Biomaterials for Non-Viral Delivery of Nucleic Acid Drugs

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Abstract

Significant progress has been made in the field of biomaterials for non-viral delivery of macromolecules. A wide variety of distinct types of novel biomaterials, in combination with corresponding cutting-edge therapeutic systems and technologies, have been developed and have shown highly promising therapeutic efficacies in research studies published. Furthermore, a number of these technologies are rapidly progressing to and through clinical trial. Specifically, substantial progress related to the development of drug delivery technologies including lipid nanoparticulate formulations, polymer nanoparticulate formulations, and other types of nanoparticulate formulations may lead to the development of substantial libraries of novel, highly effective, next-generation therapeutic technologies. The motivation of this thesis is to review these state-of-the-art technologies.

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Chapter 1: Introduction

There are a number of diverse types of nucleic acid drugs that have been delivered with advanced nanoparticle technologies in translational *in vivo* models. Numerous therapeutic viral and non-viral biologics such as RNAi, siRNA, mRNA, and various genome editing systems including CRISPR, in combination with biomaterials development, have made substantial progress towards the clinic.[1–3] Published research studies show significant potential of these new technologies to mitigate deleterious diseases such as cancer, diabetes and heart disease.[4,5,2,6]

Genome editing, for example, is a method by which patients with deleterious mutations can be permanently treated via a single injection of non-viral therapeutic nanoparticulate formulation technologies, such as CRISPR/Cas9 lipid nanoparticles.[7–10] Genome editing is an enticing technology due to the technology's potential to supplant expensive, transient enzyme/protein replacement technologies that were been developed previously for a wide variety of diseases and require multiple injections of fresh therapeutic formulation materials and components throughout the course of a patient's treatment.[11,12] Other examples of important novel biomolecular systems include siRNA and mRNA nanoparticulate formulations.[13–15,16] The payloads of these drug delivery systems consist of various RNA based, non-viral therapeutic technologies that have been developed to treat broad ranges of diseases via transient loss-of-function silencing or gain-of-function cellular processes, respectively.[11,17]

Chapter 2: Examples of Different Types of Nucleic Acid Therapeutics and Their Barriers

Some of the earliest nucleic-acid-based drugs developed by drug delivery researchers were based off of therapeutic viral-vector compounds. Viral-vectors have advanced further than non-viral gene delivery systems.[7,18] The relative ubiquity of viral genome editing technologies can be attributed to ease of packaging these technologies within easily accessible, commercialized drug delivery biomaterials and formulations.[3,7,11,19] In addition to desirable ease-of-use functionality, viral technologies, corresponding biomaterials, and constituent components have consistently exhibited extremely high transfection efficiency with respect to target diseased mammalian cells and tissues.[20,21] With viral-based therapeutic formulation technologies, transporting broad varieties of distinct therapeutic viral-vector payloads from the external environments of target cells, through the respective semi-permeable cell-membrane barriers, into the main internal cytosolic compartments of these target mammalian cells can be done much more efficiently when compared to alternative non-viral biomaterials and corresponding therapeutic technologies.[22,23]

Viral vector based therapeutics are easy to use but elicit substantial immune responses.[11,12,18] This bio-incompatibility can result in substantial immune reaction in patients against injected exogenous viral materials that have paradoxically been developed to mitigate the affected patient's progressing disease physiology. Importantly, the first mortality reported from viral gene therapy trial with adenoviral particles resulted from the patient's substantial immune reaction against the injected viral capsids.[12,24,25] Furthermore, highly undesirable

random, error-prone foreign DNA integration throughout a treated patient's genome represents an additional source of concern related to the safety of these viral therapeutic technologies.[3,18,26,27] This concern is due to the possibility of exogenous DNA components integrating into a patient's genome, potentially rendering critical cell cycle and cancer-inhibition-check processes ineffective.[28] Inhibition of cell-cycle check processes due to random foreign DNA integration may lead to cancer in a treated patient.[18,26,29] Contrastingly, non-viral alternative technologies are considered to represent safer therapeutic options due to their improved biocompatibility and transient nature.[11,12] Although these more biocompatible therapeutic formulation systems may be preferable to comparable viral CRISPR genome editing technologies, effective drug delivery systems for entirely non-viral "all-in-one" genome editing mechanisms remain elusive to researchers in the field due to a number of potential un-resolved issues.[1,12,18,30]

Although effective non-viral CRISPR genome editing systems have demonstrated limited efficacy in therapeutic studies, alternative non-viral technologies such as mRNA and siRNA therapeutics have advanced significantly.[16,31,32] However, unlike gene therapy which can be characterized by a permanent therapeutic effect through direct manipulation of a patient's genome, mRNA and siRNA technologies do not necessarily reduce the substantial cost due to required repeat administrations associated with enzyme replacement therapies. It is important to note that there are diverse and distinct varieties of genome editing therapeutics that have been developed. For example, viral-AAV-based genome editing therapeutics have been developed to act as episome structures, avoiding genome integration within the host cell's nucleus.[26,33,34] Since these AAV-based genome editing therapeutics do not integrate into the

host's genome, they do not necessarily function beyond the lifetime of the transfected cell and therefore may elicit less of an immune response as compared to integrating retrovirus AAV-based genome editing technologies.[35,36] RNA-based therapeutic technologies such as mRNA and siRNA formulations are inherently transient in nature and functionality. Importantly, a number of advanced therapeutic non-viral nanoparticles have been developed to undergo effective enhanced penetration and retention (EPR) activities in translational cancer *in vivo* models.[37]

Furthermore, RNA-based technologies such as mRNA and siRNA are highly prone to physiological degradation pathways which negatively effects their longevity and therapeutic efficacy.[38–40] It is important to note that *in vivo* oligonucleotide degradation is mostly due to enzymatic activity.[41,42] The high rate of degradation characteristic of RNA-based poly-nucleotide macromolecules is due to their molecular structure consisting of a highly reactive hydroxyl moiety covalently bonded to the second carbon (2' position) of the constituent ribose sugar. The exposed conformation of these highly reactive hydroxyl moieties of the ribose sugar makes hydrolysis of these therapeutic polynucleotides highly favorable thermodynamically when exposed to aqueous physiological environments. Therefore, these RNA-based macromolecules are characterized to exhibit short pharmacokinetic profiles compared to alternative therapeutic compounds as they are quickly reduced to non-functional oligomeric products when exposed to aqueous environments rendering them therapeutically ineffective.[38,43,44]

Contrastingly, viral vector therapeutic payload technologies prototypically consist of DNA-based components exclusively and are therefore significantly less prone to these physiological degradation pathways. The enhanced longevity of DNA-based viral components versus analogous RNA-based compounds is due to the constituent deoxyribose molecular

structure of DNA poly-nucleotide macromolecular structure, which has a significantly less

reactive proton moiety at the 2' position of the deoxyribose sugar structure (versus the highly

reactive 2' hydroxyl molecular group of the constituent ribose sugar structure of comparable

RNA poly-nucleotide macromolecules).[45] It has been demonstrated to be critical to consider

nuclease degradation of oligonucleotide therapeutic payloads.[42,46–48] Therefore, substantial

research has been pursued to date to develop novel therapeutic technologies that can

effectively mitigate rapid nuclease degradation pathways.[49,50]

To address degradability issues associated with therapeutic, non-viral RNA compounds,

substantial research efforts have been pursued to develop novel synthetic RNA

macromolecules that are less prone to high rates of degradation.[51–54] Encouragingly, cutting

edge synthetic strategies and RNA processing methodologies, such as locked nucleic acid

formation to effectively protect the highly reactive 2' hydroxyl of the ribose sugar structure,

have shown significant prospective potential.[55–57] Researchers have shown mitigation of the

high degradation rate of these modified RNA-based macromolecules substantially improves

their respective therapeutic efficacies when compared to analogous un-modified RNA

macromolecular therapeutic compounds.[44,55,58,59]

Although these results are highly promising, the extremely involved and complicated

synthetic processes required to generate these classes of modified RNA macromolecular

compounds characterized by improved longevity makes these cutting-edge therapeutic

technologies substantially more expensive than their unmodified equivalents.[44,60] These

expensive, time intensive processing methods result in these modified RNA therapeutic

macromolecules being significantly less accessible compared to more economical alternatives

such therapeutic formulations that consist of viral-vector components.[61] It is important to note that substantial progress has been made with respect to chemical modification of short RNA therapeutic payloads, such as anti-sense and siRNA to improve nuclease degradation mitigation.[62,63] However, it has been significantly more challenging to get analogous mitigation of nuclease degradation in the case of high molecular weight mRNA therapeutic payloads due to the larger size.[64]

In addition to direct synthetic alteration of the therapeutic compounds themselves to improve upon rapid degradation of non-viral RNA-based therapeutic compounds, drug delivery vehicles such as nanoparticulate formulations have been developed and utilized to entrap these classes of non-viral therapeutic molecules.[65,66] These drug delivery vehicles, formulation methodologies, and corresponding biomaterials have been developed to significantly improve upon the longevity and corresponding efficacies of wide varieties of distinct, novel RNA-based, non-viral therapeutic macromolecules.[16,67]

Furthermore, drug delivery vehicles can be altered both synthetically and physically to exhibit targeting capabilities when injected into a patient's blood stream via intravenous injections and through other physiological modes of administration.[32,67–69] Different physical and chemical characteristics of distinct varieties of therapeutic formulations consisting of non-viral RNA-based molecular mechanism payloads within drug delivery biomaterials have been designed to target and treat cells within wide varieties of tissues such as liver, lung, brain, spleen, and more.[32,68,70,71] Some of these formulations have shown substantial potential in clinical trial.[17,40,64,72] Different drug delivery vehicles developed for efficient intracellular delivery of non-viral therapeutic compounds include lipid nanoparticulate formulations,

polymer nanoparticulate formulations, and other nanoparticulate formulations.[7,11,73] These

diverse classes of drug delivery vehicles are described in detail below.

Importantly, these non-viral lipid nanoparticle drug delivery systems have been

developed to overcome the significant drug delivery challenges related intracellular

penetration. In order to transport these therapeutic molecules from the extracellular space to

their respective intracellular compartments where they act upon their physiological targets,

these lipid drug delivery systems implement advanced intracellular delivery and transfection

activities to overcome the cell membrane barrier that represents a significant obstacle.

The scope of this thesis is to describe the major advancements achieved by researchers

developing major classes of drug delivery technologies. There are three major classes of drug

delivery technologies that have been developed to deliver nucleic acid drug compounds as

described below; lipid nanoparticles, polymeric nanoparticles, as well as other types of metallic

and carbon nanotube-based nanoparticle technologies.

Chapter 3: Lipid nanoparticulate formulations for intracellular delivery *in vivo*

i) Lipid nanoparticulate formulations; physical, chemical, and thermodynamic properties

In general, constituent lipid compounds that make up heterogeneous lipid-nanoparticulate formulations are conventionally considered to be multi-blocked macromolecular biomaterials that prototypically consist of a hydrophilic "head-group" that is synthesized with to interact favorably with aqueous environments, and a hydrophobic "tail-group" which contrastingly conforms to minimize interactions at the molecular level with aqueous environments. This interfacial functionality defines the overall lipid compound as amphiphilic, with portions that simultaneously conform to both minimize and maximize unfavorable and favorable enthalpic interactions, respectively, within aqueous physiological environments. In an effort to minimize free energy of the overall heterogenous nanoparticulate formulation consisting of lipid-like biomaterials, these multi-blocked polymeric materials typically conform into spherical inter-molecular structures consisting of lipid bilayer shell formations surrounding an aqueous payload compartment within which active therapeutic bio-macromolecules reside.

These nanoparticulate formulations consisting of lipid-bilayers are quintessentially characterized by hydrophilic "head-groups" making up the external and internal shells of the lipid nanoparticle that directly interact with the aqueous physiological environments external to the nanoparticle formulation and that make up the payload containing compartment of the nanoparticle, this being where the therapeutic non-viral payload is generally modelled to reside. The hydrophobic tail groups reside within the lipid bilayer shell resulting in minimization

of thermodynamically undesirable molecular interactions between these aliphatic groups and aqueous environments within and external to the nanoparticulate formulation.

It is important to note that in general, lipid nanoparticulate drug delivery formulations are typically characterized as multilamellar structures and are considered to be "onion-like."[74] In other words, the bilayer structure repeats within the nanoparticulate formulation structure forming concentric circular patterns that completes at roughly the geometrical center within the spherical drug delivery vehicle.

ii) First lipid-nanoparticulate formulations

The United Kingdom based Bangham research group demonstrated the first preparation of un-naturally formed liposomes containing entrapped solutes for proof-of-concept pharmaceutical applications.[75–77] They demonstrated an efficient remote loading process in which they effectively swelled preformed liposomes and formulated substantial concentrations of ionic solutes within these lipid-nanoparticulate formulations once the system re-equilibrated.[77,78] They characterized these lipid nanoparticulate formulations as consisting of lipid bilayers surrounding aqueous payload structures.[74,75] These lipid-nanoparticulate formulations primarily consisted of amphiphilic phospholipids external to intra-liposomal spaces in which the ionic therapeutic solutes localized within the heterogeneous inter-molecular structure.[77,78]

From this pioneering work, related lipid nanoparticulate drug delivery systems were developed for vast categories of small molecule drugs, such as chemotherapeutics.[7,10,72–75] Importantly, small-molecule therapeutics represent important categories of non-viral therapeutic technologies that have been, and continue to be, of significant translational and clinical interest.[4,5,84] The spontaneous formation of multilamellar lipid bilayers around active

therapeutic compounds has made these formulations particularly useful for a variety of novel therapeutic applications. Early on, small molecule lipid nanoparticulate formulations were developed and characterized by hydrodynamic radii typically around 100 nanometers with low corresponding polydispersity indices, making them ideal for physiological administration and in adherence with regulations imposed by the Food and Drug Administration.[5,6,67] These relatively homogeneous characteristics of these lipid nanoparticulate formulations enabled them to function well in cancer therapeutics applications, for example.[44,64,70,71] For example many of the lipid technologies have been developed to implement an enhanced penetration and retention effect in order to more effectively act against cancerous tumor tissue *in vivo*.

iii) Liposome formation methodologies to solubilize hydrophobic therapeutics

There are a number of methodologies that have been pursued by researchers since Bangham's remote loading swelling process that have been developed and utilized to form vast and distinct libraries of lipid-nanoparticulate formulations for novel drug delivery applications.[3,64,67,75] Some of these methods include sonication, extrusion, reverse-phase evaporation and solvent injection.[86–88] Each of these lipid nanoparticulate formulation methodologies results in diverse sizes, morphologies, entrapment efficiencies, as well as other variable engineering parameters.[4,89,90] Formulations of different sizes and chemical-physical constitutions have been optimized and utilized to perform properly for different therapeutic applications.[64,79,91] Variations of these properties of lipid-nanoparticulate formulations have been shown to have significant effects on various physiological features and activities of these nanoparticles such a cytotoxicity and extra-cellular glycocalyx interactions.[90,92–95] It is also important to note that since the early lipid-nanoparticulate formulation technologies, wide

varieties of diverse lipid molecules and additive macromolecular structures have been synthesized, tested, and included within these lipid-nanoparticulate formulations that have enabled significant expansion upon potential prospective therapeutic applications.[10,16,63,66]

Importantly, lipid-nanoparticulate formulations were primarily developed to substantially improve upon the extremely poor hydrophilicity characteristics of aromatic small-molecule therapeutics within their intended aqueous physiological environments initially.[2,82] The insoluble nature of these clinically pervasive non-viral synthetic compounds on their own within their supposed physiological environments represented a major obstacle with respect to their defined therapeutic functionalities.[4,82,90] Translationally significant chemotherapeutics such as Doxorubicin, Bupivacaine, and Paclitaxel all exhibit extremely poor water-solubility characteristics on their own and are prone to volatile precipitation and solidification processes, potentially within fragile tissue structures of a prospective host patient.[4,82,90] This process of "crashing out" within a patient's physiological environment is considered to be an extremely harmful potential side effect associated with these drugs and can cause severe inflammation and auto-immune responses.[4,82,90]

Lipid nanoparticulate technologies have therefore been developed and utilized by many research groups to effectively deliver non-soluble, hydrophobic small molecule therapeutics.[2,82,96] Via properly optimized formulation processes, these synthetic non-viral small molecules, in combination with constituent lipid biomaterials, can undergo controlled self-assembly mechanisms and form effective emulsifying liposome-nanoparticulate drug delivery vehicles as described above.[89,96,97] The resultant well-formed heterogeneous lipid-nanoparticulate formulations work to effectively solubilize the previously insoluble hydrophobic

therapeutic compounds within their intended aqueous environments, leading to improved therapeutic functionalities while lessening the potential for harmful side effects within diseased physiological systems.[17,96,98] When entrapped within lipid-nanoparticulate formulations, these non-water-soluble synthetic compounds become solubilized within physiological aqueous environments, making them significantly more effective, enabling considerably improved therapeutic functionalities.[4,90,93]

iv) Physiological and biochemical barriers to non-viral lipid therapeutic functionality

For more difficult and complex non-viral therapeutic payloads, next-generation lipid nanoparticles have been further developed to exhibit broad ranges of important drug delivery functionalities.[91,99,100] Even the most advanced RNA macromolecules, for example, cannot function therapeutically without supplementary biomaterials or additional molecular modifications and can inherently exhibit no relevant utility within physiological systems on their own. Firstly, as mentioned above, RNA compounds are rapidly degraded within physiological environments into non-functional product molecules via energetically favorable and rapid hydrolysis reactions.[32,40,101] Furthermore, when applied to a prospective host's physiological system in a "naked" manner (on their own), such as via intravenous injection into a patient's circulatory system, therapeutic compounds such as DNA and RNA have no physical or chemical means to precisely accumulate within damaged or diseased tissues of the treated host.[69,102] Correspondingly, the conventional hallmark of the drug delivery field is to engineer targeted therapeutic technologies; getting therapeutically active compounds to precise sites of disease while preventing inadvertent and potentially harmful activities of these potent compounds

within unintended tissue locations throughout a host patient's physiological system represents a key goal of the field.[4,32,73]

At a cellular level, the cell-membrane is a highly controlled, semi-permeable barrier structure that has evolved over time to prevent endocytosis (cellular internalization) of undesirable, potentially harmful exogenous compounds and is particularly effective against ionic, charged macromolecules such as DNA and RNA. Therefore, genetic therapeutic macromolecules, such as DNA and RNA, have no ability to transverse the cellular membrane of a target diseased cell on their own.[103,104] Importantly, lipid-nanoparticulate formulations consisting of payload structures to house these therapeutic macromolecules enable these exogenous non-viral macromolecular compounds to cross this otherwise impermeable natural barrier efficiently, subsequently leading to effective therapeutic efficacies and activities of these non-viral macromolecules.[12,89,105,106] A number of models and theories have been extensively researched and developed to date with regards to how this endocytosis process is achieved, and there is substantial debate within the field on this point currently.

It is important for non-viral lipid nanoparticulate technologies to be able to effectively avoid the host's immune system.[17,91,100] Therefore, lipid-nanoparticulate formulations, including those formulated with non-viral RNA-based macromolecule payloads (such as siRNA and mRNA), have been rigorously developed throughout substantial research efforts attempting to effectively circumvent these critical cellular and physiological level obstacles that can ultimately prevent any achievable significant therapeutic efficacy.[17,93,107] Encouragingly, resultant advanced lipid-nanoparticulate formulations that have been engineered to more effectively evade the host's immune system and efficiently transfect target cells of interest have played

major roles in enabling functional therapeutic activities of corresponding cutting edge RNA-based, non-viral genetic compounds.[13,16,43,102]

v) Advanced lipid nanoparticulate formulations

Examples of efforts to impart targeting abilities onto lipid-nanoparticulate technologies include research and development of novel drug delivery formulations engineered to preferentially accumulate within and specifically target cells at tumor sites throughout a cancer patient's physiological system.[4,85,107] These cancer-therapeutic non-viral lipid-nanoparticulate formulations have been developed and engineered to functionally increase the circulation time of the associated formulated chemotherapeutic compounds by kinetically evading immune-system clearing mechanisms while preferentially accumulating within cancerous tissues.[4,85,108] Specifically, these chemotherapeutic formulations are methodically formed to avoid normal, non-cancerous tissues and organ systems while accumulating within the significantly vasodilated arteries adjacent to and throughout these diseased cancer sites, this being characteristic of tumor tissue pathology.[4,99,108]

The enhanced circulation half-life of these therapeutic compounds, in combination with the preferential retention of these formulations within affected diseased circulatory structures (distinctive to cancerous tissues), epitomize the "enhanced penetration and retention" (EPR) functionality quintessential to this class of therapeutic lipid-nanoparticulate technology specifically developed for novel cancer applications.[37,108,109] Importantly, the improvement of lifetime and avoidance of rapid immunological clearance afforded by the advancement of PEGylation of these lipid nanoparticulate formulations has played a major role in increasing the

retention and circulation times of these therapeutic technologies, leading to significant improvements with regards to therapeutic efficacies of these novel formulations.[37,109–111]

In that vein, another critical aspect and key milestone of subsequent versions of lipid-nanoparticulate drug delivery formulations has been the inclusion of PEGylated lipid amphiphiles within the phospholipid bilayer shell structure.[110–112] This critical materials-engineering advancement has consistently been demonstrated to be necessary for subsequent versions of lipid-nanoparticulate formulations functionalized to effectively bypass the reticulo-endothelial clearance system of a host's physiological system.[72,91,111,113,114] This advancement has enabled significantly improved circulation times and subsequent efficacies associated with some of the most therapeutically potent novel lipid-nanoparticulate formulation technologies.[111,112,115]

Functionally, these PEGylated lipids form an additional external "hairy-layer" of heteroatomic polyethylene glycol polymer blocks throughout the surface of the lipid-bilayer shell structure which coordinately act to kinetically prevent rapid accumulation and adhesion of immune-system originating opsonization peptide signals via rigorously defined polymer conformation thermodynamics.[97,112,114] Surface adhesion of these immune-derived peptide signals represents the first step towards a kinetically rapid immune-clearance response against foreign materials within a host's physiological system.[99,114] Normally, these signal peptides function to accumulate on exogenous materials that the host's immune system flags as foreign, resulting in a highly efficient immune response. These immune system response processes act to quickly sequester foreign material, preventing intermolecular interactions with resident physiological systems within the host, ultimately rendering these exogenous materials inactive

and non-functional.[91,99] In the case of foreign viral particulates, harmful bacterial entities, and other potentially deleterious exogenous materials, clearly this fast sequestering activity of the immune system of the host is a desirable response.

However, obviously in the case of administration of therapeutic technologies, rapid immune signaling, and clearance of foreign therapeutic technologies represents a major obstacle for exogenous therapeutic materials developed and administered to function within a prospective host in a therapeutic manner.[91,99,116] The PEGylation layer formed via the addition of chemically mixed amphiphilic biomaterials kinetically blocks these immune-signaling proteins from accumulating on the surface of the lipid nanoparticulate formulations.[114,116] This molecular-level kinetic blocking process effectively prevents rapid clearance of these materials from the host's physiological system, significantly improving the effective lifetimes of these therapeutic lipid-nanoparticulate formulations and their respective efficacies.[100,110,111]

The hydrophilic "head" groups of constituent amphiphilic lipid macromolecules can be conjugated with additional ligands such as antibodies, targeting peptides, and other polymeric biomaterial oligomers for even more expansive varieties of diverse targeting and transfection strategies, enabling substantially more potential prospective therapeutic applications.[71,85,117,118] These types of generalizable and combinatorial modifications can have significant effects on the chemical and physical functionalities of these lipid-nanoparticulate formulations within a host's physiological system.[119,120] For example, diverse libraries of lipid-nanoparticulate formulations have been engineered to accumulate within specific arterial structures within a host patient's liver, pulmonary system, brain, and structures within a patient's spleen when administered intravenously.[70,113,121,122] Researchers have also pursued a variety of tuning and modification

strategies to reproducibly alter various biomaterials parameters of innovative therapeutic lipid-nanoparticulate formulations such as hydrodynamic radii distributions, PEGylation densities, rheological properties, and electrostatic properties to achieve more precise targeted accumulation of formulations at specific tissue locations within diseased physiological models.[73,105,120]

Furthermore, particular materials-engineering parameters of these lipid-nanoparticulate formulations have been pursued to preferentially transfect specific cell types within these macroscale physiological organ systems.[32,67,123,124] For example, non-viral RNA-based lipid nanoparticulate formulations have been developed to preferentially transfect particular immune cell types, including B-cells and T-cells, in addition to endothelial cells, hepatocytes, as well as other cell-types of interest within their resident organ systems adding additional levels of precision to these innovative therapeutic technologies.[83,84,89,90] Researchers have shown different combinations of physical aspects of these nanoparticulate formulations, including size and zeta-potential, as well as the addition of targeting ligands, enable these lipid nanoparticulate technologies to achieve various precision targeting functionalities at the cellular level.[6,107,117,127] These novel, tunable formulations have demonstrated substantial utility and have been strategically applied to broad varieties of innovative therapeutic strategies, some of which are currently being pursued for clinical translation.[4,32,128] These diverse targeting functionalities enabled by novel engineering approaches to lipid-nanoparticulate technology have effectively broadened prospective potential therapeutic applications substantially beyond the initial tumor accumulation functionalities.[15,64,68,129]

In addition to designing broad ranges of lipid nanoparticulate formulations with diverse targeting functionalities, engineering lipid-nanoparticulate formulations for optimized release of their therapeutic macromolecular payloads at the right kinetic point and physiological location has been another critical area of research and development.[67,108,130] In this vein, various triggering strategies have been developed to date to ensure optimized release of active therapeutic biomolecules within a host patient's physiological system.[108,130,131] These strategies typically involve spontaneous formation of defects within the amphiphilic bilayer of the administered lipid nanoparticulate formulation in a kinetically controlled manner via exposure to physiological, and/or timed external, cues such as pH differences and electromagnetic light wave application.[23,37,109,132] Liposomes can be formed with amphiphilic lipid macromolecules that respond to light or thermal changes by controlled and relatively rapid degradation, for example.[133–135] Some of these triggerable release methods for lipid-nanoparticulate technologies are currently being tested in clinical trial.[136,137] However, it is important to note that many of these triggered release strategies are currently at relatively preliminary stages of research and development. Consequently, the effectiveness of different particular modes of controllable triggerable release mechanisms for different lipid nanoparticulate formulation technologies is a source of debate within the field currently.

Examples of controlled release strategies being pursued include methods of exploiting unique physical-chemical aspects of targeted physiological environments such as acidity, molarity, and characteristic structures of local glycocalyx constituents which make up the surrounding extracellular matrices of particular organ systems of interest and are responsible for associated inter-molecular interactions.[138–140] For example, pore-forming lipid

nanoparticulate formulations have been developed to undergo conformational transitions to hexagonal structures when accumulated within physiological locations of lower pH levels compared to average physiological pH levels as exemplified by cancerous diseased tissue compared to normal tissue, respectively.[131,138,141] Correspondingly, once these lipid-nanoparticulate formulations reach a more acidic physiological environment, they undergo this particular degradation transition and release their therapeutic payload in a kinetically controlled manner as a result.[101,141] A substantial amount of research and development has been pursued to date to develop pH-sensitive lipid-nanoparticulate formulations that have shown significant therapeutic potential.[104,135,136]

One interesting controlled-release mechanism that is currently being pursued involves engineering lipid-nanoparticulate formulations with advanced electromagnetic radiation-triggerable release functionalities.[108,132,142] These classes of lipid-nanoparticulate formulations are typically designed to exhibit light-triggerable defect formation and degradation within their constituent lipid bilayers, theoretically enabling highly controllable release kinetics for their active therapeutic agent payloads.[143,144] Substantial research and development efforts have been pursued by researchers to generate innovative designer lipid surfactant polymers synthesized with photo-reactive molecular groups engineered to undergo light-triggered chemical changes when exposed to timed activation of electromagnetic waves of the proper corresponding wavelengths and frequencies.[108,143,145]

Although these innovative materials are very exciting and creative novel technological advancements with substantial prospective clinical potential, concerns related to the possibility of deleterious side effects caused by extended tissue exposure to electromagnetic radiation

sources have arisen.[146,147] Furthermore, photo-sensitive lipid macromolecules typically exhibit

significantly limited molecular stability when compared to more conventional, less-advanced

lipid macromolecules due to their relatively weaker chemical structure. The limited molecular

integrities exhibited by these macromolecules have been shown to negatively affect the

circulation life-time and integrities of nanoparticulate formulations formed from these types of

photo-reactive biomaterials.[147] Unfortunately, toxicity concerns and limited molecular stability

characteristics associated with these light-triggerable lipid-nanoparticulate formulations and

their constitutive photo-sensitive lipid macromolecules exemplify major obstacles that continue

to limit achievable therapeutic utilities with these novel technologies.[146,148]

     Importantly, non-viral therapeutic macromolecules contained within lipid

nanoparticulate drug delivery formulations represent advantageous alternatives to viral analogs

with respect to their corresponding therapeutic utilities.[1,11,12] Compared to viral therapeutic

formulations, non-viral nanoparticulate technologies have been shown to exhibit significantly

lessened immune responses against administered therapeutic biomaterials.[11,18] Additionally,

non-viral formulation therapeutic technologies enable significantly greater potential payload

capacity compared to viral formulations.[12,149] Due to an enhanced payload capacity, non-viral

therapeutic formulations can be engineered to enable significantly greater combinatorial

packaging designs for more effective, multi-functional therapeutic macromolecular

systems.[12,149] The tunable functionalities associated with non-viral lipid nanoparticulate

formulations can be taken advantage of to engineer vast and diverse libraries of novel

therapeutic biomaterials with various targeting abilities, significantly improved circulation half-

lives, and combinatorial therapeutic activities. The generalizable and modifiable nature that

characterizes non-viral nanoparticulate formulations enables substantial flexibility of the architecture and design of these formulations. Contrastingly, viral therapeutic formulation technologies are not modifiable and therefore cannot be tuned to exhibit broad ranges of functionalities as exemplified by superior non-viral nanoparticulate therapeutic alternatives.

Researchers have made substantial strides modelling and analyzing diverse lipid-nanoparticulate structures. Furthermore, researchers in the drug delivery field have achieved a thorough understanding of the underlying kinetics and thermodynamics related to the spontaneous formation processes of these lipid-nanoparticulate formulations from their constituent biomaterials.[72,79,86] This foundational research has led to the development of a number of diverse and distinct lipid-nanoparticulate drug delivery formulation libraries that have been developed by researchers for various novel therapeutic applications.[1,15,64] Vast libraries of diverse biomaterials and polymeric macromolecules classified to exhibit lipid-like surfactant functionalities have been developed and used in novel formulations of various non-viral lipid nanoparticulate technologies for broad ranges of therapeutic applications.[5,17,129]

Examples of therapeutic payloads researchers have formulated within these lipid-nanoparticulate drug delivery formulations include siRNA macromolecular compounds, RNAi macromolecular compounds, mRNA macromolecular compounds, and anti-sense oligonucleotide compounds.[15,150–152] Non-viral therapeutic nanoparticulate formulations consisting of small interfering RNA (siRNA) macromolecular compounds have been developed to function by silencing pathological genes expressed in diseased cells.[13,32,150] These non-viral lipid-nanoparticle systems are currently at the forefront for enabling clinical progression of genetic therapeutic technologies and are considered to represent the greatest prospective

potential towards clinical translation to date.[14,15,150] Non-viral lipid-nanoparticulate

formulations have been developed for biomedical applications including therapeutic mitigation

of severe cardiovascular disease, mitigation of lung diseases such as pulmonary arterial

hypertension, cancer immunotherapeutic applications, non-viral vaccine therapeutic

applications, among others.[1,11,15] Encouragingly, a number of non-viral lipid-nanoparticulate

drug delivery formulations are rapidly progressing to and through clinical trial

currently.[31,39,64,150]

vi) Therapeutic Advancement of Lipid-Nanoparticle Drug Delivery formulations

Significant advancement of the bioavailability of nucleic acid drugs has been achieved by

researchers developing lipid nanoparticular drug delivery therapeutics. A number of lipid-

nanoparticulate therapeutics have been developed to mitigate deleterious cancerous

phenotypes by implementing advanced enhanced penetration and retention activities (EPR)

when administered *in vivo*.[105] In addition, substantial advancements have been made with

respect to diverse combinations of targeting abilities that have been imparted to particular

advanced lipid-nanoparticulate technologies. These lipid-nanoparticle technologies have been

developed by drug delivery researchers to effectively deliver nucleic acid drugs by employing

targeting ligands as well as other advanced targeting mechanisms to specifically accumulate

within particular physiological coordinates when implemented within *in vivo* disease models.[79]

Importantly, substantial recent translational progress has been made to date with

respect to the development and research pursuits of innovative lipid nanoparticulate

formulations specifically for clinically relevant therapeutic applications.[153–156] Examples of these

important therapeutic nanoparticulate technologies that are currently being pursued by drug

delivery researchers include the Onpattro siRNA nanoformulations, translational mRNA delivery research efforts, and DNA therapeutic drug delivery research efforts, in addition to many other clinically relevant and innovative engineering efforts.[64,150,157,158]

There are a number of clinically relevant benefits specifically related to the implementation of well-engineered nanoparticulate-based drug delivery technologies developed to effectively deliver therapeutic macromolecules when dispensed within treated human patients.[159–162] In particular, one major advantage of employing advanced drug delivery materials and potent formulation technologies for therapeutic applications, as compared to administering exogenous macromolecular drugs on their own within a treated human patient, is the enablement of desirable corresponding effective targeting functionalities.[67,163,164] Respectively, extensive research and engineering efforts have been pursued to date by drug delivery researchers to create novel, innovative therapeutic nanoparticulate platforms developed to effectively evade a treated patient's immune system and accumulate within the right tissue structures when administered within that treated patient's physiology.[165–167]

In addition, these therapeutic nanoparticulate formulation technologies have also been developed to prevent any significant inadvertent release of their potent exogenous genetic macromolecular agent payloads within unintended tissue locations and organ structures.[168,169] Any macromolecular therapeutic technologies, including those engineered to function genetically, that are not designed to exhibit effective targeting functionalities *in vivo* are likely to result in highly undesirable off-target drug activities potentially leading to severely deleterious physiological toxicities.[170,171] Furthermore, advanced drug delivery technologies have been developed to exhibit slow and controlled release functionalities that can be

specifically and reproducibly optimized for particular therapeutic clinical applications.[172,173] For example, particular iterations of these cutting-edge non-viral nanoparticulate formulations have been developed to enable significantly enhanced temporal therapeutic efficacy windows, facilitating substantial broadening of potential prospective translational strategies for effective mitigation of deleterious diseases.[174,175]

Importantly, this particular property of certain classes of non-viral therapeutic drug delivery technologies has been suggested to have the potential to play a significant role in improving patient compliance throughout their courses of treatment.[71,176] It is obviously significantly more convenient for ailing patients to take single dosages of their medications throughout greater portions of their therapeutic courses of prescribed treatments in comparison to alternative continual, frequent, and costly administrations of more primitive, "naked" macromolecular drugs. In addition, in some cases, methods of therapeutic diagnoses and treatments can be at least mildly painful, further contributing to potentially problematic patient aversion and their resultant non-compliance, ultimately rendering their courses of treatment ineffective.[177,178]

Non-viral macromolecular therapeutics formulated within lipid-nanoparticulate drug delivery systems represent desirable classes of cutting-edge translational technologies with the potential to impactfully mitigate various deleterious genetic diseases such as cancerous tumors, cardiovascular disease, in addition to various subsets of rare genetic diseases and their corresponding injurious physiological phenotypes.[73,179,180] In that vein, many diverse and distinct classes of cutting-edge, polymeric drug delivery surfactant materials in addition to non-viral genetic lipid-nanoparticulate formulation platforms have been extensively researched,

engineered, and developed by drug delivery researchers for clinical utility to date. For example,

the Anderson lab at MIT developed a number of distinctive multi-block polymeric lipid material

libraries to form effective non-viral nanoparticulate formulations for the treatment of diverse

genetic diseases and acute physiological abnormalities.[181,182]

Chapter 4: Polymer nanoparticulate formulations for intracellular delivery _in vivo_

i) Polymer-nanoparticulate formulations as compared to lipid-nanoparticulate formulations

Polymer-nanoparticulate formulations, as compared to lipid-nanoparticulate formulations, are significantly different with regards to physical, chemical, and morphological characteristics. Although a number of processing strategies have been developed to generate diverse lipid-nanoparticulate formulations from various surfactant lipid biomaterials as mentioned above, structure conformation is determined solely by inter-molecular hydrophobic interactions between "tail" structures and favorable hydrophilic interactions between the highly water-soluble "head" moieties that make up these diblock biomacromolecules and their corresponding external aqueous environments or aqueous payload compartments within their respective macro-molecular lipid-nanoparticulate formulations. Therefore, lipid and lipid-like polymeric biomaterials used for therapeutic nanoparticle formulations are inherently limited thermodynamically due to their constituent hydrophilic and hydrophobic diblock structures and therefore must conform to spherical bilayer inter-molecular formations in order to minimize their overall free energies.

Contrastingly, polymeric nanoparticulate materials can be designed to conform to enthalpically interact via more diverse combinations of inter-molecular forces. Not only can these polymeric biomaterials be designed and synthesized to interact inter-molecularly in many unique and distinct ways, they can also be functionalized in a variety of interesting ways to cooperatively interact with molecular components of their intended physiological environments to afford even greater broadening of possible enabled drug delivery functionalities.[90,142,208,209]

More specifically, polymeric biomaterials used to generate polymer-nanoparticulate formulations can be engineered to exhibit much more complex combinations of intermolecular forces beyond solely interfacial hydrophobicity, such as ionic and electrostatic inter-molecular interactions.[210,211]

ii) Polymer nanoparticulate technologies; physical, chemical, and thermodynamic properties

In addition to complex combinatorial multi-blocked polymer structures with simultaneous hydrophobic and hydrophilic enthalpic interactions occurring, these polymeric units are also constantly interacting thermodynamically between units within themselves, with other equivalent polymeric molecules, and with ionic components within their aqueous environments via additional inter-molecular interactions of various strengths. Though important for polyplex formation, these electrostatic interactions that are relied upon for complexation of these types of nanoparticles are significantly less robust, especially in physiological environments, than the hydrophobic effects of aliphatic tail groups utilized to generate lipid-nanoparticulate formulations with internalized therapeutic agents.

It is important to note that the stable complexation process of nucleic acid carrying polyplex nanoparticles is not necessarily solely based upon energetically favorable interactions between the anionic backbone of the therapeutic agent and the cationic electrolyte polymer. More significantly, stability of the resultant polyplex formulation is mostly due to the enormous entropy gain from counterion release from the charged polycation interacting with the anionic units of the entrapped macromolecular genetic agent.[212–215] Furthermore, polyplexes formed from various types of polycations can exhibit substantially diverse physical and chemical characteristics that include distinct ranges of hydrodynamic radii, radius of gyration, and zeta

potential.[216–218] In the case of polymer-nanoparticulate formulations, the zeta-potential is varied by altering the ratio of moles of anionic phosphate backbone units of the genetic oligonucleotide to the charged cationic units of the polymeric biomaterials.[219,220] If a polyplex contains a greater proportion of anionic units than cationic units, then the polyplex will tend to exhibit a more anionic surface charge and vice versa.[185]

Due to the diversity of continuous simultaneous interactions between the polymers, entrapped therapeutic biomacromolecules, and components within their physiological environments, these polymeric molecules are constantly in motion.[221,222] They reptate and swell throughout each other while attempting to ultimately find their energetically minimized equilibrium conformation within their macro-molecular structures; a process theorized to require infinitely greater timeframes than the timeframes associated with the respective observable lifetimes of existence of these polymeric materials within their physiological environments in some cases. As a result of the inherent physical and chemical complexities associated with multi-blocked polymeric materials used for therapeutic nanoparticulate formulations, compared to lipid-nanoparticulate formulation strategies, it is extremely difficult to form tight distributions of hydrodynamic radii within formulated batches of polymer-nanoparticulate formulations.[223] It follows that it can also be extremely difficult to ensure reproducible physical and chemical characteristics when generating different batches of the same therapeutic polymer-nanoparticulate formulations and therefore can be very difficult to scale up to generate the required quantities for clinical trials.[224]

iii) Polymer-nanoparticulate formulations; inherent formation challenges

In this vein, substantial broadening of potential structure formation possibilities may seem like an obvious key advantage for therapeutic nanoparticulate technologies formulated from synthetic polymeric biomaterials as compared to alternative classes of drug delivery vehicles, however, designer polymers engineered to exhibit numerous combinatorial inter-molecular interactions with each other are highly difficult to control and model with respect to their resultant inter-molecular conformations.[225–227] Impressive computational predictions of how these highly complex macromolecular multi-blocked polymeric structures will form and interact with the required precision and accuracy are just coming to fruition.[228] However, veritable pervasive utility and validation of these models continues to be highly desirable and remains to be relatively elusive throughout the field currently. As a result, compared to the lipid-nanoparticulate therapeutic formulations described above, polymer-nanoparticulate formulations may continue to be significantly more unwieldly and unpredictable in terms of polydispersity, morphology, and glycocalyx inter-molecular interactions than alternative nanoparticulate formulations.[229] Furthermore polymer-nanoparticulate formulation methodologies tend to be relatively complex and generally require harsh organic solvents and extreme processing steps that can inadvertently degrade the entrapped active non-viral therapeutic agent.[230,231]

Furthermore, lipid-nanoparticulate formulations can be reproducibly formed to be extremely robust (in the general case) due to the relative vitrification and kinetic trapping properties inherent to their model lipid-bilayer structures and their standardized heterogenous aliphatic macromolecular compositions.[232] Well-formed lipid-nanoparticulate formulations do not significantly change rheologically or morphologically when exposed to different aqueous

environments either external to, or within, the intended physiological system and when administered unless exposed to extreme osmolarities and acidities or harsh detergents are added.[233] This characteristic rigidity in structure due to the kinetically robust nature of lipid-nanoparticulate formulation processes represents a major advantage with regards to the therapeutic utility of these classes of nanoparticles compared to polymer-nanoparticulate drug delivery technologies.[234,235]

iv) Basic polymer-nanoparticulate formulations

In the case of delivering genetic modulating payloads such as DNA and RNA agents, polyplex nanoparticulate formation is a standard approach to form effective polymer-nanoparticulate therapeutic formulations.[18,28,202] For example, it is common practice to take advantage of favorable enthalpic electrostatic interactions between cationic polyelectrolyte biomaterials and anionic oligonucleotide macromolecules.[236–239] Polyamines with primary, secondary or tertiary amine moieties along and/or extended from the backbone cationic polymer structure are typical examples of electrolytes that would be used to form oligonucleotide containing polyplex nanoparticles.[240,241]

For these polymeric materials to function satisfactorily *in vivo*, they need to be engineered to be nontoxic, non-immunogenic, non-mutagenic, and biocompatible.[90,242] Therefore, one critical chemical characteristic of biomaterials engineered to form non-viral polymer nanoparticulate formulations is that they need to be functionalized to be cleared from a treated host's physiological system in an innocuous and efficient manner.[243] In order to do this, researchers have developed standard polymeric materials for the formulation of non-viral

polyplex nanoparticulate formulations that are functionalized to be biodegradable or bioeliminable when dispensed within their respective physiological environments.[222,244–246]

v) Biodegradable and bioeliminable polymer-nanoparticulate formulations

Correspondingly, a number of elegant synthetic strategies have been developed to enable reproducible generation of biodegradable polymeric materials with heteroatomic backbones that can undergo predictable hydrolysis when utilized in model physiological environments.[245,247,248] High molecular weight polymer materials used to generate therapeutic non-viral polyplex formulations are insoluble in most relevant aqueous solvents, including physiological solutions. When these polymer materials are functionalized with water-cleavable bonds, hydrolysis reactions can result in continual sequential halving of these formulated polymer materials.[249,250] When these high molecular weight macromolecular polymers are hydrolyzed sufficiently into low molecular weight oligomeric compounds that are below a threshold hydrodynamic radius, they become highly soluble within physiological environments and can then be easily cleared from a host's physiological system in an innocuous manner.[251,252] Examples of biodegradable polymeric materials that have been developed and utilized in therapeutic applications include heteroatomic polymers with ester linkages such as poly(lactide-co-glycolide); this being one of the first biodegradable polymer materials used to generate veritable drug delivery formulations.[253–257] Backbone ester linkages can be hydrolyzed by water molecules and completely broken down into carbon dioxide and water products within physiological environments.[250]

Importantly, there are a few standard heteroatomic backbone structures that are utilized for predictable relative rates of hydrolysis and resultant polymer degradation

kinetics.[245,248,253,258] Biodegradable polymers synthesized with anhydride units along their backbones are susceptible to the most rapid catabolic reactions when dispensed within physiological environments. These classes of drug-delivery polymers are therefore used when rapid drug release is desirable and they have also been designed to exhibit tunable pH responsive degradation and resultant triggerable controlled release of non-viral therapeutics.[259–261] Backbone ester linkages are hydrolyzed at relatively rapid rates as well when administered in physiological environments, but are cleaved by water molecules at significantly slower rates than anhydride groups.[262] Biodegradable polyplex biomaterials engineered with polymers containing carbonate and amide backbone units are hydrolyzed at the slowest kinetic rates when exposed to water molecules within model physiological solvents and environments due to their characteristically higher glass transition temperatures, crystallinity and lower free volume throughout these materials.[240] They are used when slow release kinetics are desired for their respective genetic therapeutic payloads and are also commonly synthesized into branched structures such as dendrimers.[263–266]

Bioeliminable materials are also biocompatible polymer materials that have been developed and utilized to generate potent polymer nanoparticulate formulations for therapeutics.[267–269] These are polymers that do not undergo hydrolysis reactions. They remain at high molecular weights throughout their respective timeframes of therapeutic utility and are usually removed from the host's physiological system via natural waste clearance mechanisms. Usually these materials are eliminated through the kidneys or are filtered out from the extracellular fluid by the host's liver organ system.[270,271] Examples of bioeliminable polymers that have been used for therapeutic polymer nanoparticulate formulations include low

molecular weight dextran sulfate and polyethylene glycol.[272–275] Importantly, these polymer

backbones can also be heteroatomic like those described above for biodegradable polymer

materials. For example, polyethylene glycol polymers contain periodic ether bonds along their

backbone, however, these molecular units are not susceptible to hydrolysis reactions and

therefore are not cleaved when exposed to water.[276]

vi) Polymer-nanoparticulate formulations with controlled release functionalities

Precisely engineered controlled release functionalities are particularly important with respect

to therapeutic utility of polymer-nanoparticulate formulations.[256,277] Substantial research and

development efforts have been pursued to generate polymeric biomaterials that reproducibly

exhibit particular sets of controlled release mechanisms.[222,278] Controlled release parameters

represent another critical aspect of study within the drug delivery field along with targeted drug

delivery.[248,258,279] Optimizing the controlled release kinetics of particular polymer

nanoparticulate formulations is critical for maintaining an effective dosage within a

physiological system over a particular therapeutic timeframe.[280,281]

Furthermore, developing polymer-nanoparticulate formulations that can controllably

release important non-viral therapeutic agents over extended periods of time in a sustained

manner can help mitigate potential issues with patient compliance.[282] In addition, as in the case

of lipid-nanoparticulate formulations, controlled release kinetics in the case of polymer-

nanoparticulate formulations can help ensure that the active genetic agent is released at the

site of disease while avoiding potentially harmful reactions within normal tissue and organ

systems.[73,283] Also, optimization of controlled release kinetics of polymer nanoparticulate

formulations can effectively protect a therapeutic macromolecule for extensive premature

degradation.[67,284] As mentioned above, RNA macromolecules, for example, tend to degrade rapidly when dispensed within physiological environments and can quickly become non-functional if flagged by the host's immune system as exogenous.[275,285]

A number of standardized drug release functionalities have been implemented in distinct classes of non-viral nanoparticulate formulations that have been utilized for therapeutic applications.[286–290] For example, a solid matrix can be formed around an aqueous compartment that can be loaded with the active non-viral therapeutic compound such as in the case of nanogel drug delivery vehicles.[217,291,292] In this case, the polymers typically used are not biodegradable and the diffusion of the therapeutic out of the nanoparticle into the extracellular environment is determined by predictable and reliable diffusion kinetics.[293,294] Similar to the case of liposomes formulated with small-molecule therapeutic agents, this formulation approach is a good strategy to deliver non-viral water-insoluble drugs such as poorly soluble therapeutic peptides as well as other active macromolecular agents to aqueous tissue and organ environments.[295–297]

In addition, very concentrated amounts of oligonucleotide drugs can be loaded into these polymer-nanoparticulate formulations with related formulation processing methodologies, enabling long lasting therapeutic effects when dispensed within physiological systems.[298,299] However, one potential major problem with these classes of drug delivery formulations is the possibility of toxic levels of therapeutic compounds inadvertently being released as a burst.[300–302] This can happen when unintended defects are formed within the spherical polymer layer and can result in extremely harmful side effects due to dose dumping within a treated host.[303,304]

Another commonly implemented formulation design for controllably releasing non-viral polymer-nanoparticulate formulations is an eroding polymer matrix biomaterials structure.[248,277,305] Eroding polymer matrix nanoparticulate formulations can controllably degrade and release their entrapped non-viral therapeutic agents based on the types of heteroatomic polymer macromolecules used.[184,306,307] This formulation strategy is beneficial in that there is less of a chance of inadvertent burst release of the potent therapeutic agent when administered within a physiological system. The two main modes of erosion (degradation) that define distinct classes of these polymer matrix nanoparticulate formulations are surface erosion and bulk erosion.[308,309] Polymer nanoparticulate formulations designed to undergo surface erosion exhibit zero order drug release.[310,311] This means that the therapeutic agent is released within the intended physiological environment in a highly controlled, steady manner. The polymers used to formulate surface eroding non-viral polymer nanoparticulate formulations are typically types of poly-anhydrides, which degrade at relatively rapid rates.[312,313] Therefore, the lifetimes of these particular drug delivery systems are relatively short, and the therapeutic effect is kinetically limited.

In comparison, nanoparticulate formulations that have been engineered to undergo bulk erosion mechanisms are typically formed from polymers synthesized with poly-ester backbone units, and consequently can exhibit significantly longer lifetimes within their respective physiological systems.[309,310,314] Therefore, these bulk eroding nanoparticulate formulations can be designed to demonstrate effective therapeutic efficacies over longer periods of time compared to alternative therapeutic surface eroding polymer nanoparticulate formulations. However, these bulk eroding polymer nanoparticulate formulations have been

characterized to release their therapeutic macromolecular agents in an unsteady manner due to their spontaneous degradation mechanisms and corresponding reaction kinetics which can be highly undesirable for therapeutic applications.[293]

Specifically, as these bulk eroding poly-ester materials are hydrolyzed by water molecules within their aqueous physiological environments, they can rapidly catabolize to produce acid products that can act to significantly alter the pH of the local environment within the polymer matrix.[308,315] This alteration of pH within the bulk eroding material can act to inadvertently enhance the degradation kinetics of the surrounding ester bonds.[256,257] This can result in an exponential increase in the reaction kinetics of degradation of the constituent poly-ester biomaterials as well as the corresponding drug release rates as the pH throughout the material significantly decreases over time.[318,319] This change in pH can also detrimentally affect the structural integrity of the therapeutic compounds entrapped within their respective polymer matrices, potentially rendering these genetic therapeutics non-functional over time. Alterations to the molecular structures of these polymer matrices, such as addition of periodic bulky side groups to slow the diffusion of the surrounding water molecules throughout these materials, have been studied and implemented by researchers to better control the degradation kinetics of these bulk erosion polymer biomaterials while more effectively protecting the activities of the entrapped therapeutic genetic compounds.[248,310,320,321]

vii) Advanced polymer-nanoparticulate formulations

As suggested above, the stability of polyplexes in general is a concern when utilizing these classes of non-viral nanoparticulate formulations *in vivo*.[223,322] When these polyplexes are exposed to physiological environments characterized by high molarities and/or undesirable pH

ranges, they can exhibit prohibitively limited stabilities and degrade before exhibiting any significant therapeutic efficacies. A number of materials engineering strategies have been applied to improve the stability of systemically administered polyplex nanoparticles formulated for macromolecular drug delivery such as PEGylation, addition of stealth protein coronas, as well as modulating size and surface charge characteristics of non-viral polymeric nanoparticulate formulations.[115,223,229,322] Importantly, diverse physiological environments can also result in deleterious aggregation of these nanoparticles which can lead to volatile immune responses from the treated host, similar to those described above in the case of non-viral lipid-nanoparticulate formulations.[280,323,324]

Other challenges with utilizing non-viral polyplex nanoparticulate formulation technologies include potential issues and concerns with regards to critical therapeutic functionalities. If a polyplex is designed to effectively remain stable when exposed to various model physiological environments, how will the internalized oligo-nucleotide payload ever controllably release? This remains to be a highly contested source of debate within the field currently.[325–328] Contrastingly, in the case of lipid-nanoparticulate formulations, once the liposome has reached a targeted cell, efficient internalization is achieved through a subsequently triggered endocytosis pathway.[329–331] Methods of internalization of polyplexes, on the other hand, are potentially significantly less consistent even from cell to cell within a single targeted tissue type. Another substantial concern with respect to pervasive commercialized cationic polymer biomaterials used to form non-viral polymer nanoparticulate formulations for therapeutic applications, is the characteristic cytotoxicity of some of these synthetic, un-natural macromolecules that can inherently limit therapeutic utilities of these types of drug delivery

technologies.[332,333] Free cationic polymer materials, such as poly-ethylenimine, can be highly cytotoxic and can cause detrimental membrane disruption.[334,335]

Although expansive research and development efforts have been pursued to date to generate vast libraries and varieties of non-viral polymer-nanoparticulate formulation technologies, in general, polymer-based formulations have been demonstrated to be less therapeutically effective and biocompatible *in vivo* when compared to therapeutic lipid-nanoparticulate alternatives.[336–338] However, polymer-nanoparticulate formulations can afford considerably greater synthetic options and can therefore be designed to enable significantly broader potential prospective materials functionalities as compared to lipid-nanoparticulate formulations.

Importantly, lipid-nanoparticulate formulations are limited to intracellular applications and therapeutic strategies; they can only affect a cell internally after endocytosis. Contrastingly, polymer-nanoparticulate technologies can be designed to remain within targeted extracellular environments, enabling even more diverse potential therapeutic functionalities.[339–341] For example, polymer-nanoparticulate drug delivery vehicles have been designed to entrap growth factor proteins to function as stem cell homing signals, directing pluripotent cells to damaged sites within a diseased organ system.[342,343] Polymer-based nanoparticulate formulations can be synthesized to exhibit more sophisticated timed degradability functionalities, multilayered combinatorial drug release capabilities, and can be formulated from wide varieties of controllable self-assembly formation methodologies and processes leading to vast libraries of distinct ranges of nanoparticulate structures.[174,241,244,284] Encouragingly, many varieties of

polymer nanoparticulate formulations that have been designed to function in diverse and

distinct ways are currently progressing rapidly to and through clinical trials.[345–348]

Chapter 5: Nanoparticles for diagnostics and imaging relevant to intracellular delivery *in vivo*

There are a number of distinct classes of other nanoparticulate formulations that have been developed for therapeutic applications that are not classified as lipid or polymer nanoparticulate formulations.

i) Advanced nanoparticulate technologies for biomedical imaging functionalities

One example of exciting nanoparticle technologies being rigorously pursued to date include the development of formulations that exhibit unique and precise biomedical imaging functionalities. Biomedical imaging nanoparticulate technologies have been designed with diverse surface chemistries, distinct magnetic properties, and have be designed to absorb and emit specific wavelengths of electromagnetic radiation in order to effectively function.[321,349,350] Many iterations of these unique, novel nanoparticulate technologies have also been developed with specific surface chemistry properties.[351–353] These functional advancements have effectively enabled these exciting nanoparticle technologies to more precisely target tissue and organ systems of interest while being endocytosed by the correct cell type within the host's physiological system.[354–356]

Currently, nanoparticles being developed for therapeutic molecular imaging applications related to the diagnosis and treatment of cancer.[357,358] The most important aspects of novel formulations developed for imaging and diagnostic functionalities in general relate to their characteristic water-solubilities, biocompatibilities, surface charges, and hydrodynamic radii enabled by the chosen constituent nanomaterials.[350,359,360] For example, as was in the case of other classes of nanoparticles mentioned above, researchers have pursued synthetic

PEGylation strategies so these nanoparticles can effectively evade phagocytosis and subsequent inactivation by cells of the host's immune system while increasing the circulation lifetimes of these types of formulations.[361,362]

Biomedical imaging nanoparticulate formulations formed from metallic materials have demonstrated immense potential therapeutic utility to date.[349,350,363] Metallic imaging nanoparticles can be functionalized to exhibit highly potent X-ray absorption properties and are non-toxic *in vivo* at low dosages over short therapeutic windows.[350] In particular, nanoparticles formed from gold metallic materials are considered to be highly promising therapeutic formulations as they are particularly bioinert and also exhibit extremely efficient X-ray absorption properties.[364–366] Furthermore, gold nanoparticles are substantially denser, leading to enhanced imaging functionalities compared to other possible metal nanomaterials that have been pursued for therapeutic imaging applications such as iodine.[350,367,368]

ii) Surface functionalization strategies for metallic nanoparticles for biomedical imaging

As suggested above, a key area of study is surface functionalization of these nanoparticles with molecular targeting moieties and epitope ligands.[369–371] Enabling targeting functionalities on to these nanomaterials is critical for effective and precise accumulation of these materials within specific physiological structures and tissue environments.[355,370,372] With properly enabled targeting functionalities, these biomedical imaging nanomaterials can demonstrate significantly enhanced contrast images as compared to less advances nanoparticulate technologies.[373,374] Improvements in contrast functionalities by advancing targeting abilities of these biomedical imagine formulations has greatly improved potential prospective therapeutic utilities of these exciting nanoparticulate technologies for cancer diagnostics and therapeutics.[350,351,365]

In this vein, in order to target diseased tissues in *in vivo* cancer models effectively, biomedical imaging nanoparticulate technologies have been developed to exhibit high affinities towards overexpressed surface receptors characteristic of cancer cells.[375,376] These targeted diagnostic imaging nanoparticles have been developed with innovative and empirically validated peptide moieties conjugated to the surface and expansive varieties of targeting ligands.[350,375] Although substantial progress has been made with respect to development of biomedical imaging nanoparticle technologies along these lines, veritable clinical utility remains suspect considering the immense cost of these particular synthetic strategies in addition to limited shelf lives of these particular heterogenous macromolecular formulations.[363,375,377]

Alternatively, conjugation of small organic targeting molecules to the surface of biomedical imaging nanoparticulate formulations to impart precise targeting capabilities on to these next generation theragnostic technologies has been demonstrated to successfully enable significantly enhanced contrast functionalities while being more economical.[350,378] This innovative approach to accurate and precise accumulation of these nanomaterials within the proper tissue systems within an *in vivo* cancer model has demonstrated substantial potential therapeutic utility while providing a significantly more economical alternative to expansive synthetic and storage processes required for more complex biomacromolecule targeting ligands and peptides.[379,380] These small-organic-targeting molecular approaches that have been extensively pursued by drug delivery researchers to enable advanced precision accumulation properties onto these imaging nanoparticulate formulation technologies also allows more defined surface densities of these targeting moieties over the surfaces of these nanomaterials.[379,381,382] Tunable surface densities of these nanoparticulate formulations

enables the generation of imaging materials functionalized with even more specific binding affinities leading to even more precise targeting of specific cell types *in vivo*.[379]

iii) Iron-oxide drug-delivery nanoparticulate technologies

In particular, novel formulations generated from magnetic iron oxide nanomaterials have become significantly pervasive biomedical imaging technologies developed and used by drug delivery researchers.[159,383] In combination with advanced MRI techniques, novel iron oxide nanoparticulate formulations have been utilized to elucidate characteristics of a wide variety of deleterious diseases such as autoimmune diseases, cancer theragnostic, and tagged cell-fate determination in mammalian *in vivo* models in real time.[384–386] In order to generate batches of low polydispersity iron oxide nanoparticles, a number of synthesis strategies have been developed to date. These formation methodologies include sono-chemical synthesis, precipitation techniques, and microemulsion formulation strategies.[23,387,388] It has been demonstrated that techniques which utilize hydrophobic ligands in combination with organic solvents are some of the most effective ways to create low polydispersity iron oxide nanoparticulate formulations with consistent shapes and morphological features.[350]

Iron oxide nanoparticulate formulations are typically generated within hydrophobic conditions and are therefore not directly biocompatible.[389,390] Therefore, strategies have been pursued by drug delivery researchers to generate iterations of new iron oxide nanoparticle formulations with multifunctional ligand systems that enable transfer of these nanomaterials from hydrophobic to aqueous solvents.[391,392] This, as well as other related key materials engineering advancements have led to significantly more biocompatible iron oxide nanoparticles that have been demonstrated to be extremely useful for broad ranges of

therapeutic and diagnostic applications.[393,394] For example, researchers demonstrated substantially enhanced colloidal stabilities of iron oxide nanoparticles when using a novel co-precipitation formulation process.[389,395] Some iterations of these innovative particles were also functionalized with reactive primary amines, enabling even greater combinations of chemical and physical therapeutic functionalities *in vivo*.[350]

It has been shown that variations in properties of iron oxide nanoparticles significantly affect the resultant functionalities of these materials and their respective formulations.[353,396,397] In addition to enabling effective imaging functionalities, iron oxide nanomaterials have also been developed to exhibit wide ranges of magnetic properties.[398,399] It has been shown that the hydrodynamic radius of these types of metallic nanoparticles can have a substantial effect on the magnetic resonance and magnetism properties of these formulations.[400,401] Researchers have shown that optimizing the hydrodynamic radii of iron oxide nanoparticles in a controlled, low polydispersity manner can lead to enhanced magnetism properties as well as significantly improved image contrast and quality.[373,400,402] Furthermore, doping these iron-oxide nanoparticulate formulations with other metal elements can also significantly enhance magnetic features of these materials while improving their contrast functionalities.[395] For example, Lee *et al*. showed that doping iron oxide nanoparticles with $MnFe_2O_4$ exhibited significantly greater magnetic susceptibility than other formulated iron oxide nanoparticles.[350]

As with the previously described nanoparticles that have been developed for drug delivery applications *in vivo*, a wide variety of surface modification strategies have been pursued by researchers to improve and expand upon potential therapeutic functionalities enabled by novel magnetic nanomaterials.[351,353,403] For example, iron oxide nanoparticles have

been developed with PEGylated surface chemistries.[404–406] PEGylated iron oxide nanoparticles have shown significantly enhanced circulation times *in vivo* compared to analogous, unmodified iron oxide nanoparticles.[404,407] Magneto-fluorescent iron oxide nanoparticles have also been developed with multi-modal imaging functionalities via innovative synthetic conjugation methods.[408,409]

Iron oxide nanoparticulate formulations can be used to generate informative images and data sets with magnetic resonance imaging techniques.[352,363,386] MRI imaging functionality is highly desirable in clinical settings due to its inherent non-invasive nature.[410,411] Importantly, many iterations of iron oxide nanoparticle technologies that have been published on to date have not shown significantly toxicity *in vivo* at the concentrations administered.[412,413] Iron oxide nanoparticulate technologies have become highly desirable MRI imaging agents due to their biocompatibility and the high-quality images that are achievable with low to moderate doses of these magnetic materials.[414] In combination with advanced MRI imaging techniques, corresponding iron oxide nanoparticulate formulations have been developed to generate accurate images of soft tissue structures *in vivo* of high contrast.[411,415] These MRI clinical methodologies are considered to be significantly preferable as compared to radionuclide-based imaging strategies as the latter can lead to substantially detrimental toxicity *in vivo* due to the high doses of radiation required.[350]

Novel iron oxide nanoparticulate technologies have been developed to exhibit broad ranges of diagnostic and research functionalities *in vivo* to date. Precise targeting functionalities have been engineered into some of these innovative magnetic nanoparticles, for example.[416–418] Iron oxide nanoparticles have been formulated with conjugated carcinoembryonic antigen

monoclonal antibodies which enabled selective accumulation of these nanoparticles within cancerous tissues of diseased *in vivo* models.[350,375] Cross-linked iron oxide magnetic nanoparticles have also been formed with surface conjugated Annexin-V-Labeled macromolecules, which demonstrated preferential targeting of apoptotic cells when administered *in vivo*.[419,420]

In addition to sophisticated targeting and imaging functionalities, effective macromolecular nanoparticulate drug delivery vehicles have been developed from constituent iron oxide nanomaterials.[421] Researchers have shown that magnetic nanoparticles can effectively transfect target cell types with genetic materials such as DNA and RNA. For example, magnetic nanoparticles have been coated with polyethyleneimine polymers to efficiently carry out targeted non-viral gene delivery in mammalian cell types.[422] Due to their biocompatibility, low tissue toxicity, and potent magnetic properties, iron oxide nanoparticles have also been developed by researchers to deliver chemotherapeutic agents in *in vivo* cancer disease models.[416] Targeted chemotherapeutic iron oxide nanoparticulate formulations have also been developed with layers of covalently attached folate moieties on the surface and demonstrated potent, precise theragnostic efficacies *in vivo*.[423] Clearly, magnetic drug delivery formulations, such as those generated from iron oxide nanomaterials, continue to demonstrate immense clinical potential as they can be engineered to function effectively in an number of therapeutic ways.

iv) Quantum dot drug-delivery nanoparticulate technologies

Another significantly pervasive class of imaging nanoparticulate formulations are formed from quantum nanodot materials.[424–426] These materials are fluorescent semiconductor nanocrystals

and are typically between 1 and 100 nm with respect to their hydrodynamic radii.[350,427,428] In comparison to alternative fluorescent dies, quantum nanodot materials have been developed by researchers to exhibit near unity quantum yields and are characteristically bright *in vivo* imaging technologies.[350,429,430] Quantum dots are typically formed to exhibit broad absorption properties while emitting extremely narrow linewidths in corresponding emission spectra.[431,432] Quantum dot materials are continuously fluorescent and are tunable with long fluorescence lifetimes compared to comparable fluorescent dyes, > 100 ns versus 1 to 5 ns, respectively, making them extremely useful for cancer therapeutics applications.[350,433,434]

Quantum dot materials were first developed and synthesized by Onuschenko and Efros in 1982.[350,435] After this initial pioneering work, substantial efforts have been pursued by researchers to enable significantly broader spectrum functionalities and improve biocompatibility properties of subsequent generations of these technologies.[426,436–438] Initially, quantum dots were synthesized from cadmium sulfide, cadmium selenide, or cadmium telluride materials.[350] Researchers have also developed quantum dots out of these types of materials in the form of shells containing metalloid crystalline cores for cancer imaging applications.[439–441] Shells that have been synthesized and formed around quantum dot nanoparticulate materials that contain metalloid crystalline cores have also been made from ZnS, this materials advancement in particular having greatly enhanced potential functional spectrum ranges of these fluorescent semiconductors.[442–444] Researchers have also successfully developed ZnO core/shell quantum dot formulations that have shown significantly improved photographic imaging by significantly diminishing the potential for photobleaching issues as previously demonstrated by alternative iterations of these quantum dot nanomaterials.[350,445,446]

Colloidal stability of quantum dot imaging materials represents a major challenge associated with these particular therapeutic nanoparticulate technologies.[447–449] Particularly in aqueous solvents, such as those analogous to physiological environments, these quantum dot nanoparticles can have difficulty remaining suspended and soluble within their respective solutions.[450,451] Furthermore, in general synthesis and chemical processing methodologies to generate quantum dot nanomaterials are characteristically hydrophobic, making direct transfer of these nanomaterials to physiological administration a significant obstacle.[350,452] Modifying the surfaces of these quantum dot nanoparticles with stabilizing macromolecules and chemical moieties is one strategy that researchers have used to significantly enhance the water-solubility and stability of these biomedical imaging nanoparticulate technologies within their physiological environments.[453,454]

Therapeutic and diagnostic applications involving the utilization of quantum dot nanoparticulate technologies are becoming increasingly pervasive throughout the drug delivery field.[424,426,455] Quantum dot nanomaterials have been developed to exhibit extremely high-resolution images with enhanced sensitivities that have been demonstrated to be extremely useful sources of therapeutically relevant empirical data *in vivo*.[456–458] Furthermore, quantum dot imaging technologies enable high quality imaging data with relatively inexpensive photographic equipment while being inherently non-invasive, enabling highly reliable and robust data sets.[456,459,460] One challenge with using quantum dots *in vivo*, however, has been the limited tissue penetration depth of acquirable fluorescent images when using these semi-conductive nanomaterials.[425,461,462] Due to the limited depth of possible penetration through *in vivo*, high resolution images of internal organs of interest for therapeutic applications may be

unachievable with quantum dot technologies. Encouragingly, a number of promising research efforts are currently being pursued to improve upon achievable tissue penetration with fluorescent imaging technologies.[463,464]

A number of interesting quantum dot formulation technologies have been developed to deliver non-viral macromolecular therapeutics *in vivo*. For example, the Bhatia Lab at MIT developed multifunctional quantum dot nanoparticulate drug delivery vehicles conjugated with targeting peptides specific for cancer cells that also entrapped active siRNA gene effector agents.[465] These innovative nanoparticulate technologies demonstrated potent therapeutic efficacies in mammalian cells while exhibiting bright, easily detectable fluorescent signals.[465] Non-viral quantum dot based drug delivery formulations were also developed to produce effective therapeutic efficacies and bright fluorescent signals when targeted to mammalian brain-derived cell types.[466] Quantum dot intracellular non-viral nanoparticulate formulations have been developed by researchers to effectively deliver therapeutic siRNA genetic agents to therapeutically silence a number of disease pathways including sphingomyelinase pathways and disease pathways involving MMP-9.[466,467]

iv) Carbon nanotube drug-delivery nanoparticulate technologies

Carbon nanotubes represent another important class of drug delivery nanoparticulate technologies. Carbon nanotubes were discovered in 1991 and have become extremely interesting to drug delivery researchers for therapeutic and diagnostic applications due to their highly unique molecular compositions and morphologies.[468–470] In comparison to alternative nanomaterials that have been used for therapeutic applications *in vivo*, carbon nanotube materials have been characterized to exhibit substantial thermal and electrical conductivities

and are of significantly higher tensile strengths.[471,472] It is important to note that in addition to being researched for their potential prospective utilities in biomedical applications, carbon nanotube materials have been developed and used to advance and engineer a variety of innovative technologies in other fields as well, such as novel nanoscale transistors, components in various composite materials, and to form more effective tips for scanning microscopy applications.[350]

In particular, single-walled carbon nanotubes are currently being investigated for their pronounced near-infrared fluorescence properties that have been demonstrated to be extremely useful for therapeutic and diagnostic applications *in vivo*.[473–475] Importantly, near-infrared fluorescence represents a particularly desirable spectral range for drug delivery imaging strategies *in vivo* and corresponding therapeutic applications of interest due to the lack of inherent background autofluorescence in that range from the surrounding blood cells and tissue structures. This background autofluorescence is inherent to all physiological systems *in vivo* and has been consistently demonstrated to preclude any significant therapeutic utility from a number of alternative fluorescent agents developed to date.

Contrastingly, this unique and desirable near-infrared electromagnetic spectral range, in combination with broad ranges of generalizable surface modification strategies that have been developed by researchers to date, has enabled the advancement and production of extremely effective novel *in vivo* imaging agents, biological sensors, and diagnostic platforms based off of single-walled carbon nanotube materials.[476–478] Furthermore, single-walled carbon nanotubes can be synthesized with a specific molecular chirality.[479,480] It has been shown that tuning the chirality of different iterations and batches of single-walled carbon nanotubes can lead to a

diversity of distinct metallic and semiconducting properties.[481,482] Carbon nanotubes can also be engineered to emit triggerable near-infrared fluorescence in the presence of altered pH levels, molarities, and temperatures, further expanding upon possible therapeutic and diagnostic functionalities.[483–485]

Importantly, there are two major concerns associated with using carbon nanotube nanomaterials in biomedical applications. Carbon nanotube materials universally exhibit extremely poor solubilities in physiological environments and can also cause substantial toxicity when administered *in vivo*, even when dispensed at low dosages.[486–488] These carbon nanotube materials are inherently non-biocompatible, non-biodegradable, and non-bioeliminable when administered *in vivo*.[468] Unfortunately, these undesirable molecular characteristics inherent to carbon nanotube materials continue to significantly limit their potential clinical utility.[489] However, there have been a number of encouraging research efforts pursued to date focused on the development of surface functionalized carbon-nanotube materials that have exhibited significantly improved biocompatibilities and corresponding solubilities in various aqueous solvents.[490,491]

Although cytotoxicity of these carbon nanotube materials remains a significant concern in the drug delivery field, these nanoparticulate formulation technologies have been successfully developed to exhibit wide ranges of exciting therapeutic functionalities in mammalian *in vivo* disease models.[492–495] For example, using electrostatic intermolecular interactions, researchers have been able to surface modify carbon nanotube materials with biological macromolecules that can function as targeting ligands.[496–498] Researchers have developed single-walled carbon nanotubes functionalized with streptavidin that subsequently

demonstrated preferential targeted intracellular delivery to promyelocytic leukemia cells.[499,500]

Researchers have also developed surface modified single-walled carbon nanotubes that demonstrated targeted endocytosis by T-cells residing within tumor tissues *in vivo*.[501]

Single-walled carbon nanotubes have been developed for broad ranges of imaging functionalities *in vivo* as well.[473,502,503] For example, researchers have developed FITC conjugated carbon nanotubes functionalized to exhibit multi-modal imaging capabilities *in vivo*.[504,505] Interestingly, versions of these carbon nanotubes were developed to empirically evaluate the cytotoxicity of the carbon nanotubes when located intracellularly within target cell types.[506] Researchers also surface modified multi-walled carbon nanotubes with diethylenetriaminepentaacetic dianhydride for dynamic imaging functionalities *in vivo* and demonstrated encouraging potential utility of these graphene materials for systemic cancer diagnostic evaluation and treatment.[507–509] It was also demonstrated that iterations of these conjugated carbon nanotube materials were eliminable as they were detected in the excreted urine from treated *in vivo* cancer models.[350,510]

A number of carbon nanotube based drug delivery vehicles for non-viral therapeutics and diagnostic applications have been developed to date. [495,510] Carbon nanotube materials can be functionalized as described above to target specific cell types. Supramolecular nanotube-based structures have also been formed and developed by drug delivery researchers to enable intracellular delivery of a number of macromolecular therapeutic agents of interest.[511,512] Carbon nanotubes have also been developed to deliver siRNA therapeutics to targeted cancer cells by carboxylation modification of the graphene surface.[513] Non-viral carbon nanotube formulations have also been developed to intracellularly deliver siRNA therapeutics precisely to

antigen presenting cells, such as dendritic cells, and other immune cells such as T-cells both *in vitro* and *in vivo*.[514,515] Although there continue to be a number of concerns related to the toxicities and biocompatibilities these high tensile materials, their unique ranges of potentially engineerable functionalities specifically for theragnostic applications, which include diagnostic imaging and non-viral drug delivery, make these carbon nanoparticulate drug delivery vehicles extremely attractive candidates for clinical translation currently.[492,496,497]

Chapter 6: Conclusion and Outlook

Substantially exhaustive and expansive research efforts have been pursued by numerous drug delivery researchers and related commercial entities. Although impressive developments have been demonstrated throughout the field thus far, considerable advancement and innovation remains to be achieved by researchers focused on enabling more consistent development of clinically useful drug delivery technologies.[28,272,516–518] Exhibited efficacies of many of these non-viral nanoparticulate technologies remain severely limited and inconsistent when implemented in some of the most important, clinically relevant, translational *in vivo* systems.[519–521]

For example, resultant cancer therapeutic efficacies achieved by non-viral genetic nanoparticulate technologies remain significantly limited and inconsistent in some of the most relevant *in vivo* models and translational studies.[522,523] Inherent physiological barriers are considered to be key obstacles that even some of the most current and advanced non-viral therapeutic nanoparticulate formulations cannot sufficiently overcome.[524,525] The individualized, heterogenous genetic and physiological natures characteristic of diseased tumor tissues may be aspects of cancer phenotypes that need to be considered more thoroughly by drug delivery researchers.[521,526,527] Modular, generalizable biomaterials and therapeutic nanoparticulate formulations designed to co-deliver combinatorial sets of genetic macromolecular agents may help these classes of technologies to more effectively overcome these significant barriers, enabling more efficient cancer therapeutic activities.[18,189,528]

Nuclear transport relates to another inherent physiological barrier to achievable therapeutic efficacies that continues to lack in available innovative drug delivery solutions with

respect to novel non-viral nanoparticulate formulations. The nuclear membrane structure

represents a specific natural barrier that acts to ultimately prevent effective genome editing,

enhancing, and silencing activities from many of the most current molecular complexes that

have been administered in *in vivo* systems.[529,530] High molecular weight, charged, exogenous

synthetic molecular mechanisms cannot physically pass through the nuclear membranes of cell

types targeted in *in vivo* systems.[531,532] This is due to the highly selective nuclear pore structures

responsible for efficiently directing transport of all compounds from the cellular cytosolic space

to the nuclear space, and vice versa, within a prospective cell targeted by drug delivery

mechanisms.[533–535]

These highly sophisticated, natural, hydrogel-containing nuclear pore channel structures

act as extremely selective combination-locked doors, blocking any inadvertent access to the

thoroughly protected genetic material of the somatic cell of interest.[536–539] It is important to

recognize that the nuclear membrane is a significantly more formidable barrier compared to

the cell membrane. The cell-membrane barrier is generally considered to be surmountable by

drug delivery technologies, or exogenous foreign entities such as viruses, engineered or formed

to undergo efficient and generalizable fusogenic and/or endocytosis mechanisms.

In that vein, if genome manipulating entities are not engineered or formulated to easily

pass through the nuclear pores, they cannot function therapeutically in significantly effective

ways *in vivo*.[540,541] Correspondingly, many research groups and companies have pursued

exhaustive and expensive research and development efforts to develop multi-tagged genome

editing macromolecular mechanisms.[542,543] However, the most common, conventionally

pervasive nuclear localization sequences that have been pursued thus far have been based on

tags that cannot possibly function to induce transport high of molecular weight genome editing

mechanisms via the required progressive intermolecular interactions throughout the

corresponding mammalian cell nuclear pore channel structures.[544,545] This is mainly because

these nuclear localization tags do not function intracellularly to activate the proper transport

mechanisms. Furthermore, high molecular weight genome manipulation entities with

significant surface charges cannot interact in the required enthalpically favorable ways with the

hydrophobic crosslink points within the nuclear pores.[546,547] Therefore, adding two or even

three nuclear localization signals throughout the molecular structures of cutting-edge active

therapeutic agents can have minimal to no effect on enhancing the resultant genome editing

efficacies *in vivo*.

Toxicity issues represent major problems that continue to hinder veritable rapid

progress of non-viral nanoparticulate technologies to and through clinical trials.[548–550]

Conventionally, toxicity refers to a number of distinct, unrelated aspects associated with the

administration of synthetic materials and technologies into mammalian hosts, many of which

have been alluded to throughout this thesis in various contexts. For example, non-viral systems

that are not designed properly, and that do not efficiently operate within their intended

physiological environments, may be prone to erroneous genomic manipulations throughout the

genomes of transfected cell populations.[18,551,552] These types of mistakes are typically

conventionally classified as "off-target" effects and are considered to be major sources of

toxicities specifically related to genome editing, and to genome manipulation mechanisms.[553–555] Erroneous genomic manipulations in critical cell cycle check loci within the genetic material

of the host mammalian cell, for example, can lead to catastrophic mutations leading to deleterious cancerous phenotypes in some cases.[556,557]

Many theoretical genetic models, molecular models, and prediction algorithms have been developed to date in parallel with cutting edge non-viral genome editing technologies to try to estimate coordinates and types of errors that should result from particular therapeutic formulations.[558–561] However, there continues to be significant controversy throughout the field currently as to which algorithms and approaches are best to evaluate erroneous genetic manipulations and their respective frequencies with respect to different genome editing technologies.[562,563] Therefore, little consensus within the drug delivery field has been reached on how to reliably take these potential "off-target" issues into account when designing novel non-viral genome editing nanoparticulate formulations for therapeutic applications.[564]

Another category of toxicity issues of significant importance to novel non-viral nanoparticulate formulation technologies relates to the inadvertent release of potent active therapeutic compounds in unintended tissue locations within the treated *in vivo* mammalian host.[565,566] Active agents released by poorly designed drug delivery vehicles in erroneous locations within their intended physiological systems can induce significantly harmful immune reactions that can lead to substantial disease morphologies and phenotypes.[567,568] Therapeutic agents that fail in this way can result in compounding harmful progressions of diseases and represent major sources of toxicities.[569,570] Encouragingly, substantial progress has been made by drug delivery researchers enabling significantly improved targeting and controlled release functionalities with many cutting edge, non-viral nanoparticulate technologies that have been developed for clinical applications.[571]

Material toxicities also represent another major source of concern in the field of drug delivery. Many synthetic polymers and biomaterials that have been developed for drug delivery and theragnostic applications are non-biodegradable and non-bioeliminable and therefore cannot be rapidly cleared by the treated *in vivo* mammalian host.[281,572] For example, unnatural materials such as high tensile strength carbon nanotubes, biomaterials that consist of prevalent aromatic backbones throughout their molecular structures, and macromolecular polymeric entities that exhibit poor water-solubility characteristics can all lead to highly toxic immune reactions when administered within *in vivo* systems.[573–575]

To address concerns related to improvements in biocompatibilities of advanced, novel non-viral nanoparticulate therapeutic platforms, substantial libraries of synthetic biodegradable materials, such as poly-anhydrides and poly-ester polymers have been and continue to be pursued.[576–578] Many of these cutting-edge biocompatible materials have been thoroughly developed by drug delivery researchers to efficiently deliver non-viral therapeutic compounds to targeted cell types *in vivo* in a significantly more innocuous manner when compared to less advanced nanoparticulate formulations.[579–581] However, as mentioned above, in the particular case of poly-ester drug delivery materials that have been developed by drug delivery researchers to undergo acid-catalyzed hydrolysis reactions, these particular degradation mechanisms can lead to substantial alterations of local pH levels and can cause toxic prevalence of tissue acidosis in critical tissue structures within treated patients.[315]

Another aspect of toxicity that is less well studied to date relates to hitting the proper cell types within targeted tissues.[67,582] Within targeted organs or diseased tissue structures, such as the lung or liver, there are numerous types of cells, such as immune cells, endothelial

cells, epithelial cells, and more, all resident within these complex systems.[583–585] Very few examples of current biomaterials have been designed with capabilities that enable them to accurately, reproducibly, and significantly target a single cell subtype within particular organ systems.[67] For example, limited success has been achieved thus far to develop therapeutic nanoparticles that function to accumulate sufficiently within injured tissues of treated lungs affected by cystic fibrosis due to physiological barriers blocking access to the diseased cell types that most current non-viral nanoparticulate technologies are not designed to overcome.[586,587] It remains extremely difficult to attempt to design drug delivery systems with this level of selectivity within targeted tissues. However, in order to significantly mitigate the vast majority of deleterious diseases of interest to drug delivery researchers, organ specific targeting is rarely sufficient. It is also critical to hit the proper cell types within targeted organ systems as well, in order to induce the required targeted therapeutic activities. Furthermore, analytical methods remain lacking in real-time precision making it very difficult to track and subsequently optimize cell-level localization of injected non-viral therapeutic compounds.[588–590]

Furthermore, nanoparticle tagging efforts to imbue these technologies with identifiable fluorescent signals, or quantum dots, can act to significantly confound the physiological path these materials would take in the absence of conjugation to these extra, high molecular weight, chemically distinct tracking moieties.[591,592] In addition, enabling consistent, clinically efficacious therapeutic activities of non-viral nanoparticulate technologies in translationally critical tissue structures beyond the liver continues to remain elusive to drug delivery researchers and experimental clinicians currently.[64,593] Therefore, targeting critical, diseased cell types in organ

systems beyond those which reside within the liver organ system remains unachievable by most current drug delivery vehicles.[594,595]

Although significant work, advancement, and innovation remains to develop classes of non-viral nanoparticulate technologies that exhibit consistent, veritable clinical utilities to date, encouraging progress has been made by drug delivery researchers and experimental clinicians thus far. Substantial progress related to the development of state-of-the-art drug delivery technologies including lipid nanoparticulate formulations, polymer nanoparticulate formulations, and other types of nanoparticulate formulations has been achieved. Libraries of novel, highly effective, next-generation drug delivery technologies have been created that have shown highly promising efficiencies in many translational research studies published on to date. Although there are significant pervasive concerns related to the safety and veritable therapeutic efficacy of current non-viral nanoparticulate therapeutic drug delivery technologies, encouragingly, a number of clinical trials are being pursued.

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