FBS.

24 hours after initial treatment addition, a static co-culture was initiated. As described in section 7.2.4, PA01 bacteria was added to all wells except those corresponding to treatment 7 at

a concentration of 10^5 CFU/mL in gentamicin free LHC-8 media. After one hour incubation, supernatant was removed and immediately replaced with gentamicin free LHC-8 with 0.4% arginine. After incubation at 37°C, 5% CO2 for 1 or 4 hours, supernatant samples were collected and saved. Upon supernatant removal, as directed by manufacturer instructions, 500 µL of LHC-8 media and 250 µL QuantiGene working lysis mixture was added to each well. The volume was mixed by pipetting up and down 10 times, and cells were incubated for 30 minutes at 55°C per manufacturer instructions to complete cell lysis. Upon completion of incubation, cell lysates were placed into microcentrifuge tubes and stored at -80°C.

7.2.8 Quantitative analysis of inflammatory cytokine expression and secretion

A QuantiGene Plex 2.0 plex reagent system was used to amplify and quantify mRNA expression levels of GMCSF, IL-6, and IL-8. The kit was run on the Luminex 200 system, and all steps were carried out according to manufacturer instructions. Briefly, the reagent system employed the use of beads with varying degrees of fluorescence intensity, each different intensity representing a different analyte. The beads were coated with capture probes and hybridization extenders. mRNA is amplified, and labeled with a probe that binds streptavidin-phycoerythrin (SAPE). The Luminex 200 system was then used to separate the different bead intensities, and quantify the amount of SAPE fluorescence associated with each bead. The SAPE fluorescence intensity allows for quantification of mRNA levels. Here, we looked a 5 different analytes, including cytokine IL-6, chemokine IL-8, and growth factor GMCSF, as well as two housekeeping genes, PPIB and ACTB. The housekeeping genes were used to normalized levels of IL-6, IL-8, and GMCSF in each sample. Samples were run in duplicate, and each experiment was repeated independently three times.

IL-6, IL-8, and GMCSF ELISA kits were purchased from Peprotech (Rocky Hill, NJ) and run according to manufacturer's instructions to quantify levels of the respective proteins in collected supernatant. Samples were run in duplicate, and each experiment was repeated independently three times.

7.2.9 Statistical analysis

Protein and mRNA levels were expressed as fold changes relative to a control. The control was composed of pooled values to increase variance of the "divide by" control. Data are presented as mean ± standard deviation. To determine effect of exposure of IB31 cells to PA01 bacteria, Students T test was used to compare inflammatory protein mRNA expression between groups treated with bacteria, and groups without exposure to PA01. We have shown previously, unloaded NP and NP coated with a scrambled ODN sequence to not have a significant impact on inflammatory protein suppression[174]. To confirm, Student's t test was performed between the NP and control, and NPSCO and control groups. NP and NPSCO were then combined with the control group to create the pooled control. Likewise, as we have shown Lipo-ODN induces cytotoxicity on this cell line, we elected not to include this group in statistical analysis. Therefore, groups of interest became the pooled control, ODN alone, and NPODN. One way ANOVA, followed by a Holm-Sidak test for multiple comparisons was performed to determine significance. All statistical testing was performed with an alpha value of 0.05.

7.3 Results and Discussion

7.3.1 Co-culture development

A bacterial/mammalian co-culture model consisting of IB3-1 and PA01 was successfully generated, based on an adapted procedure reported initially by Anderson et. al

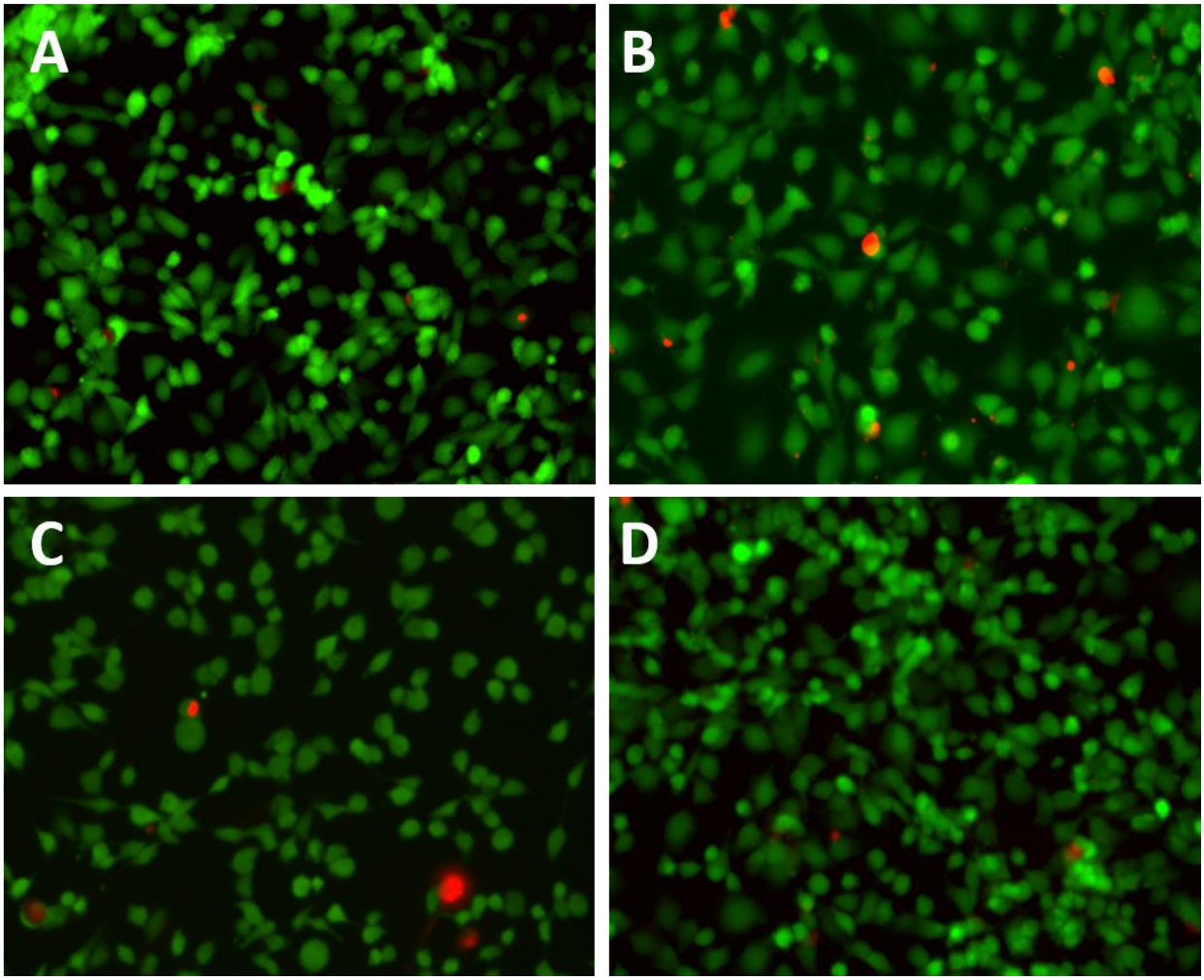

Fig. 31- After incubation with PA01 bacteria for one hour, live/dead staining was used to assess viability of IB3-1 cells. A, IB3-1 cells alone, -Arg, B., IB3-1 cells with PA01, - Arg, C., IB3-1 cells alone +Arg, D. IB3-1 cells with PA01 + Arg.

[234]. An initial screening of a PA01 bacterial dilution series ranging from 10^6 CFU/mL to 10^2 CFU/mL was performed. Live/dead staining showed IB3-1 cell health and the amount of bacteria adherence were not concentration dependent. Therefore, a bacterial concentration of 10^5 CFU/mL was chosen for all subsequent co-culture testing. The impact of arginine on mammalian cell viability in the presence of bacteria was assessed by inoculating IB3-1 cells with PA01 for 1

hour (Fig. 31) or 4 hours (Fig. 32) in gentamicin free LHC-8 media with or without 0.4% arginine. Arginine has been previously reported to facilitate bacterial attachment while maintaining mammalian cell health[234]. At one hour, the addition of arginine did not appear to have an effect on cell health, (Fig. 31); however, as shown in Fig. 32, the impact of arginine at 4 hours was apparent. PAO1 exposure without arginine supplementation enhanced mammalian cell

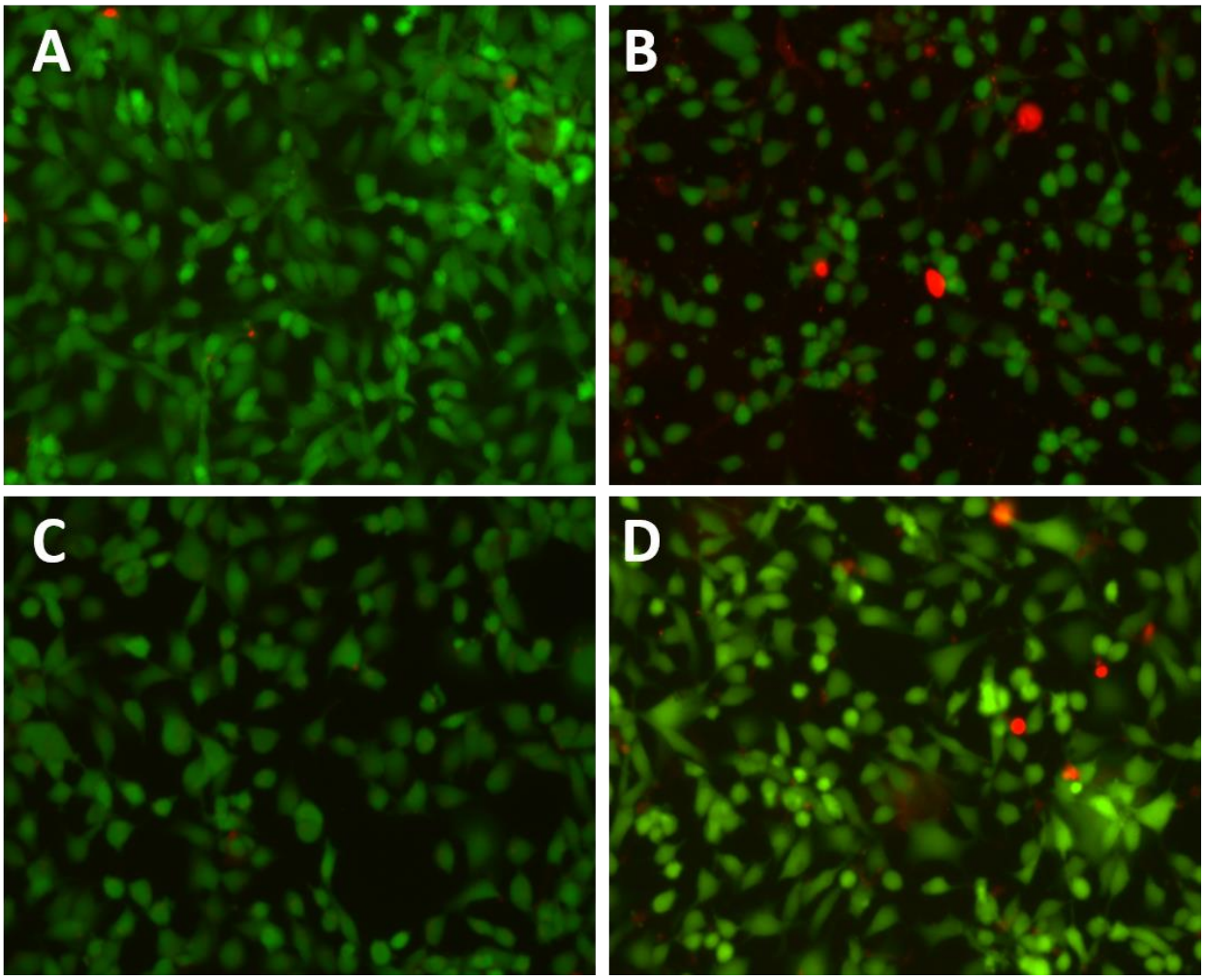

Fig. 32- After incubation with PA01 bacteria for four hours, live/dead staining was used to assess viability of IB3-1 cells. A, IB3-1 cells alone, -Arg, B., IB3-1 cells with PA01, - Arg, C., IB3-1 cells alone +Arg, D. IB3-1 cells with PA01 + Arg.

death and increased the characteristics associated dying cells, such as rounding.

7.3.2 PA01 induction of cytokine expression in IB3-1

To validate the use of the PA01-IB3-1 co-culture as a mimic for the *in vivo* inflammatory environment, the induction of inflammation was confirmed by comparing levels of IL-6, IL-8,

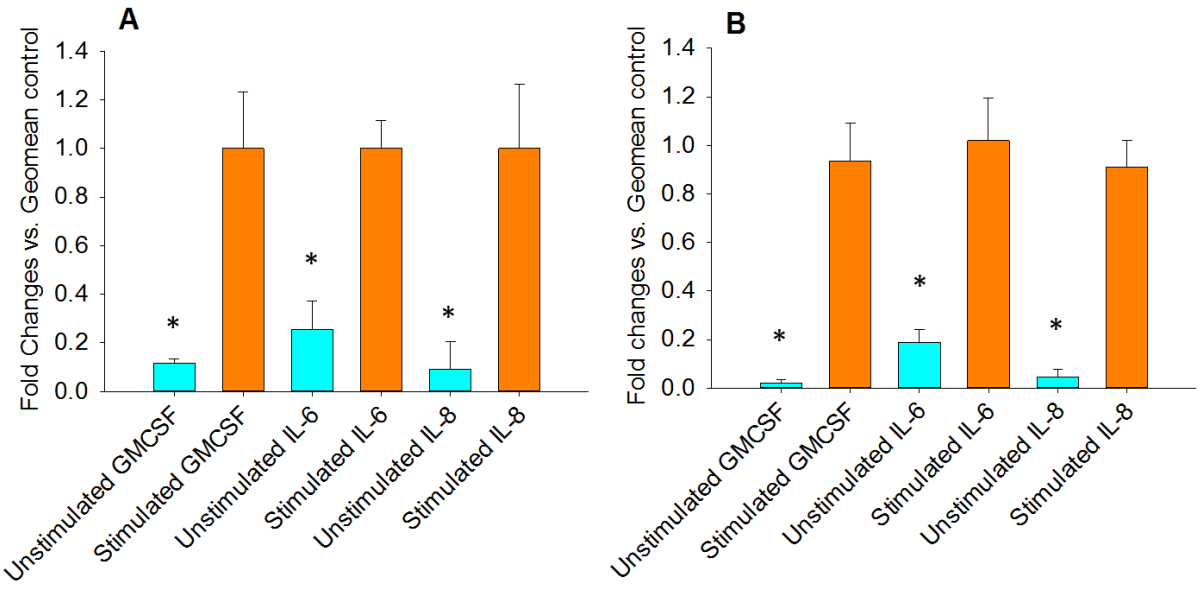

Fig. 33- Confirmation of induction of cytokine expression by IB31 cells in response to incubation with PA01 at one hour (A) and four hours (B). Results are expressed as fold changes relative to a stimulated pooled control. * Represents significant difference (alpha value 0.05) between stimulated (with bacteria) and unstimulated groups, determined by Student's T Test.

and GMCSF mRNA in groups of IB3-1 cells exposed to PA01 to un-infected control cells at one and four hours. As shown in Fig. 33, at one and four hours, levels of IL-6, IL-8, and GMCSF of unstimulated IB3-1 cell groups were significantly lower than those of stimulated IB3-1 cell groups, indicating the bacterial addition did indeed have a potent inflammatory effect on IB3-1 cells.

7.3.3 Preparation and characterization of ODN coated PSA-TMC nanoparticles

As reported previously, PSA-TMC nanoparticles alone exhibit a size of close to 100 nm (~115 nm) and a zeta potential of ~ 37 mV. When coated with 20 BP ODN, the size increases to approximately 165 nm diameter, while the zeta potential decreases to 23 mV. Although the zeta potential was reduced by the presence of the nucleic acids, the overall positive surface charge of

the nanoparticles is expected to facilitate increased interaction with the negatively charged cell membrane. Furthermore, positively charged chitosan is known to have mucoadhesive properties, allowing for increased retention time in the mucus of the CF lung epithelium [235].

7.3.4 Efficacy of ODN coated PSA-TMC nanoparticles: mRNA expression

To further validate the use of PSA-TMC nanoparticles as ODN delivery vehicles, a co-culture environment, established as described above, was used. The expression of three

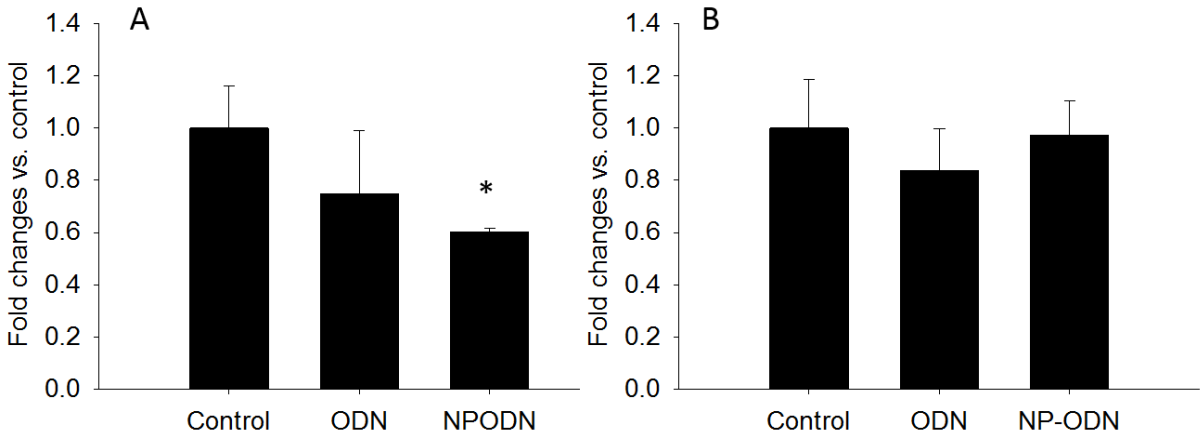

Fig. 34-Effect of ODN and NPODN treatment on GMCSF expression in IB3-1 cells in an IB3-1/PA01 co-culture model. mRNA levels are expressed relative to a pooled control, and all data are presented as mean ± standard deviation. * indicates a significant difference of $p<0.05$ relative -to the untreated control group.

proinflammatory mediators by IB3-1 cells upon exposure to PA01 and administration of ODN or NP-ODN at 1 and 4 hour time points was assessed with a QuantiGene Assay. Relative to a pooled control group, a significant decrease in GMCSF was observed at one hour when the cells were pre-treated with NPODN, as shown in Fig. 34A. However, significant differences between groups were not observed at 4 hours (Fig. 34B). GMCSF has recently been shown to enhance IL-6 production when in combination with interferon gamma (INF-γ) in CF lungs cells after P. aeruginosa infection [236]. In a healthy individual, this would likely serve to help eradicate the infection, increasing the functionality of macrophages and therefore phagocytic activity, however

in a CF lung, increased immune cell activity contributes to chronic inflammation and eventual tissue damage [237]. Thus, as observed with the ODN coated nanoparticles presented herein, agents that cause a reduction in GMCSF may serve as a viable treatment option for mitigation

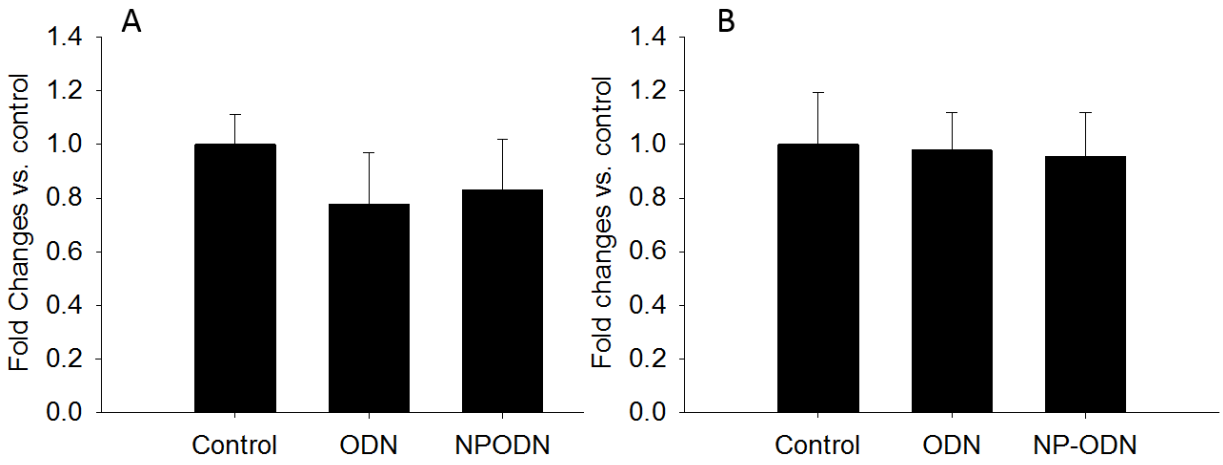

Fig. 35- Effect of ODN and NPODN treatment on IL-6 expression in IB3-1 cells in an IB3-1/PA01 co-culture model. mRNA levels are expressed relative to a pooled control, and all data are presented as mean ± standard deviation. * indicates a significant difference of $p<0.05$ relative to the untreated control group.

the long term destruction caused by inflammation.

In contrast to GMCSF, non-significant decreases in the expression of IL-6 (Fig 35) and IL-8 (Fig. 36) at one hour were observed upon pre-treatment of cells with ODN-coated nanoparticles. The results obtained for the expression levels of IL-6 and IL-8 were surprising, as we have previously seen significant decreases in these secretion of these proteins at longer time points. A literature search revealed other reports of discrepancies between cytokine expression and secretion in *in vitro* models [238, 239]. These reports, combined with previously published data reporting significant decreases in protein secretion of IL-6 and IL-8 from IB3-1 cells when treated with NPODN at time points of 24 and 48 hours prompted further investigation of IL-6

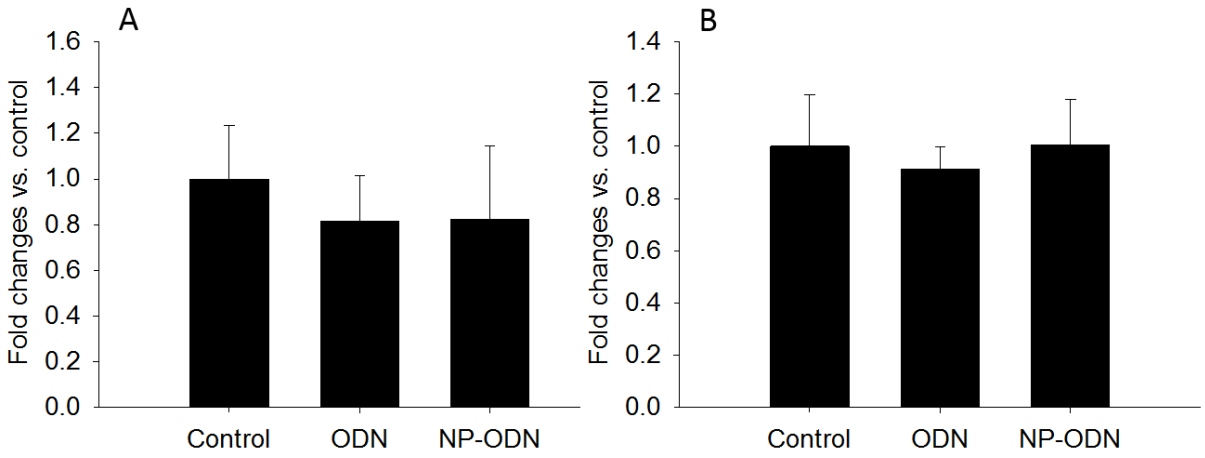

Fig. 36- Effect of ODN and NPODN treatment on IL-8 expression in IB3-1 cells in an IB3-1/PA01 co-culture model. mRNA levels are expressed relative to a pooled control, and all data are presented as mean ± standard deviation. * indicates a significant difference of $p<0.05$ relative to the untreated control group.

and IL-8 secretion [174].

7.3.5 Efficacy of ODN coated PSA-TMC nanoparticles: protein secretion

Changes in the secretion of GMCSF, IL-6, and IL-8 byIB3-1 cells within the co-culture environment in response to ODN and NPODN treatment were investigated at time-points of one

and four hours. GMCSF protein levels were below the limit of detection, as expected based on low levels of mRNA observed, thus only the results for IL-6 and IL-8 are reported herein.

In regards to IL-6, at one hour, NPODN treatment significantly reduced protein secretion from IB3-1 cells, relative to a pooled control (Fig.37A). A similar trend was observed at 4 hours;

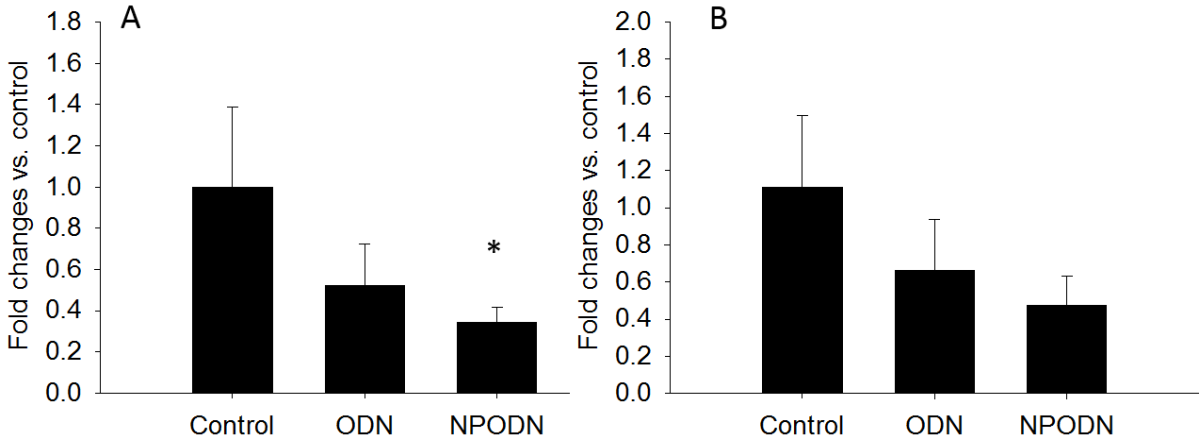

Fig. 37- Effect of ODN and NPODN treatment on IL-6 secretion in IB3-1 cells in an IB3-1/PA01 co-culture model. Protein levels are expressed relative to a pooled control, and all data are presented as mean ± standard deviation. * indicates a significant difference of $p<0.05$ relative to the untreated control group.

however, the reduction was not significant. The observed differences between IL-6 expression and secretion are consistent with results reported previously for other CF models. For example, in a CF pancreatic model, an approximately double dose of inflammatory stimulus was required to induce an increase in IL-6 expression relative to what was required to induce enhanced IL-6 secretion [239]. This suggests that secretion of inflammatory proteins, such as IL-6, may be more responsive to inhibitory treatments when compared to expression patterns of the same inflammatory proteins.

In regards to IL-8 secretion, at both one and four hours, protein levels were significantly reduced when treated with NPODN compared to both ODN alone and an untreated control (Fig.

38). Although a reduction in IL-8 secretion was observed upon treatment with ODN alone at 4 hours, the difference was not significant. Recent work by Gambari et. al reported a decrease in IL-8 mRNA expression of about 25% in IB3-1 cells when treated with an anti NF-κB agent. However, IB3-1 cells that received the same dose of the treatment exhibited 50% decrease in cytokine secretion, relative to untreated controls [238]. Similar to IL-6, IL-8 secretion may be more easily manipulated than IL-8 expression.

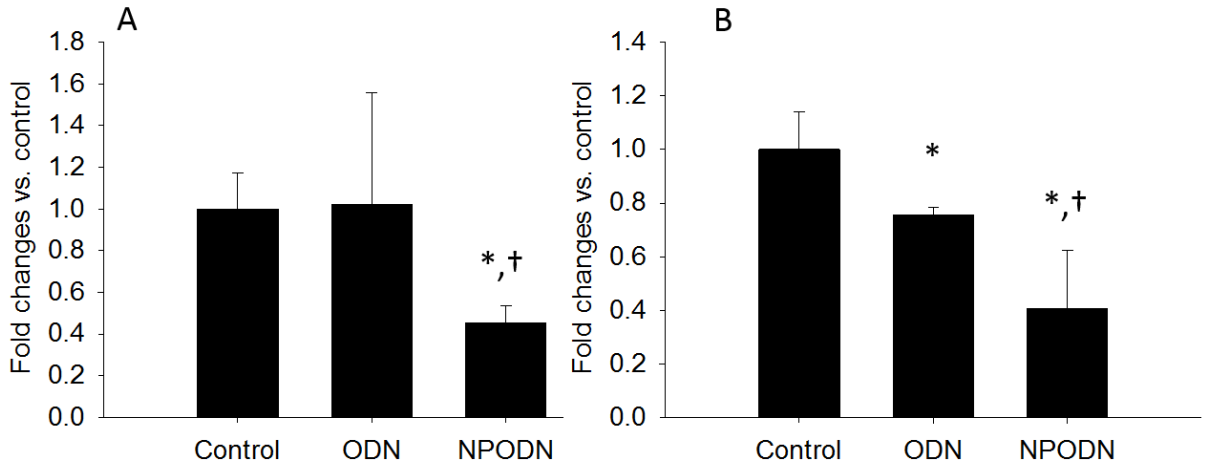

Fig. 38- Effect of ODN and NPODN treatment on IL-8 secretion in IB3-1 cells in an IB3-1/PA01 co-culture model. Protein levels are expressed relative to a pooled control, and all data are presented as mean ± standard deviation. * indicates a significant difference of $p<0.05$ relative to the untreated control group. †represents a significant difference of $p<0.05$ relative to the ODN treated group.

The levels of secreted IL-6 and IL-8 correspond well to previous studies showing reductions of these protein levels at 24 and 48 hours in response to treatment with decoy ODN delivered via PSA-TMC nanoparticles. We expect that the increase in decoy ODN efficacy is due to increased cellular uptake associated with the PSA-TMC carrier system.

Studies have shown that the inflammatory response in the CF lung is excessive, relative to levels of bacterial infection [240]. Excessive amounts of neutrophils recruited to the area by

high IL-8 levels are so vast that they cannot be cleared by macrophages, leading to cell death and debris build-up. In CF lung epithelia, it has been shown that the death of neutrophils due to bacterial infection leads to the release of DNA, which further contributes to mucus viscosity and impaired mucociliary clearance [228, 240]. Therefore, the use of decoy ODN must be carefully regulated as to not introduce excess DNA to the environment and contribute to disease pathology. Due to these restrictions, the amount of ODN loaded onto the PSA-TMC nanoparticles was kept low. As previously reported, every one mg of PSA-TMC contains approximately 750 ng of ODN. This is considerably less DNA being applied in vitro than other researchers attempting to deliver decoy ODN's in a CF model [241, 242]. The effective anti-inflammatory activity by lower amounts of ODN than previously reported provide evidence that PSA-TMC is a safe and efficacious delivery system for nucleic acid based drugs.

7.4 Conclusions and Future Work

In this study, a bacterial/mammalian co-culture in vitro model of CF was successful generated, and used to demonstrate that PSA-TMC can successfully deliver and enhance therapeutic efficacy of NF-kB decoy ODN's in a complex CF model. Although gene expression was mainly unaffected at the time points examined here, IL-6 and IL-8 protein secretion proved to be significantly lower in groups treated with ODN coated PSA-TMC at both one and four hour time points. This reduction in protein secretion is in agreement with previous studies, and suggests that PSA-TMC nanoparticles are suitable carriers for small nucleic acid based therapeutics.

8. Summary and Future Work

To conclude, this dissertation has featured an introduction detailing the need for an improved delivery system for nucleic acid based therapeutics (chapter one), followed by a chapter extensively describing challenges faced when designing drug delivery systems (chapter two). After introducing two pathologies chosen as a focus for experiments (chapter three), a polysaccharide based nanoparticle system with potential for carrying nucleic acid based drug was introduced (chapter four). Chapters five, six, and seven contained experimental methods, results and conclusions of *in vitro* efficacy experiments using a non-cytotoxic, non-immunogenic, polysaccharide based nanocarrier system for delivery of nucleic acid based therapeutics; specifically a transcription factor decoy.

The PSA-TMC nanoparticle system proved able to successfully incorporate transcription factor decoy oligonucleotides, likely via electrostatic interactions with the positively charged surface of the particles. Furthermore, the DMARD methotrexate was able to be incorporated into PSA-TMC nanoparticles as previously investigated, however this time with the addition of ODN coated on the surface. This illustrates potential for a dual treatment approach to controlling inflammation in RA patients.

PSA-TMC nanoparticles were initially designed to enhance RA treatments, however preliminary studies showed a lack of efficacy and an excess of variability in cytokine secretion when MTX loaded PSA-TMC nanoparticles were introduced to an RA *in vitro* model. Based on studies claiming enhanced efficacy of chemotherapeutics when administered in combination with anti NF-κB agents, PSA-TMC loaded with MTX and decoy ODN were tested on RA in vitro models. Initially, a cell line model was investigated (chapter five). Upon seeing success in the cell line, a primary cell model was used to obtain more physiologically relevant results. While

the primary cells yielded slightly different cytokine secretion results than the cell line, in general, combining MTX therapy with a decoy ODN resulted in significant reduction of inflammatory cytokines in both RA models.

Initial *in vitro* efficacy experiments using a simple CF model revealed using PSA-TMC as a delivery vehicle for an NF-κB decoy ODN resulted in significantly enhanced functionality of the decoy, as determined through reduction in cytokine secretion at both 24 and 48 hour time points (chapter six). Furthermore, the reduction in cytokine secretion was presumed to be due to a decrease in NF-κB activity, as determined via a luciferase reporter assay, confirming delivery and activity of the decoy ODN. To create a more accurate *in vitro* environment of a CF lung, a mammalian/bacterial co-culture model was generated, and also used to examine efficacy of PSA-TMC mediated NF-κB decoy ODN delivery (chapter seven). While a co-culture model was successfully generated, due to cell viability constraints, the time points investigated using this model were shorter than the previous model where instead of bacterial infection, inflammation was induced using soluble inflammatory mediators. Therefore, to obtain a clear picture of what was happening in this model at these time points, gene expression as well as cytokine secretion was investigated. Unexpectedly, with the exception of GMCSF at one hour, which yielded significantly lower levels compared to an untreated control when treated with PSA-TMC-ODN a reduction in expression of inflammatory genes was not observed at one and four hours. On the other hand, cytokine secretion analysis revealed significant reductions of cytokine levels at both one and four hours in groups treated with PSA-TMC-ODN.

In addition to providing a model for investigation of PSA-TMC-ODN efficacy in studies described in this thesis, the bacterial/mammalian co-culture model can be a useful tool for other *in vitro* studies. PA01 bacteria can produce different virulence factors based on different growth

conditions, allowing for a comprehensive study of bacterial/mammalian interactions under multiple bacterial phenotype conditions. In addition, future preliminary testing of drug candidates for CF can make use of the co-culture model.

Future work involving the PSA-TMC-ODN in applications aimed at treating either RA, CF, or other inflammatory conditions first and foremost involves *in vivo* testing. Despite best efforts at mimicking disease conditions *in vitro*, *in vivo* pathologies can drastically differ from cell culture models. *In vivo* testing must be done do determine both safety and efficacy of this particle system. As a mouse model has been previously used by the Bader lab to investigate efficacy of another PSA based DMARD loaded nanocarrier system for RA treatment. The mice tolerated the dosing well, and showed signs of decreased disease progression after treatment with the DMARD loaded carriers (unpublished data). Therefore, this same type of model is a logical choice for further investigation of PSA-TMC loaded with both ODN and MTX. Drug delivery systems for nucleic therapies have been examined in CF *in vivo* models as well. Based on previous successes using the rat model, this is recommended for future studies involving PSA-TMC-ODN and CF treatment efficacy.

In addition to *in vivo* work, further studies regarding how PSA-TMC acts as a protective carrier for the ODN should also be conducted. A protocol for simulated enzymatic degradation has been established, however DNA detection was not achieved likely due to the small amounts and short base pair sequences attempted to be detected. Further studies will require extensive knowledge of DNA measuring techniques, such as advanced gel electrophoresis, PCR, and potentially mass spectroscopy.

To conclude, the work described in this dissertation provides compelling evidence for the use of polysaccharide based nanoparticle system PSA-TMC as a nanocarrier delivery system for

small nucleic acids, demonstrated by the application of a transcription factor decoy ODN in several different *in vitro* models of inflammatory diseases.

9. References

1. Niidome, T. and L. Huang, *Gene therapy progress and prospects: Nonviral vectors.* Gene Therapy, 2002. **9**(24): p. 1647-1652.
2. Urban, E. and C.R. Noe, *Structural modifications of antisense oligonucleotides.* Farmaco, 2003. **58**(3): p. 243-258.
3. Shoji, Y. and H. Nakashima, *Current status of delivery systems to improve target efficacy of oligonucleotides.* Current Pharmaceutical Design, 2004. **10**(7): p. 785-796.
4. Finotti A., B.M., Bezzerri V., Nicolis E., Lampronti I., Dechecchi M. C., Mancini I., Cabrini G., Saviano M., Avitabile C., Romanelli A., Gambari R., *Effects of decoy molecules targeting NFkappaB transcription factors in cystic fibrosis IB3-1 cells.* Artificial DNA: PNA and XNA, 2012. **2**(3): p. 97-104.
5. Elsabahy M., N.A., Foldvari M., *Non viral nucleic acid delivery: Key challenges and future diretions.* Current Drug Delivery, 2011. **8**: p. 10.
6. Zhang, N., P.R. Wardwell, and R.A. Bader, *Polysaccharide-based micelles for drug delivery.* Pharmaceutics, 2013. **5**(2): p. 329-52.
7. Bader, R.A. and P.R. Wardwell, *Polysialic acid: overcoming the hurdles of drug delivery.* Ther Deliv, 2014. **5**(3): p. 235-7.
8. Buschmann, M.D., et al., *Chitosans for delivery of nucleic acids.* Adv Drug Deliv Rev, 2013. **65**(9): p. 1234-70.
9. Imbuluzqueta, E.G., Carlos; Ariza, Javier; Blanco-Prieto, Maria, *Drug delivery systems for potential treatment of intracellular bacterial infections.* Frontiers in Bioscience, 2010. **15**: p. 397-417.
10. Zuwała-Jagiełło, J., *Endocytosis mediated by receptors-function and participation in oral drug delivery.* 2003. **57**: p. 275-291.
11. Ranade, V., *Drug delviery systems 5A. Oral Drug delivery.* J. Clin. Pharmacol, 1991. **31**.
12. Langer RS, P.N., *Present and future applications of biomaterials in controlled drug delivery systems.* Biomaterials, 1981. **2**.
13. Meera George, A.E., *Polyionic hydrocolliods for the intestinal delivery of protein drugs: Alginate and chitosan-a review.* Journal of controlled release, 2006. **114**: p. 1-14.
14. Wawrezinieick, A.P., JM; Wuthrich, P, *Oral bioavailiability and drug/carrier particulate systems* Medicine and Sciences, 2008. **24**: p. 659-664.
15. Jain, K., *Strategies and technologies for drug delivery systems.* TiPS, 1998. **19**.
16. Smith, A., *Drug delivery systems in the 20th century: Merely scratching the surface.* Pharmaceutical Science and Technology Today, 1999: p. 225-227.
17. Rosen, H.A., Thierry, *The rise and rise of drug delivery.* Nature Reviews Drug Discovery, 2005.
18. Edwards, D.B.-J., Abdelaziz; Langer, Robert. , *Recent advances in pulmonary drug delivery using large, porous, inhaled particles.* J. Appl. Physiol. , 1998. **85**: p. 379-385.
19. McConnell, E.F., Hala; Basit, Abdul. , *Gut instincts: Explorations in intestinal physiology and drug delivery.* International Journal of Pharmaceutics, 2008. **364**: p. 213-226.
20. Mustara, G.D., S.M., *Approaches to oral delivery for challenging molecules.* Clinical Reviews in Therapeutic Drug Carrier Systems, 2006. **23**: p. 111-135.
21. Guy, R., *Current status and future prospects of transdermal drug delivery.* Pharmaceutical Research, 1996. **13**: p. 1765-1769.
22. Thies, C., *Microencapsulation.* Kirk-Othmer Encyclopedia of Chemical Technology, 2005.

23. Hoffman, A., *The origin and evolution of "controlled" drug delivery systems.* Journal of controlled release, 2008. **132**: p. 153-163.
24. S. S. Venkatraman, L.L.M., J. V. Natarajan, S. Chattopadhyay, Front. Biosci (Schol Ed), 2010. **2**: p. 801-814.
25. V. Weissig, S.V.B., S. M. Cheng, G.G. D'Souza, J Liposome res, 2006. **16**: p. 249-264.
26. G.K. Khuller, M.K., S. Sharma, Curr Pharm Des, 2004. **10**(3263-3274).
27. Park, J.W., Breast Cancer Res, 2002. **4**: p. 95-99.
28. M. Langner, T.E.K., Pol J Pharmacol, 1999. **51**: p. 211-222.
29. Agoram, B.W., Walter; Bolger, Michael. , *Predicting the impact of physiological and biochemical processes on oral drug bioavailability.* Advanced Drug Delivery Reviews, 2001. **50**: p. 541-567.
30. Madera, J., *Regulation of the movement of solutes across tight junctions.* Annu. Rev. Physiol., 1998. **60**: p. 143-159.
31. Gabor, F.B., E.; Weissenboeck, A.; Wirth, M, *The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery.* Advanced Drug Delivery Reviews, 2004. **56**: p. 459-480.
32. Jones, B.G., Greogry, *Physiology and pathophysiology of apoptosis in epithelial cells of the liver, pancreas, and intestine.* Am. J. Physiol. Gastrointest. Liver. Physiol., 1997. **273**.
33. Veltman, K.H., Stephanie; Chichon, Christoph; Sonnenborn, Ulrich; Schmidt,Marcus. , *Identification of specific mrRNAs targeting proteins of the apical junctional complex that stimulate the probiotic effect of E. coli Nissle 1917 on T84 epithelial cells.* The International Journal of Biochemistry and Cell Biology, 2012. **44**: p. 341-349.
34. Whitehead, K.M., Samir, *Mechanistric Analysis of chemical Permeation Enhancers for Oral Drug Delivery.* Pharmaceutical Research, 2008. **25**.
35. Deli, M., *Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery.* Biochimica et. Biophysics Acta, 2009. **1788**: p. 892-910.
36. Wang, W., et al., *Human zonulin, a potential modulator of intestinal tight junctions.* Journal of Cell Science, 2000. **113**(24): p. 4435-4440.
37. Tavelin, S., et al., *A New Principle for Tight Junction Modulation Based on Occludin Peptides.* Molecular Pharmacology, 2003. **64**(6): p. 1530-1540.
38. Benet, L.I., Takashi; Zhang, Yuanchao; Silverman, Jeffrey; Wacher, Vincent, *Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery.* Journal of controlled release, 1999. **62**: p. 25-31.
39. Clark, M.A., B.H. Hirst, and M.A. Jepson, *Lectin-mediated mucosal delivery of drugs and microparticles.* Advanced Drug Delivery Reviews, 2000. **43**(2-3): p. 207-223.
40. Bonnefille, P., et al., *The use of isolated enterocytes to study Phase I intestinal drug metabolism: Validation with rat and pig intestine.* Fundamental and Clinical Pharmacology, 2011. **25**(1): p. 104-114.
41. Nursat, A.G., M; Turner, J.R.; Colgan, S.P, *Rho protein regulates tight junctions and perijunctional actin organization in polarized epithelia.* Proc. Natl. Acad. Sci. USA, 1995. **92**: p. 10629-10633.
42. Prausnitz, M.R. and R. Langer, *Transdermal drug delivery.* Nature Biotechnology, 2008. **26**(11): p. 1261-1268.
43. Asbill, C.E.-K., Ayman; Michniak, Bozena, *Enhancement of Transdermal Drug Delivery: Chemical and Physical Approaches.* Critical Reviews in Therapeutic Drug Carrier Systems, 2000. **17**(6): p. 621-658.
44. Kooiman, K., et al. *Transiently increased endothelial layer permeability by ultrasound-activated microbubbles*. in *Proceedings - IEEE Ultrasonics Symposium*. 2006.
45. Hajitou, A., R. Pasqualini, and W. Arap, *Vascular targeting: Recent advances and therapeutic perspectives.* Trends in Cardiovascular Medicine, 2006. **16**(3): p. 80-88.

46. Charoenphol, P., et al., *Targeting therapeutics to the vascular wall in atherosclerosis-Carrier size matters.* Atherosclerosis, 2011. **217**(2): p. 364-370.
47. Molema, G., L.F.M.H. De Leij, and D.K.F. Meijer, *Tumor vascular endothelium: Barrier or target in tumor directed drug delivery and immunotherapy.* Pharmaceutical Research, 1997. **14**(1): p. 2-10.
48. Norris, D.P., Navneet; Sinko, Patrick. , *The effect of physical barriers and properties on the oral absorption of particulates.* Advanced Drug Delivery Reviews, 1998. **34**: p. 135-143.
49. Bhat, P.F., Douglas; Donovan, Maureen *The limiting role of mucus in drug absorption: drug permeation through mucus solution.* International Journal of pharmaceutics, 1995. **126**: p. 179-187.
50. Bhat, P.F., Douglas; Donovan, Maureen, *Drug binding to gastric mucus glycoproteins.* International Journal of Pharmaceutics, 1996. **134**: p. 15-25.
51. Hillery, A.L., Andew; Swarbrick, James, *Drug Delivery and Targeting for Pharmacists and Pharmaceutical Scientists.* 2001: p. 2-47.
52. Mahato, R.N., Ajit, *Pharmaceutical Dosage Forms and Drug Delivery*, R. Mahato, Editor., CRC Press.
53. Jones, A.G., Mark; Duncan, Ruth, *Understanding endocytotic pathways and intracellular trafficking: A prerequisite for effective design of advanced drug delivery systems.* Advanced Drug Delivery Reviews, 2003. **55**: p. 1353-1357.
54. Y. Masaoka, Y.T., M. Kataoka, S. Sakuma, S. Yamashita, *Site of drug absorption after oral administration: assesment of membrane permeability and luminal concentration of drugs in each segment of gastrointestinal tract.* Eur. J. Pharm. Sci., 2006. **29**: p. 240-250.
55. Y. Chen, L.L., *Modern methods for delivery of drugs across the blood brain barrier.* Advanced Drug Delivery Reviews, 2011.
56. Scherrmann, J.M., *Drug delivery to the brain via the blood brain barrier.* Vascular Pharmacology, 2002. **28**: p. 349-354.
57. Wolka, A.H., Jason; Davis, Thomas, *Pain and the blood brain barrier: obstacles to drug delivery.* Advanced Drug Delivery Reviews, 2003. **55**: p. 987-1006.
58. Abbott, J.R., Ignacio, *Transporting therapeutics across the blood brain barrier.* Molecular Medicine Today, 1996.
59. Drees, A., et al., *The multidrug resistance protein BMDP/ABCG2: A new and highly relevant efflux pump at the blood-brain barrier*, in *International Congress Series*, A.G. Boer, Editor. 2005. p. 154-168.
60. Taylor, E.M., *The impact of efflux transporters in the brain on the development of drugs for CNS disorders.* Clinical Pharmacokinetics, 2002. **41**: p. 81-92.
61. Tsuji, A. and I. Tamai, *Blood-brain barrier function of P-glycoprotein.* Advanced Drug Delivery Reviews, 1997. **25**(2-3): p. 287-298.
62. Veber, D.J.S.C., Hung-Yuan; Smith, Brian; Ward, Keith; Kopple, Kenneth, *Molecular properties that influence the oral bioavailability of drug candidates.* J. Med. Chem., 2002. **45**: p. 2615-2623.
63. Schmucker, D.L., *Liver function and phase 1 drug metabolism in the elderly: A paradox.* Drugs and Aging, 2001. **18**: p. 837-851.
64. Liddel, C.S., Catherine, *Hepatic Metabolism of Drugs.* Gastro Hep. **2**: p. 241-249.
65. Park, B.K., et al., *The role of metabolic activation in drug-induced hepatotoxicity*. 2005. p. 177-202.
66. Benet, L.W., Chi-Yuan; Hebert, Mary; Wacher, Vincent, *Intestinal drug metabolism and antitransport processes: A potential paradigm shift in oral drug delivery.* Journal of controlled release, 1996. **39**: p. 139--143.

67. Zhang, Y.B., Leslie, *The gut as a barrier to drug absorption: The comined forle of Cytochrome P4503A and P-glycoprotein.* Clinical Pharmacokinetics, 2001. **40**: p. 159-168.
68. Zhou, S., et al., *Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs.* Clinical Pharmacokinetics, 2005. **44**(3): p. 279-304.
69. Wacher, V.S., Laurent; Benet, Leslie, *Active secretion and enterocytic drug metabolism barriers to drug absorption.* Advanced Drug Delivery Reviews, 1996. **20**: p. 99-112.
70. Kwan, K.C., *Oral bioavailability and first pass effects.* Drug Delivery Today, 1997. **25**: p. 1329-1336.
71. Petrak, K., *Essential properties of drug-targeting delivery systems.* Drug Delivery Today, 2005. **10**.
72. Singh, R.M., P; Lalla, J.K, *A diffusion controlled drug delivery system for theophylline.* Drug Development and Industrial Pharmacy, 1994. **20**: p. 1225-1238.
73. Leichty, W.K., David; Slaughter, Brandon; Peppas, , *Polymers for drug delivery systems.* Annu. Rev. Chem. Biomed. Eng., 2010. **1**: p. 149-173.
74. Berkland, C.K., Matt; Narasimhan, Balaji; Kim, Kevin; Pack, Daniel, *Microsphere size, precipitation kinetics, and drug distribution control drug releae from biodegradable polyanhydride microspheres.* Journal of controlled release, 2004. **94**: p. 129-141.
75. Soppimath, K.A., Tejraj M.; Kulkarni, Anandrao; Rudzinski, Walter, *Biodegradable polymeric nanoparticles as drug delivery devices.* Journal of controlled release, 2001. **70**: p. 1-20.
76. Minko, T., *Soluble polymer conjugates for drug delivery.* Drug Discovery Today: Technologies, 2005. **2**.
77. Torchillin, V., *Tumor delivery of macromolecular drugs based on the EPR effect.* Advanced Drug Delivery Reviews, 2011. **63**: p. 131-135.
78. Muller, R.K.C., *Challenges and solutions for the delivery of biotech drugs: A reveiw of drug nanocrystal technologies and lipid nanoparticles.* Journal Of Biotechnology, 2004. **113**: p. 151-170.
79. Crommelin, D.J.A.S., G.; Storm, G, *Active targeting with particulate carrier systems in the blood compartment.* Advanced Drug Delivery Reviews, 1995. **17**: p. 49-60.
80. Knop, K., et al., *Poly(ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives.* Angewandte Chemie International Edition, 2010. **49**(36): p. 6288-6308.
81. Plapied, L.D., Nicolas; des Rieux, Anne; Preat, Veronique, *Fate of polymeric nanocarriers for oral drug delivery.* Current Opinion in Colloid and Interface Science, 2011. **16**: p. 228-237.
82. Oerlemans C, B.W., Bos M, Storm G, Nijsen JF, Hennick WE, *Polymeric micelles in anticancer therapy: targeting, imaging, and triggered release.* Pharm Res., 2010. **12**: p. 2569-89.
83. Kataoka, K.H., Atsushi; Nagasaki, Yukio, *Block copolymer micelles for drug delivery: design, characterization, and biological significance.* Advanced Drug Delivery Reviews, 2001. **47**: p. 113-131.
84. Singh R, L.J.J., *Nanoparticle based targeted drug delivery.* Exp. Mol. Pathol., 2009. **86**: p. 215-223.
85. Singh, R. and J.W. Lillard Jr, *Nanoparticle-based targeted drug delivery.* Experimental and Molecular Pathology, 2009. **86**(3): p. 215-223.
86. Todoroff, J.V., Rita, *Fate of nanomedicines in the lungs.* Current Opinion in Colloid and Interface Science, 2011. **16**: p. 246-254.
87. Sharma, A.S., Uma, *Liposomes in drug delivery: progress and limitations.* International Journal of Pharmaceutics, 1997. **154**: p. 123-140.

88. Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A, *Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half lives in vivo.* Biochimica et. Biophysics Acta, 1991. **1066**: p. 29-36.
89. Lukyanov, A.N., et al., *Tumor-targeted liposomes: doxorubicin-loaded long-circulating liposomes modified with anti-cancer antibody.* Journal of Controlled Release, 2004. **100**(1): p. 135-144.
90. Rosenecker, J., S. Huth, and C. Rudolph, *Gene therapy for cystic fibrosis lung disease: Current status and future perspectives.* Current Opinion in Molecular Therapeutics, 2006. **8**(5): p. 439-445.
91. Greenwald, R.C., Yun; Mcguire, Jeffrey; Conover, Charles, *Effective drug delivery by PEGylated drug conjugates.* Advanced Drug Delivery Reviews, 2003. **55**: p. 217-250.
92. Milas, L.M., Kathryn; Hunter, Nancy; Li, Chun; Wallace, Sidney. , *Poly(L-glutamic acid)-paclitaxel conjugate is a potent enhancer of turmor radiocurability.* International Journal of Radiation Oncology* Biology* Physics, 2003. **55**: p. 707-712.
93. Elvira, C.G., Alberto; Roman, Julio; Cifuentes, Alejandro, *Covalent Polymer-Drug conjugates.* Molecules, 2005. **10**: p. 114-125.
94. Dash, A. and G. Cudworth Ii, *Therapeutic applications of implantable drug delivery systems.* Journal of Pharmacological and Toxicological Methods, 1998. **40**(1): p. 1-12.
95. Xu, L. and T. Anchordoquy, *Drug delivery trends in clinical trials and translational medicine: Challenges and opportunities in the delivery of nucleic acid-based therapeutics.* Journal of Pharmaceutical Sciences, 2011. **100**(1): p. 38-52.
96. Kay, M.A., J.C. Glorioso, and L. Naldini, *Viral vectors for gene therapy: The art of turning infectious agents into vehicles of therapeutics.* Nature Medicine, 2001. **7**(1): p. 33-40.
97. Boulaiz H., M.J.A., Prados J., Melguzio C., Aranega A., *Non viral and viral vectors for gene therapy.* Cellular and Molecular Biology, 2005. **51**: p. 19.
98. Ratko, T.A., et al., *Clinical gene therapy for nonmalignant disease.* The American Journal of Medicine, 2003. **115**(7): p. 560-569.
99. Daya, S. and K.I. Berns, *Gene therapy using adeno-associated virus vectors.* Clinical Microbiology Reviews, 2008. **21**(4): p. 583-593.
100. Jorgensen, C. and F. Apparailly, *Prospects for gene therapy in inflammatory arthritis.* Best Pract Res Clin Rheumatol, 2010. **24**(4): p. 541-52.
101. Fabre, S. and F. Apparailly, *Gene therapy for rheumatoid arthritis: current status and future prospects.* BioDrugs, 2011. **25**(6): p. 381-91.
102. Ilies, M.A., W.A. Seitz, and A.T. Balaban, *Cationic lipids in gene delivery: Principles, vector design and therapeutical applications.* Current Pharmaceutical Design, 2002. **8**(27): p. 2441-2473.
103. Hoekstra, D., et al., *Gene delivery by cationic lipids: In and out of an endosome.* Biochemical Society Transactions, 2007. **35**(1): p. 68-71.
104. Thomas, M., et al., *Non-viral siRNA delivery to the lung.* Advanced Drug Delivery Reviews, 2007. **59**(2–3): p. 124-133.
105. Dokka, S., et al., *Oxygen radical-mediated pulmonary toxicity induced by some cationic liposomes.* Pharmaceutical Research, 2000. **17**(5): p. 521-525.
106. Lv, H., et al., *Toxicity of cationic lipids and cationic polymers in gene delivery.* Journal of Controlled Release, 2006. **114**(1): p. 100-109.
107. Kim, Y.H., et al., *Polyethylenimine with acid-labile linkages as a biodegradable gene carrier.* Journal of Controlled Release, 2005. **103**(1): p. 209-219.
108. Aradhya, S. and D.L. Nelson, *NF-κB signaling and human disease.* Current Opinion in Genetics & Development, 2001. **11**(3): p. 300-306.

109. Kumar, A., et al., *Nuclear factor-kappaB: its role in health and disease.* J Mol Med (Berl), 2004. **82**(7): p. 434-48.
110. Makarov, S.S., *NF-KB in rheumatoid arthritis: A pivotal regulator of inflammation, hyperplasia, and tissue destruction.* Arthritis Research, 2001. **3**(4): p. 200-206.
111. Hayden M.S, G.S., *Shared principles in NF-kB signaling.* Cell, 2008. **132**.
112. Zvereff, V.V., et al., *Cystic fibrosis carrier screening in a North American population.* Genetics in Medicine, 2014. **16**(7): p. 539-546.
113. Buchanan, P.J., et al., *Role of CFTR, Pseudomonas aeruginosa and Toll-like receptors in cystic fibrosis lung inflammation.* Biochemical Society Transactions, 2009. **37**(4): p. 863-867.
114. Kurbatova, P., et al., *Model of mucociliary clearance in cystic fibrosis lungs.* Journal of Theoretical Biology, 2015. **372**(0): p. 81-88.
115. Jacquot, J., et al., *Airway epithelial cell inflammatory signalling in cystic fibrosis.* The International Journal of Biochemistry & Cell Biology, 2008. **40**(9): p. 1703-1715.
116. Stecenko, A.A., et al., *Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells.* Inflammation, 2001. **25**(3): p. 145-155.
117. Dauletbaev, N., et al., *Ibuprofen modulates NF-κB activity but not IL-8 production in cystic fibrosis respiratory epithelial cells.* Respiration, 2010. **79**(3): p. 234-242.
118. Deligianni, E., et al., *Pseudomonas aeruginosa cystic fibrosis isolates of similar RAPD genotype exhibit diversity in biofilm forming ability in vitro.* BMC Microbiology, 2010. **10**.
119. Ahmad M. Z., A.S., Mallik N., Anwar M., Tabassum W., Ahmad F. J., *Application of Decoy Oligonucletodies as Novel Therapeutic Strategy: A Contemporary Overview.* Current Drug Discovery Technologies, 2013. **10**: p. 71-84.
120. Wardwell PR, W.D., Garner DL, Bader RA, *Pseudomonas Quinolone Signal Modulates Cystic Fibrosis Epithelial Cell Response through the Toll-Like Receptor 4.* SOJ Immunology, 2015. **3**(1): p. 1-5.
121. Tsukuda, Y., et al., *Ganglioside GM3 Has an Essential Role in the Pathogenesis and Progression of Rheumatoid Arthritis.* PLoS ONE, 2012. **7**(6): p. e40136.
122. Venkatesha, S.H., et al., *Cytokine-modulating strategies and newer cytokine targets for arthritis therapy.* International Journal of Molecular Sciences, 2015. **16**(1): p. 887-906.
123. Turner, J.D. and A. Filer, *The role of the synovial fibroblast in rheumatoid arthritis pathogenesis.* Current Opinion in Rheumatology, 2015. **27**(2): p. 175-182.
124. Isomäki, P. and J. Punnonen, *Pro- and anti-inflammatory cytokines in rheumatoid arthritis.* Annals of Medicine, 1997. **29**(6): p. 499-507.
125. Georganas, C., et al., *Regulation of IL-6 and IL-8 Expression in Rheumatoid Arthritis Synovial Fibroblasts: the Dominant Role for NF- B But Not C/EBP or c-Jun.* The Journal of Immunology, 2000. **165**(12): p. 7199-7206.
126. Gerards, A.H., et al., *Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis.* Rheumatology, 2003. **42**(10): p. 1189-1196.
127. De Keyser, F., *Choice of Biologic Therapy for Patients with Rheumatoid Arthritis: The Infection Perspective.* Current Rheumatology Reviews, 2011. **7**: p. 77-87.
128. Wagoner, K.L. and R.A. Bader, *Evaluation of SV40-transformed synovial fibroblasts in the study of rheumatoid arthritis pathogenesis.* Rheumatol Int, 2012. **32**(7): p. 1885-91.
129. Büning, H., M. Braun-Falco, and M. Hallek, *Progress in the use of adeno-associated viral vectors for gene therapy.* Cells Tissues Organs, 2004. **177**(3): p. 139-150.
130. Glebova, K.V., et al., *Therapeutic siRNAs and nonviral systems for their delivery.* Molecular Biology, 2012. **46**(3): p. 335-348.

131. Gomes, C.P., et al., *Translating chitosan to clinical delivery of nucleic acid-based drugs.* Mrs Bulletin, 2014. **39**(1): p. 60-70.
132. Nitta, S.K. and K. Numata, *Biopolymer-based nanoparticles for drug/gene delivery and tissue engineering.* Int J Mol Sci, 2013. **14**(1): p. 1629-54.
133. Zillies, J. and C. Coester, *Evaluating gelatin based nanoparticles as a carrier system for double stranded oligonucleotides.* J Pharm Pharm Sci, 2005. **7**(4): p. 17-21.
134. Csaba, N., M. Koping-Hoggard, and M.J. Alonso, *Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery.* Int J Pharm, 2009. **382**(1-2): p. 205-14.
135. Gregoriadis, G., et al., *Polysialic acids: potential role in therapeutic constructs.* Biotechnol Genet Eng Rev, 1999. **16**: p. 203-15.
136. Gregoriadis, G., et al., *Polysialic acids: potential in improving the stability and pharmacokinetics of proteins and other therapeutics.* Cell Mol Life Sci, 2000. **57**(13-14): p. 1964-9.
137. Gregoriadis, G., et al., *Improving the therapeutic efficacy of peptides and proteins: A role for polysialic acids.* International Journal of Pharmaceutics, 2005. **300**(1-2): p. 125-130.
138. Semple S.C. Harasym T.O., C.K.A., Ansell S. M., Klimuk S.K., Hope M.J., *Immunogenicity and Rapid Blood Clearance of Liposomes Containing Polyethylene Glycol-Lipid Conjugates and Nucleic Acids.* The Journal of Pharmacology and Experimental Therapeutics, 2005. **312**(3): p. 1020-1026.
139. Hamad, I., et al., *Poly(ethylene glycol)s generate complement activation products in human serum through increased alternative pathway turnover and a MASP-2-dependent process.* Molecular Immunology, 2008. **46**(2): p. 225-232.
140. Mao, S., W. Sun, and T. Kissel, *Chitosan-based formulations for delivery of DNA and siRNA.* Advanced Drug Delivery Reviews, 2010. **62**(1): p. 12-27.
141. Knop K., H.R., Fischer D., Schubert U., *Poly(ethylene glycol) in Drug Delivery: Pros and Cons as Well as Potential Alternatives.* Angewandte Chemie, 2010. **49**: p. 6288-6308.
142. Webster R., D.E., Harris P., Siegel N., Stadler J., Tilbury L, Smith D., *PEGylated proteins: Evaluation of Their Safety in the Absense of Definitive Metabolism Studies.* Metabolism and Disposition, 2007. **35**(1).
143. Caliceti, P. and F.M. Veronese, *Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates.* Adv Drug Deliv Rev, 2003. **55**(10): p. 1261-77.
144. Knop, K., et al., *Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives.* Angew Chem Int Ed Engl, 2010. **49**(36): p. 6288-308.
145. Zhang, N., P.R. Wardwell, and R.A. Bader, *In Vitro Efficacy of Polysaccharide-Based Nanoparticles Containing Disease-Modifying Antirheumatic Drugs.* Pharm Res, 2014.
146. De Stefano, D.D.R.G., Maiuri M. C., Ungaro F., Quaglia F., Iuvone T., Cinelli M. P., La Rotonda M. I., Carnuccio R., *Oligonucleotide decoy to NF-kB slowly released from PLGA microspheres reduced chronic inflammation in rat.* Pharmacological Research, 2009. **60**: p. 33-40.
147. Csaba N., K.-H.M., Alonso M. J., *Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery.* International Journal of Pharmaceutics, 2009. **382**: p. 205-214.
148. De Rosa, G. and M.I. La Rotonda, *Nano and microtechnologies for the delivery of oligonucleotides with gene silencing properties.* Molecules, 2009. **14**(8): p. 2801-23.
149. Zhang, N., Bader, R.A., *Synthesis and characterization of polysialic acid-N-trimethyl chitosan nanoparticles for drug delivery.* Nano LIFE, 2012. **2**(3).
150. Bordi, F., et al., *Chitosan–DNA complexes: Effect of molecular parameters on the efficiency of delivery.* Colloids and Surfaces A: Physicochemical and Engineering Aspects, 2014. **460**(0): p. 184-190.

151. Min, S.H., K.C. Park, and Y.I. Yeom, *Chitosan-mediated non-viral gene delivery with improved serum stability and reduced cytotoxicity.* Biotechnology and Bioprocess Engineering, 2015. **19**(6): p. 1077-1082.
152. Lin, W.J. and W.Y. Hsu, *Pegylation effect of chitosan based polyplex on DNA transfection.* Carbohydrate Polymers, 2015. **120**(0): p. 7-14.
153. Jiang, X., et al., *Chitosan-g-PEG/DNA complexes deliver gene to the rat liver via intrabiliary and intraportal infusions.* Journal of Gene Medicine, 2006. **8**(4): p. 477-487.
154. Zhang, X., et al., *Preparation of arginine modified PEI-conjugated chitosan copolymer for DNA delivery.* Carbohydrate Polymers, 2015. **122**: p. 53-59.
155. Xu, T., S. Wang, and Z. Shao, *Insight into polycation chain length affecting transfection efficiency by O-methyl-free N,N,N-trimethyl chitosans as gene carriers.* Pharmaceutical Research, 2014. **31**(4): p. 895-907.
156. Zhang N., B.R., *Synthesis and characterization of polysialic acid-N-trimethyl chitosan nanoparticles for drug delivery.* NanoLife, 2012.
157. Wilson, D.R., et al., *Synthesis and evaluation of cyclosporine A-loaded polysialic acid-polycaprolactone micelles for rheumatoid arthritis.* Eur J Pharm Sci, 2014. **51**: p. 146-56.
158. Bader, R.A., A.L. Silvers, and N. Zhang, *Polysialic Acid-Based Micelles for Encapsulation of Hydrophobic Drugs.* Biomacromolecules, 2011. **12**(2): p. 314-320.
159. Boddohi, S., et al., *Polysaccharide-based polyelectrolyte complex nanoparticles from chitosan, heparin, and hyaluronan.* Biomacromolecules, 2009. **10**(6): p. 1402-9.
160. Janes, K.A., P. Calvo, and M.J. Alonso, *Polysaccharide colloidal particles as delivery systems for macromolecules.* Adv Drug Deliv Rev, 2001. **47**(1): p. 83-97.
161. Nagpal, K., S.K. Singh, and D.N. Mishra, *Chitosan nanoparticles: a promising system in novel drug delivery.* Chem Pharm Bull (Tokyo), 2010. **58**(11): p. 1423-30.
162. Nafee, N., et al., *Chitosan-coated PLGA nanoparticles for DNA/RNA delivery: effect of the formulation parameters on complexation and transfection of antisense oligonucleotides.* Nanomedicine, 2007. **3**(3): p. 173-83.
163. Sieval, A.B., et al., *Preparation and NMR characterization of highly substituted N-trimethyl chitosan chloride.* Carbohydrate Polymers, 1998. **36**(2-3): p. 157-165.
164. Dragan, E.S., M. Mihai, and S. Schwarz, *Complex nanoparticles based on chitosan and ionic/nonionic strong polyanions: formation, stability, and application.* ACS Appl Mater Interfaces, 2009. **1**(6): p. 1231-40.
165. Fredheim, G.E., *Polyelectrolyte Complexes: INteractions between lignosulfonate and chitosan.* Biomacromolecules, 2003. **4**: p. 232-239.
166. Otero, M. and M.B. Goldring, *Cells of the synovium in rheumatoid arthritis. Chondrocytes.* Arthritis Research and Therapy, 2007. **9**(5).
167. Sung, J.-Y., et al., *Methotrexate suppresses the interleukin-6 induced generation of reactive oxygen species in the synoviocytes of rheumatoid arthritis.* Immunopharmacology, 2000. **47**(1): p. 35-44.
168. Neumann, E., et al., *Rheumatoid arthritis progression mediated by activated synovial fibroblasts.* Trends in Molecular Medicine, 2010. **16**(10): p. 458-468.
169. van Loo, G. and R. Beyaert, *Negative regulation of NF-κB and its involvement in rheumatoid arthritis.* Arthritis Research and Therapy, 2011. **13**(3).
170. Georganas, C., et al., *Regulation of IL-6 and IL-8 expression in rheumatoid arthritis synovial fibroblasts: The dominant role for NF-κB but not C/EBPβ or c-Jun.* Journal of Immunology, 2000. **165**(12): p. 7199-7206.

171. Roman-Blas, J.A. and S.A. Jimenez, *NF-κB as a potential therapeutic target in osteoarthritis and rheumatoid arthritis.* Osteoarthritis and Cartilage, 2006. **14**(9): p. 839-848.
172. Morishita, R., et al., *Molecular therapy to inhibit NFκB activation by transcription factor decoy oligonucleotides.* Current Opinion in Pharmacology, 2004. **4**(2): p. 139-146.
173. Pietersz G.A., T.C.-K., Apostolopoulos V., *Structure and Design of Polycationic Carriers for Gene Delivery.* Mini-Reviews in Medicinal Chemistry, 2006. **6**: p. 1285-1298.
174. Wardwell P.R., B.R.A., *Immunomodulation of cystic fibrosis epithelial cells via NF-kB decoy oligonucleotide-coated polysaccharide nanoparticles.* Journal of Biomedical Materials Part A, 2014.
175. Zimmermann, T., et al., *Isolation and characterization of rheumatoid arthritis synovial fibroblasts from primary culture - Primary culture cells markedly differ from fourth-passage cells.* Arthritis Research, 2001. **3**(1): p. 72-76.
176. Choi EM, L.Y., *Luteloin suppresses IL-1 Beta induced cytokines and MMPs production via p39 MAPK, JNK, NF-KappaB and AP-1 activation in human synovial sarcoma line, W982.* Food Chem Toxicol, 2010. **48**(10): p. 2607-11.
177. Choi, E.M. and Y.S. Lee, *Effects of hesperetin on the production of inflammatory mediators in IL-1β treated human synovial cells.* Cellular Immunology, 2010. **264**(1): p. 1-3.
178. Hashizume, M. and M. Mihara, *The Roles of Interleukin-6 in the Pathogenesis of Rheumatoid Arthritis.* Arthritis, 2011. **2011**: p. 765624.
179. Feldmann, M., F.M. Brennan, and R.N. Maini, *Role of cytokines in rheumatoid arthritis*, in *Annual Review of Immunology*. 1996. p. 397-440.
180. Majumdar, S. and B.B. Aggarwal, *Methotrexate suppresses NF-κB activation through inhibition of IκBα phosphorylation and degradation.* Journal of Immunology, 2001. **167**(5): p. 2911-2920.
181. Simmonds, R.E. and B.M. Foxwell, *Signalling, inflammation and arthritis: NF-κB and its relevance to arthritis and inflammation.* Rheumatology, 2008. **47**(5): p. 584-590.
182. Kamimura, K., et al., *Advances in gene delivery systems.* Pharmaceutical Medicine, 2011. **25**(5): p. 293-306.
183. Duncan, R., *The dawning era of polymer therapeutics.* Nature Reviews Drug Discovery, 2003. **2**(5): p. 347-360.
184. Moghimi, S.M., A.C. Hunter, and J.C. Murray, *Long-circulating and target-specific nanoparticles: Theory to practice.* Pharmacological Reviews, 2001. **53**(2): p. 283-318.
185. Xu, H., et al., *Distribution of lymphatic vessels in normal and arthritic human synovial tissues.* Ann Rheum Dis, 2003. **62**(12): p. 1227-9.
186. Afuwape, A.O., S. Kiriakidis, and E.M. Paleolog, *The role of the angiogenic molecule VEGF in the pathogenesis of rheumatoid arthritis.* Histology and Histopathology, 2002. **17**(3): p. 961-972.
187. Wechalekar, M.D. and M.D. Smith, *Utility of arthroscopic guided synovial biopsy in understanding synovial tissue pathology in health and disease states.* World Journal of Orthopaedics, 2014. **5**(5): p. 566-573.
188. Loetscher, P., et al., *Monocyte chemoattractant protein 1 and interleukin 8 production by rheumatoid synoviocytes. Effects of anti-rheumatic drugs.* Cytokine, 1994. **6**(2): p. 162-170.
189. Kraan, M.C., et al., *Inhibition of neutrophil migration soon after initiation of treatment with leflunomide or methotrexate in patients with rheumatoid arthritis: Findings in a prospective, randomized, double-blind clinical trial in fifteen patients.* Arthritis and Rheumatism, 2000. **43**(7): p. 1488-1495.
190. Nishina, N., et al., *Reduction of plasma IL-6 but not TNF-α by methotrexate in patients with early rheumatoid arthritis: A potential biomarker for radiographic progression.* Clinical Rheumatology, 2013. **32**(11): p. 1661-1666.

191. Inoue, H., et al., *An investigation of cell proliferation and soluble mediators induced by interleukin 1β in human synovial fibroblasts: Comparative response in osteoarthritis and rheumatoid arthritis.* Inflammation Research, 2001. **50**(2): p. 65-72.
192. Goekoop-Ruiterman, Y.P.M., et al., *Comparison of Treatment Strategies in Early Rheumatoid ArthritisA Randomized Trial.* Annals of Internal Medicine, 2007. **146**(6): p. 406-415.
193. Kim, H.Y., et al., *Efficacy of concurrent administration of cilostazol and methotrexate in rheumatoid arthritis: Pharmacologic and clinical significance.* Life Sciences, 2012. **91**(7–8): p. 250-257.
194. Sheppard, M.N. and A.G. Nicholson, *The pathology of cystic fibrosis.* Current Diagnostic Pathology, 2002. **8**(1): p. 50-59.
195. Gambari, R., et al., *Decoy oligodeoxyribonucleotides and peptide nucleic acids–DNA chimeras targeting nuclear factor kappa-B: Inhibition of IL-8 gene expression in cystic fibrosis cells infected with Pseudomonas aeruginosa.* Biochemical Pharmacology, 2010. **80**(12): p. 1887-1894.
196. Verhaeghe, C., et al., *Role of IKK and ERK pathways in intrinsic inflammation of cystic fibrosis airways.* Biochemical Pharmacology, 2007. **73**(12): p. 1982-1994.
197. Blackwell, T.S. and J.W. Christman, *The Role of Nuclear Factor- κ B in Cytokine Gene Regulation.* American Journal of Respiratory Cell and Molecular Biology, 1997. **17**(1): p. 3-9.
198. Joseph, T., D. Look, and T. Ferkol, *NF-κB activation and sustained IL-8 gene expression in primary cultures of cystic fibrosis airway epithelial cells stimulated with Pseudomonas aeruginosa.* American Journal of Physiology - Lung Cellular and Molecular Physiology, 2005. **288**(3 32-3): p. L471-L479.
199. Kawamura, I., et al., *Intratumoral injection of oligonucleotides to the NFκB binding site inhibits cachexia in a mouse tumor model.* Gene Therapy, 1999. **6**(1): p. 91-97.
200. Bonfield, T.L., M.W. Konstan, and M. Berger, *Altered respiratory epithelial cell cytokine production in cystic fibrosis.* Journal of Allergy and Clinical Immunology, 1999. **104**(1): p. 72-78.
201. Greene, C.M., et al., *TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells.* Journal of Immunology, 2005. **174**(3): p. 1638-1646.
202. De Stefano, D., et al., *Sustained inhibition of IL-6 and IL-8 expression by decoy ODN to NF-κB delivered through respirable large porous particles in LPS-stimulated cystic fibrosis bronchial cells.* Journal of Gene Medicine, 2011. **13**(4): p. 200-208.
203. De Stefano D., D.R.G., Carnuccio R., *NFkB decoy oligonucleotides.* Current Opinion in Molecular Therapeutics, 2010. **12**(2): p. 203-213.
204. Borgatti, M., et al., *Induction of IL-6 gene expression in a CF bronchial epithelial cell line by Pseudomonas aeruginosa is dependent on transcription factors belonging to the Sp1 superfamily.* Biochemical and Biophysical Research Communications, 2007. **357**(4): p. 977-983.
205. Borgatti, M., et al., *Silencing of genes coding for transcription factors: Biological effects of decoy oligonucleotides on cystic fibrosis bronchial epithelial cells.* Minerva Biotecnologica, 2008. **20**(2): p. 79-83.
206. Griesenbach, U., et al., *Anti-inflammatory gene therapy directed at the airway epithelium.* Gene Therapy, 2000. **7**(4): p. 306-313.
207. Kunugiza, Y., et al., *Inhibitory effect of ribbon-type NF-kappaB decoy oligodeoxynucleotides on osteoclast induction and activity in vitro and in vivo.* Arthritis Res Ther, 2006. **8**(4): p. R103.
208. Cabrini, G., et al., *Targeting transcription factor activity as a strategy to inhibit pro-inflammatory genes involved in cystic fibrosis: decoy oligonucleotides and low-molecular weight compounds.* Curr Med Chem, 2010. **17**(35): p. 4392-404.
209. Bezzerri, V., et al., *Mapping the transcriptional machinery of the IL-8 gene in human bronchial epithelial cells.* J Immunol, 2011. **187**(11): p. 6069-81.

210. Li, Y., et al., *Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-kappaB and endothelial inflammatory response.* Carbohydr Polym, 2014. **99**: p. 568-78.
211. Nadjar, A., et al., *NF kappa B activates in vivo the synthesis of inducible Cox-2 in the brain.* Journal of Cerebral Blood Flow and Metabolism, 2005. **25**(8): p. 1047-1059.
212. Hochberg, Y. and Y. Benjamini, *More Powerful Procedures for Multiple Significance Testing.* Statistics in Medicine, 1990. **9**(7): p. 811-818.
213. Holm, S., *A Simple Sequentially Rejective Multiple Test Procedure.* Scandinavian Journal of Statistics, 1979. **6**(2): p. 65-70.
214. Paats, M.S., et al., *Cytokines in nasal lavages and plasma and their correlation with clinical parameters in cystic fibrosis.* Journal of Cystic Fibrosis, (0).
215. Hartl, D., et al., *Innate immunity in cystic fibrosis lung disease.* Journal of Cystic Fibrosis, 2012. **11**(5): p. 363-382.
216. Zhang, S., et al., *Cationic lipids and polymers mediated vectors for delivery of siRNA.* Journal of Controlled Release, 2007. **123**(1): p. 1-10.
217. Tseng, Y.-C., S. Mozumdar, and L. Huang, *Lipid-based systemic delivery of siRNA.* Advanced Drug Delivery Reviews, 2009. **61**(9): p. 721-731.
218. Makarov, S., *NF-kB as a therapeutic target in chronic inflammation: recent advances.* Molecular Medicine Today, 2000. **6**: p. 441-448.
219. Croston, G.E., Z. Cao, and D.V. Goeddel, *NF-κB activation by interleukin-1 (IL-1) requires an IL-1 receptor- associated protein kinase activity.* Journal of Biological Chemistry, 1995. **270**(28): p. 16514-16517.
220. Muselet-Charlier, C., et al., *Enhanced IL-1β-induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF-κB signaling.* Biochemical and Biophysical Research Communications, 2007. **357**(2): p. 402-407.
221. Stefan Y., M.V., Pricop D., Neagu A., Mihasan M., Hritcu L., *Attenuated effects of chitosan-capped gold nanoparticles on LPS-induced toxicity in laboratory rats.* Materials Science and Engineering C, 2013. **33**: p. 550-556.
222. Ji Q., D.J., Yu X., Xu Q., Wu H., Pan J., *Modulation of pro-inflammatory mediators in LPS-stimulated human periodontal ligament cells by chitosan and quaternized chitosan.* Carbohydrate Polymers, 2013. **92**: p. 824-829.
223. Naberezhnykh G. A., G.V.I., Likhatskaya G. N., Bratskaya S. Yu., Solov'eva T. F., *Interaction of N-Acytlated and N-Alkylated Chitosans Included in Liposomes with Lipopolysaccharide of Gram-Negative Bacteria.* Biochemistry (Moscow), 2013. **78**(3): p. 301-308.
224. Tomita T., T.H., Tomita N., Morishita R., Keneko M., Shi K., Takahi K., Nakase T., Kaneda T., Yoshikawa H., Ochi T., *Transcription factor decoy for NFkB inhibits cytokine and adhesion molecule expressions in synovial cells derived from rheumatoid arthritis.* Rheumatology, 2000. **39**: p. 749-757.
225. Tranter, M., et al., *In vivo delivery of nucleic acids via glycopolymer vehicles affords therapeutic infarct size reduction in vivo.* Mol Ther, 2012. **20**(3): p. 601-8.
226. Tomita, T., et al., *Suppressed severity of collagen-induced arthritis by in vivo transfection of nuclear factor kappaB decoy oligodeoxynucleotides as a gene therapy.* Arthritis Rheum, 1999. **42**(12): p. 2532-42.
227. Palomo, J., et al., *Role of IL-1β in experimental cystic fibrosis upon P. aeruginosa Infection.* PLoS ONE, 2014. **9**(12).
228. Dhooghe, B., et al., *Lung inflammation in cystic fibrosis: Pathogenesis and novel therapies.* Clinical Biochemistry, 2014. **47**(7–8): p. 539-546.

229. Bozinovski, S., et al., *Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFκB and AP-1 in vivo.* Journal of Biological Chemistry, 2002. **277**(45): p. 42808-42814.
230. Moser, C., et al., *Serum concentrations of GM-CSF and G-CSF correlate with the Th1/Th2 cytokine response in cystic fibrosis patients with chronic Pseudomonas aeruginosa lung infection.* APMIS, 2005. **113**(6): p. 400-409.
231. Schroeder, T.H., et al., *CFTR is a pattern recognition molecule that extracts Pseudomonas aeruginosa LPS from the outer membrane into epithelial cells and activates NF-κB translocation.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(10): p. 6907-6912.
232. Singh, A., et al., *Macrophage-targeted delivery systems for nucleic acid therapy of inflammatory diseases.* Journal of Controlled Release, 2014. **190**(0): p. 515-530.
233. Saranya, N., et al., *Chitosan and its derivatives for gene delivery.* International Journal of Biological Macromolecules, 2011. **48**(2): p. 234-238.
234. Anderson G.G., M.-M.S., Stanton B.A., O'Toole G.A., *In vitro analysis of Tobramycin-Treated Pseudomonas Aeruginosa Biofilms on Cystic Fibrosis -Derived Airway Epithelial Cells.* Infect Immun, 2008. **76**(4): p. 1423-1433.
235. Sosnik, A., J. das Neves, and B. Sarmento, *Mucoadhesive polymers in the design of nano-drug delivery systems for administration by non-parenteral routes: A review.* Progress in Polymer Science, 2014. **39**(12): p. 2030-2075.
236. Singh, S., et al., *Granulocyte-Macrophage Colony Stimulatory Factor Enhances the Pro-Inflammatory Response of Interferon-γ-Treated Macrophages to Pseudomonas aeruginosa Infection.* PLoS ONE, 2015. **10**(2).
237. Limoli, D.H., et al., *Cationic antimicrobial peptides promote microbial mutagenesis and pathoadaptation in chronic infections.* PLoS Pathog, 2014. **10**(4): p. e1004083.
238. Gambari, R., et al., *Corilagin is a potent inhibitor of NF-kappaB activity and downregulates TNF-alpha induced expression of IL-8 gene in cystic fibrosis IB3-1 cells.* International Immunopharmacology, 2012. **13**(3): p. 308-315.
239. Aoki, H., et al., *Existence of autocrine loop between interleukin-6 and transforming growth factor-β1 in activated rat pancreatic stellate cells.* Journal of Cellular Biochemistry, 2006. **99**(1): p. 221-228.
240. Nichols, D., J. Chmiel, and M. Berger, *Chronic inflammation in the cystic fibrosis lung: Alterations in inter- and intracellular signaling.* Clinical Reviews in Allergy and Immunology, 2008. **34**(2): p. 146-162.
241. De Stefano, D., et al., *Sustained inhibition of IL-6 and IL-8 expression by decoy ODN to NF-kappaB delivered through respirable large porous particles in LPS-stimulated cystic fibrosis bronchial cells.* J Gene Med, 2011. **13**(4): p. 200-8.
242. Gambari, R., et al., *Decoy oligodeoxyribonucleotides and peptide nucleic acids-DNA chimeras targeting nuclear factor kappa-B: inhibition of IL-8 gene expression in cystic fibrosis cells infected with Pseudomonas aeruginosa.* Biochem Pharmacol, 2010. **80**(12): p. 1887-94.

10. Biographical Data (CV)

SELECTED PUBLICATIONS

Journal Articles

1. **Wardwell P.R.,** Bader R.A. Investigation of the immune response of *Pseudomonas aeruginosa* infected IB3-1 lung epithelial cells using NF-κB decoy ODN coated polysaccharide based nanoparticles. *ACS Biomaterials Science and Engineering*, 2015, submitted
2. **Wardwell P.R**., Bader R.A. Modulation of the immune response via NF-κB decoy oligonucleotide coated polysaccharide based nanoparticles in *in vitro* models of rheumatoid arthritis. *Arthritis Research and Therapy*. 2015, under review
3. **Wardwell P.R.**, Wilson D.R., Garner D.L., Bader R.A. Pseudomonas Quinolone Signal Modulates Cystic Fibrosis Epithelial Cell Response through the Toll-Like Receptor 4. *SOJ Immunology.* 2015, accepted.
4. **Wardwell P.R.**, Bader R.A. Immunomodulation of Cystic Fibrosis Epithelial Cells via NF-kB Decoy Oligonucleotide Coated Polysaccharide Nanoparticles. *Journal of Biomedical Materials Research Part A*. DOI: 10.1002/jbm.a.35296
5. Zhang N., **Wardwell P.R**., Bader R.A. In Vitro Efficacy of Polysaccharide-Based Nanoparticles Containing Disease-Modifying Antirheumatic Drugs. *Pharmaceutical Research*. 2014,
6. Bader R.A., **Wardwell P.R**. Polysialic Acid: Overcoming the Hurdles of Drug Delivery. *Therapeutic Delivery*. 2014, 5(3).
7. Zhang N., **Wardwell P. R.**, Bader R. A. Polysaccharide Based Micelles for Drug Delivery. *Pharmaceutics*, 5(2): 2013. 329-352.

Patent

1. R.A. Bader, N. Zhang, **P.R. Wardwell**, "Polysialic acid-based N-trimethyl chitosan gel nanoparticles for systemic drug delivery." U.S. Patent 20120294904, published November 22, 2012. Syracuse University

Book Chapter

1. **P. R. Wardwell**, R.A. Bader "Challenges of Drug Delivery" in *Engineering Polymer Systems for Improved Drug Delivery*, John Wiley & Sons, Inc., R.A. Bader and D.A. Putnam (Ed). 2014.

SELECTED TALKS

1. **P.R. Wardwell,** R.A. Bader. Modulation of the immune response via the NF-kB signaling pathway in rheumatoid arthritis in *in vitro* models. Society for Biomaterials Annual Meeting and Exposition, Charlotte, NC 2015

2. **P.R. Wardwell,** R.A. Bader. Immunomodulation of Cystic Fibrosis Epithelial Cells via NF-κB Decoy Oligonucleotide Coated Polysaccharide Nanoparticles. Society for Biomaterials Annual Meeting and Exposition, Denver, CO 2014

3. **P.R. Wardwell**, R.M. Iyer, P.N. Borer, M.P. McPike, M.B. Forstner, R.A. Bader. Polysialic Acid-N-Trimethyl Chitosan Nanoparticles for Oligonucleotide Delivery. Society for Biomaterials Annual Meeting and Exposition, Boston, MA 2013.

RELEVANT EXPERIENCE

Graduate Research

PhD Thesis: Control of inflammation using drug delivery strategies in *in vitro* models **2011-Present**

Research Assistant, Department of Biomedical and Chemical Engineering, Syracuse University
Advisor: Dr. Rebecca Bader

Overarching goal of the thesis work was to improve drug delivery strategies for mediating inflammation using polymer based nanocarriers. The inflammatory process in disease states is complex, and research required an in depth understanding of normal and disrupted cell signaling pathways and their potential for manipulation.

- Synthesized and characterized polysaccharide based nanoparticles loaded with decoy oligonucleotides
- Implemented *in vitro* models for rheumatoid arthritis, requiring primary cell isolation, and cystic fibrosis, requiring development of a bacterial/mammalian cell co-culture
- Designed and carried out *in vitro* efficacy experiments to determine a potential use for polysaccharide nanoparticles as drug delivery vehicles for DNA based therapeutics
- One manuscript directly regarding this work has been published, with two more in progress. In addition, collaborative contributions to other projects have resulted in multiple other published manuscripts. For a complete list, see page three.

Business Development

Entrepreneurial Lead **April-May 2014**

National Science Foundation Innovation Corps. Program

A major roadblock in the translation process from University bench top to industry product is a lack of understanding of what makes a viable and successful product. The I-Corps program has been designed to help University researchers have a better understanding of the market which their product will enter, helping to bridge the translation gap from academic benchtop to commercial product. Key tasks performed as the Entrepreneurial Lead in this program:

- Performed market research and customer validation to determine if commercialization of a nanocarrier based drug delivery platform was a viable option.
- Interacted with over 80 researchers, business development professionals, and CEO's at pharmaceutical companies ranging in size from start-up to Fortune 500 level.
- Attended weekly web-based meetings with other teams to share findings, eventually concluding the technology being investigated was not ready to be taken to market.

Participating in this program provided exposure to research outside of academia, and helped further define career goals.

Teaching and Mentoring

Mentor **June 2014- Feb. 2015**

National Science Foundation REM Program, Syracuse University, Syracuse NY

- Led an undergraduate student in a project requiring knowledge of cell culture and microbiology. One on one training, collaborative conversations, and general guidance were essential aspects of this experience.

Teaching Assistant **2011-May 2012**

Department of Biomedical and Chemical Engineering, Syracuse University, Syracuse NY

- Mass and Energy Balances (sophomore level): Assisted with grading homework and exams, held office hours weekly.
- Bioengineering Physiology Laboratory (junior level): Assisted with laboratory setup, supervised and assisted students performing exercises, and graded laboratory reports.

Undergraduate Internships

Polysaccharide/drug conjugates for improved drug delivery **2010-2011**

Researcher, Department of Biomedical and Chemical Engineering, Syracuse University

- Synthesized hyaluronic acid-methotrexate conjugates rheumatoid arthritis treatment
- Designed and conducted *in vitro* testing

Diabetes-induced glycosylation of collagen **Summer 2010**

Researcher, National Science Foundation REU, Illinois Institute of Technology, Chicago, IL

- Designed and conducted experiments to determine glycosylation crosslinking in collagen.
- These experiments were later translated to a thesis project for a future graduate student.

PROFESSIONAL DEVELOPMENT AND EXTRACURRICULAR ACTIVITIES

Syracuse Biomaterials Safe Practices Team **2013-2015**

- Member of a team dedicated to developing, disseminating, and monitoring streamlined laboratory safety practices to be used in a shared laboratory space

Syracuse University Women in Science and Engineering (WiSE) **2012-2014**
Future Professionals Program (FPP)

- Participated in a program focused on career development and persistence of women in science and technology

Syracuse University Cross Country and Track and Field Teams **2007-2010**

- Athlete in a NCAA Division One program for three years