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CHARACTERIZING AND EXPLOITING THE ENDOCYTIC PATHWAY FOR MACROMOLECULAR DELIVERY

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Characterizing and Exploiting the Endocytic Pathway for Macromolecular Delivery

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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In memory of Philip Bost.

“I should’ve been a cowboy.”

POPULAR SCIENCE SUMMARY OF THE THESIS

There are a handful of historical medical advancements which we can easily look back on and point out as revolutionary to humanity. These include the discovery of penicillin, the identification of surgical anesthesia, and the development of vaccines, just to name a few. In 2003, another major accomplishment occurred – the human genome was fully sequenced, meaning that the expanse of the information held within our DNA became accessible. Suddenly, humans had the technology to map the underlying sources of rare diseases which had long escaped discovery.

However, there have been hindrances in the progress we were promised in the early 2000s. We should have been able to, in theory, find new ways to re-write, mask, or replace the faulty DNA in patients' cells that cause these diseases. In reality, many of the drugs we have created in laboratories fall short of any therapeutic benefit. These hindrances stem from the immense complexities of the human body and our cells.

A promising class of drugs is RNA therapeutics. As the name implies, these drugs are made of the same molecule, RNA, that our cells use to create proteins from their DNA "blueprint." By treating cells with therapeutic RNA, we can address several problems that arise from mistakes in a patient's DNA. However, the therapeutic RNA molecules need to be internalized by cells so that they can reach the active cellular compartments, and this is no easy task.

Researchers have demonstrated cells growing in a laboratory dish can internalize certain RNA and DNA molecules under tightly-controlled conditions. Even though they are inside the cell, these drugs often do not reach the cytosol or nucleus, which are the cellular compartments where the drugs are effective. They become sequestered in non-productive cellular compartments. Further, when DNA or RNA is injected into a patient, the molecules are usually instantaneously degraded by enzymes and the remnants are excreted in urine. In the worst-case scenario, foreign DNA and RNA can trigger a severe immune response.

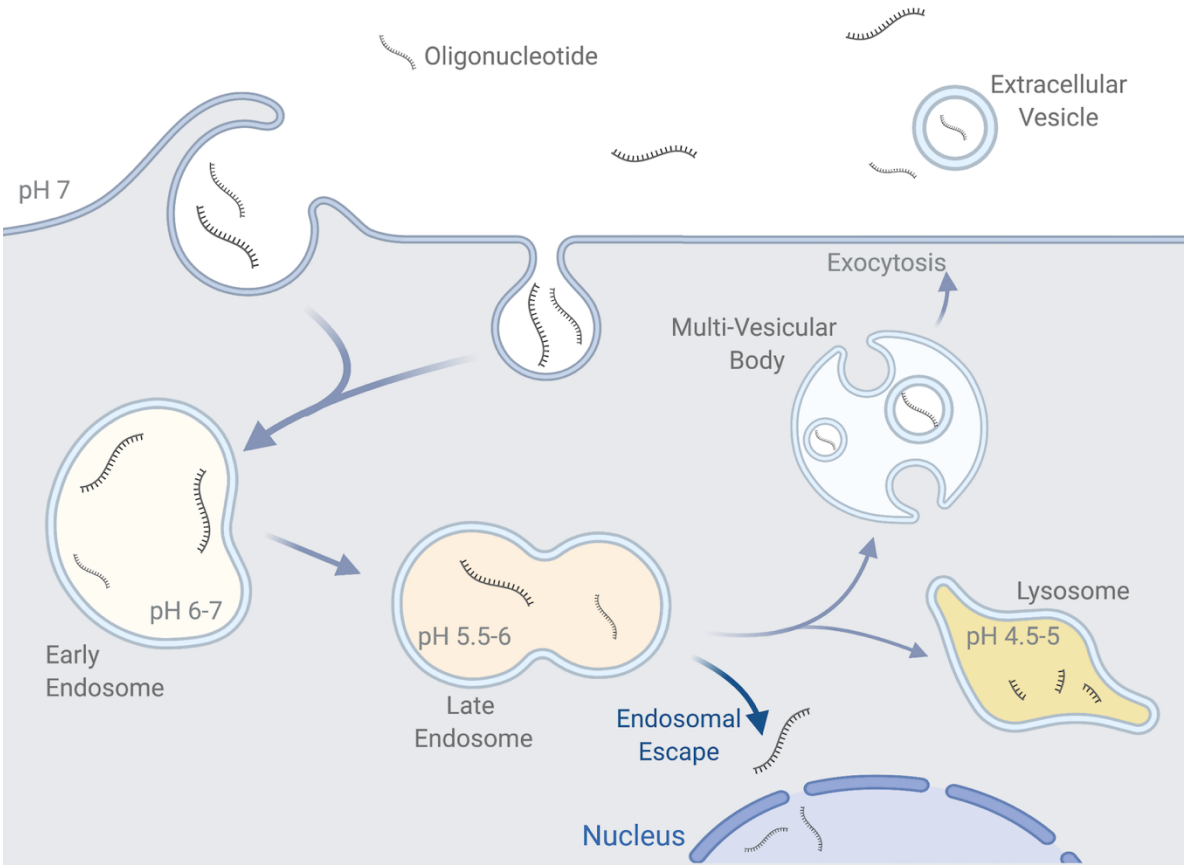
This issue has given rise to the term "drug delivery," describing how the molecules must be delivered into the required cells. The current work in this field spans several scientific disciplines, from biology to physics to chemistry. How can the therapeutic molecules be re-designed so that they are recognized by their target cells better? Can they be chemically altered to have more favorable properties? Can we encapsulate them in nanoparticles so that they evade immune detection? Or can we find another drug – a potentiator – which assists the therapeutic molecules in some way? These questions have led to the development of numerous drug delivery approaches. In this thesis, we ask two main questions:

1. How can we modify cells in our laboratory to create better, biological nanoparticles that can be used to deliver macromolecular drugs?

2. Can we identify a new molecule that acts as a potentiator of an existing genetic drug? Further, can we properly identify exactly how the new molecule works, and gain insight into the underlying biology that makes it work so well?

These two questions both depend on our understanding of a single biological pathway within the cell, the endocytic pathway. The endocytic pathway is a complex system by which cells can both internalize material from their surroundings and secrete material that they produce.

Intuitively, cells prefer to internalize familiar material. Familiar material that the cell will readily endocytose is usually biologically similar and often composed of the same molecular building blocks that the cell already contains, such as proteins and lipids. An exception here is that cells are reluctant to internalize foreign nucleic acids such as DNA and RNA. Evolutionarily, it makes sense that cells are reluctant to internalize these because foreign DNA or RNA is usually present during infection. Therefore, to treat cells with therapeutic nucleic acids, scientists need an effective approach to boost the delivery of RNA and DNA into cells.



Simplified schematic of the endocytic network. Figure adapted from Bost *et al*, 2021 and created in BioRender.

Within the endocytic pathway, therapeutic molecules will be contained within a small compartment inside the cell called an endosome. Endosomes are trafficked through the cell in a highly regulated process, often merging with other endosomes and acquiring new cargo as

they mature from an early stage to a late stage. There are three main endpoints for the cargo within an endosome. Firstly, the endosome may re-package itself into a multivesicular body, in which case its contents will be released out of the cell. Often, cells will package material into a small, membrane-bound particle called an extracellular vesicle (EV). It has been long established that cells can release EVs that contain proteins and nucleic acids, but for decades scientists wrongly assumed this was a way for the cell to get rid of "junk." In 2007, interestingly, Jan Lötvall's research group in Sweden demonstrated that these EVs are capable of delivering their cargo to other cells. With this discovery, an entire research field was born on the premise that cells can pack certain proteins into EVs, release those EVs into their environment, and recipient cells will internalize those EVs and make use of the delivered cargo. In our work, we investigate how the environment of a cell can be manipulated to encourage the cell to produce more EVs. By manipulating the EV-producing cells, we can produce therapeutic EVs that contain and deliver a desired therapeutic protein. We believe that in the near future, EVs may be used as a type of nanoparticle drug delivery system to deliver therapeutic proteins or nucleic acids.

The second endpoint of the endocytic network involves an endosome fusing with another compartment referred to as a lysosome, which contains highly degradative enzymes. This is considered a non-functional endpoint for any endosomal cargo that was meant to reach the cell cytosol or nucleus – any proteins or nucleic acids that end up in a lysosome will be quickly degraded. It is therefore necessary that any therapeutic proteins or nucleic acids contained in endosomes must escape from those endosomes before fusing with a lysosome. This escape is the third endpoint, referred to as "endosomal escape." If endosomal escape is achieved, the therapeutic cargo enters the cytosol of the cell, where it can reach its appropriate target.

Fortunately, endosomes have certain properties which can be taken advantage of to encourage endosomal escape. For example, endosomes become increasingly acidic as they mature. One of the focuses of our work has been to develop and identify new drugs which work in a manner dependent on pH. We show that by co-treating our new molecules alongside DNA or RNA, we can buffer the acidity of the endosomes, causing the endosomes to swell up and "leak." As they leak, the therapeutic DNA or RNA can escape from the endosome and become active.

While this Ph.D. thesis attempts to expand the existing knowledge within the drug delivery space, there remains much work to do. Future work will hopefully build on these findings by exploring new drug delivery approaches for genetic therapeutics across a range of diseases.

ABSTRACT

Macromolecular drugs with cytosolic or nuclear targets often exhibit low therapeutic activity due, at least in part, to their inability to escape endosomal compartments following cellular internalization. There exist a range of strategies to address this inefficient delivery by attempting to bolster the endosomal escape of the therapeutic cargo. Many of these delivery strategies apply to a broad range of molecular species, including proteins and nucleic acids. Two strategies of particular interest involve the use of extracellular vesicles (EVs) or endosomolytic small molecule compounds (SMCs). EVs are nanoscale, membrane-bound particles produced by all cell types and present in all bodily fluids. As a biological nanoparticulate species, EVs are inherently capable of delivering the material they contain to cells. Further, EVs can be modified through recombinant protein-based engineering strategies which can bestow a range of functional utilities such as fusogenicity, preferential cargo loading, and molecular targeting. However, the use of EVs as a scalable therapeutic modality is hampered by an inability to reliably mass-produce a homogenous population of these nanoparticles *in vitro*. SMCs, on the other hand, are easily synthesized at scale and can function in a stochastic manner dependent on an appropriate co-dosing strategy with their complementary therapeutic cargo. However, the mechanisms underlying SMC-mediated macromolecular delivery can be difficult to elucidate due to a lack of high-resolution characterization techniques. In this thesis, two issues - one underpinning each strategy - are investigated. First, the effects of culture media composition on the production of protein-loaded EVs *in vitro* are explored, with the ultimate aim of increasing EV output while characterizing the cellular biology driving the EV production. Certain serum components can differentially affect EV biogenesis by influencing ceramide-dependent EV biogenesis. In the second project, a functional screen of a novel family of SMCs is conducted to identify several chemical analogs in this family that demonstrate endosomolytic activity. Thereafter, super-resolution and real-time microscopic assays are employed to determine the mechanism and consequence of the novel compounds during their co-treatment with a splice-switching oligonucleotide (SSO). SSOs are clinically relevant small-RNA therapeutics that alter the production of splice variants for a given genetic transcript. The novel SMCs bolster SSO activity by disrupting the structure of endosomes in a manner dependent on the acidification of the endosomal compartments, suggesting the SMCs display a buffering capacity at certain concentrations. The findings herein strengthen the potential of each delivery strategy as a therapeutically relevant approach to functionally delivering macromolecular cargo to cells.

LIST OF SCIENTIFIC PAPERS

I. **Bost JP**, Saher O, Hagey D, Mamand DR, Liang X, Zheng W, Corso G, Gustafsson O, Görgens A, Smith CE, Zain R, El Andaloussi S, Gupta D. **Growth Media Conditions Influence the Secretion Route and Release Levels of Engineered Extracellular Vesicles**. *Adv Healthc Mater*. 2022 Mar;11(5):e2101658.

II. **Bost JP**, Ojansivu M, Munson MJ, Wesén E, Gallud A, Gupta D, Gustafsson O, Saher O, Rädler J, Higgins S, Lehto T, Holme MN, Dahlén A, Engkvist O, Strömstedt PE, Andersson S, Edvard Smith CI, Stevens MM, Esbjörner EK, Collén A, El Andaloussi S. **Novel endosomolytic compounds enable highly potent delivery of antisense oligonucleotides.** *Commun Biol*. 2022 Mar 1;5(1):185.

III. **Bost JP**, Munson MJ, Hagey D, Lennaard ABH, Roudi S, Gustafsson O, Wiklander OPB, Dahlén A, Engkvist O, Strömstedt PE, Collén A, Andersson S, El Andaloussi S. **Oligonucleotide-Enhancing Compounds Disrupt Endosomal Membranes.** *Manuscript.*

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I. **Bost JP**, Barriga H, Holme MN, Gallud A, Maugeri M, Gupta D, Lehto T, Valadi H, Esbjörner EK, Stevens MM, El-Andaloussi S. Delivery of **Oligonucleotide Therapeutics: Chemical Modifications, Lipid Nanoparticles, and Extracellular Vesicles**. *ACS Nano*. 2021 Sep 28;15(9):13993-14021.

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CONTENTS

LIST OF ABBREVIATIONS

AAV	Adeno-Associated Virus
AIDS	Acquired Immunodeficiency Syndrome
ALAS1	5'-aminolevulinate Synthase 1
ALIX	ALG-2-Interacting Protein X
ApoE	Apolipoprotein E
ARMM	ARRDC1-Mediated Microvesicle
ARRDC1	Arrestin Domain-Containing Protein-1
ASGPR	Asiologlycoprotein Receptor
ASO	Antisense Oligonucleotide
AZ	AstraZeneca
CMV	Cytomegalovirus
CPP	Cell Penetrating Peptide
DNA	Deoxyribosenucleic Acid
EMA	European Mediines Agency
EPR	Enhanced Permeability and Retention
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular Vesicle
FDA	Food & Drug Administration
GalNAc	N-Acetylgalactosamine
hATTR	Hereditary Transthyretin Amyloidosis
Hrs	Hepatocyte Growth Factor-Regulated Tyrosine Kinase Substrate
hTERT	Telomerase Reverse Transcriptase
ILV	Intraluminal Vesicle
LDL	Low-Density Lipoprotein
LNA	Locked Nucleic Acid
LNP	Lipid Nanoparticle
miRNA	MicroRNA
MSC	Mesenchymal Stem Cells
MVB	Multi-Vesicular Body
ON	Oligonucleotide
PEG	Polyethylene Glycol
PMO	Phosphorodiamidate Morpholino Oligonucleotide
PO	Phosphorodiester
PS	Phosphorothioate
RBD	RNA-Binding Domain
RNA	Ribonucleic Acid
RNAse H	Ribonuclease H
RVG	Rabies Virus Glycoprotein
siRNA	Short Inhibiting RNA
SMC	Small Molecule Compound
SSO	Splice-Switching Oligonucleotide
TSG101	Tumor Susceptibility Gene 101
TTR	Transthyretin

V-ATPase	Vacuolar-type GTPase
VPS4	Vacuolar Protein Sorting-Associated Protein 4

1. INTRODUCTION

1.1 A Brief History of the Endocytic Network

All cell types, from eukaryotes to prokaryotes, have developed complex systems for regulating the transport of molecules from their external environment to their interior compartments. There exist several ways by which small molecules and ions can enter the cell, determined by their molecular characteristics such as size, charge, hydrophobicity, etc. However, for larger molecular and biological species, there exist discrete mechanistic pathways in which cells are capable of recognizing, interacting preferentially, and specifically internalizing selected cargo. These specific pathways are collectively referred to as endocytosis and play integral roles in maintaining homeostasis at the cellular and organism levels.

The first observation of endocytosis was performed in 1882 when Elie Metchnikoff was able to observe cells engulfing pathogens using a light microscope. He deemed this process "Phagocytosis," a term originating from the Latin word meaning "to eat." Decades later, in 1931, Warren Lewis similarly observed cells creating vesicles with their membrane to internalize pockets of fluid. Lewis hypothesized that this was another mechanism by which cells could internalize nutrients and communicate, and called this process "pinocytosis," meaning "to drink." The two processes were extensively explored in the following decades and grouped together under the broader category of "endocytosis" by Christian de Duve in 1955. de Duve won the 1974 Nobel prize in Physiology or Medicine for his work characterizing these pathways, in which he identified an acidic cellular compartment that was responsible for degrading cargo. This newly-discovered compartment was named the lysosome.

The number of discoveries related to the endocytic pathway began growing exponentially over the following decades. Pivotal findings involved the identification of receptor-mediated endocytosis in 1975, giving the insight that certain biomolecules can initiate their own internalization into cells through interactions with receptors on the cell membrane. In 1980, Randy Schekman identified key genes involved in cellular transport and exocytosis in yeast. Schekman's work was corroborated by James Rothman in 1993, who hypothesized the molecular mechanism by which intracellular vesicles could initiate membrane fusion with other vesicles or with the cell's plasma membrane. Also, in 1993, Thomas C. Sudhof demonstrated that this process is tightly controlled in neurons, confirming that endocytosis and exocytosis are responsible for neurotransmitter communication. Schekman, Rothman, and Sudhof were collectively awarded the 2013 Nobel prize in Physiology or Medicine for their findings regarding these endocytic trafficking processes.

In the following years, the endocytic network has come to the forefront of medical biology. There is an extensive push across the field to fully characterize the processes driving endocytosis and exocytosis, and further, to manipulate these processes for therapeutic benefit. The term endocytosis has grown to now encompasses dozens of distinct cellular processes. As knowledge within biology, chemistry, and physics comes together and new investigative technologies develop in the laboratory, the story of endocytosis is not yet complete.

1.2 Overview of the Endocytic Network

Cells exist in a precise balance with their environment, constantly internalizing and releasing material to maintain homeostasis. This exchange plays a crucial role in a number of processes including nutrient exchange, cell signaling, developmental regulation, cell migration and adhesion, amongst others. However, the hydrophobicity of the cell membrane prevents macromolecular and nanoparticulate species from entering the cell cytosol. These cargos must be instead internalized and sorted through a highly regulated process known as endocytosis.[1] There exists a range of endocytic pathways, each seemingly optimal for a specific type of cargo. A major factor in the efficacy of any potential therapeutic is its ability to reach the proper cellular compartment. For therapeutics with intracellular targets, this generally involves crossing several lipid-membrane barriers by hitch-hiking or manipulating the endocytic network. Herein we discuss the endocytic processes relevant to the work in this thesis, including the uptake of ribonucleic acid (RNA) therapeutics and the secretion of extracellular vesicles (EVs). For simplicity, the endocytic process will be described in four parts: association, internalization, trafficking and recycling, and endosomal escape.

Association refers to the original contact between the therapeutic cargo and the target cell. This can either occur in a highly specific manner, such as in the transport of hormones, growth factors, and serum proteins (amongst numerous others), or it may happen in a stochastic manner, such is the case with certain oligonucleotides (ONs).[2,3] When specific proteins at the cellular surface are bound, they initiate a chain reaction that recruits a number of endocytosis-associated proteins to cluster at that region of the cell membrane.

Next, the associated cargo is internalized into the cell interior through one of several pathways. The internalization step is characterized by morphological changes in the plasma membrane, leading to the formation of a small (60-120 nm) membrane vesicle, termed an endosome. The most thoroughly characterized endocytic route for many cargoes is clathrin-mediated endocytosis.[4] Briefly, a specific association event, such as the binding of a receptor to its ligand, triggers membrane reorganization. Cytosolic clathrin is recruited to the interior side of the cell membrane and the membrane begins to invaginate inwards. This structure is known as a clathrin-coated pit and is subsequently subjected to scission which severs the pit from the plasma membrane, forming an endosome.

It is also possible that cargo is internalized without an association step through macropinocytosis. In this endocytic uptake pathway, the recipient cell forms a protrusion into the extracellular environment. This protrusion extends until it meets the cell membrane again, and then collapses, essentially swallowing a volume of the extracellular environment. There is evidence pointing to this process having an activating mechanism, similar to other endocytic routes. However, macropinocytosis involves the internalization of soluble, non-specific cargo as the internalized volumes can be up to 1 μm in diameter, much larger than other endocytic routes are capable of internalizing.[5]

Trafficking involves the sorting of endosomes to various intracellular compartments or the extracellular environment in a refractory manner. For endosomes containing therapeutic cargo, there are three main trafficking fates: lysosomal fusion, extracellular recycling, or endosomal escape. Once a newly formed endosome exists inside the cell, it begins a

maturation process characterized by a decrease in luminal pH, remodeling of the endosomal membrane, and translocation to the cell's interior.[6,7] The Rab protein family consists of over 60 GTPases and are key regulating proteins for the movements of endosomes throughout the cell cytosol. Rab proteins exhibit a specific localization to endosomes of a certain maturation, and through associating with kinesin motor and tether proteins, they act as an on/off switch for the radial trafficking of the endosomes.[8] Early endosomes (pH 6-7) mature into late endosomes (pH 5.5-6), which may then fuse to the highly acidic lysosomes (pH 4.5-5.5).[3] This acidification is achieved by vacuolar-type ATPase (V-ATPase), an ATP-driven complex that pumps protons into the endosomal lumen.[9]

Until recently, trafficking to lysosomes was considered a non-productive endpoint in cargo delivery, due to the high likelihood of cargo degradation by acidic lysosomal hydrolases. However, recent work has demonstrated that chemical modifications to certain cargos can make them resistant to degradation and enhance their functional delivery (discussed below).[10]

Extracellular recycling of cargo is a non-productive fate in terms of cargo delivery but can still be a useful process in regard to therapeutic design. In this process, early or late endosomes can be either redirected towards the cellular membrane to exocytose their cargo, or they may fuse with a multi-vesicular body (MVB), described in detail below under Exosome Biogenesis. Recently, it has been shown that lipid nanoparticles (LNPs) carrying therapeutic RNA can be treated to cells that internalize, remodel, and then secret hybrid nanoparticles that encapsulate the therapeutic RNA within a membrane composed of both synthetic lipids and biological membrane components.[11]

It is currently unclear what may cause a certain endosome to recycle its cargo back to the extracellular environment, although "cargo checkpoints" have been hypothesized. In this, endosomes must contain a threshold number of a given molecular cargo to be accepted further into the endosomal network.[12–14] These checkpoints are most comprehensively described in relation to clathrin-mediated endocytosis.

The final fate for endosomal cargo is the event in which the cargo escapes from the endosomal compartment and reaches the cell cytosol or nucleus. Appropriately, this outcome is termed "endosomal escape" and most drug delivery strategies seek to increase the number of endosomal escape incidents through various means. Endosomal fusion and membrane remodeling occur in a highly regulated manner, so normally there are very limited opportunities for endosomal cargo to escape. However, as endosomes mature and become more acidic, certain buffering agents can induce an osmotic inflow of water into the endosome. This causes the endosome to swell, increasing luminal pressure and increasing the incidence of endosomal membrane damage in a manner correlating to escape of endosomal cargo.[15]

Additionally, endosomal escape can occur by disruption of the endosomal membrane. This can be accomplished by exploiting the compositional differences between endosomes at different maturation stages. For example, low-density lipoprotein (LDL) cholesterol is highly abundant in the membranes of late endosomes but is present at low levels in the lysosomal membrane.[16] Treating cells with high concentrations of LDL cholesterol has been shown to alter endosomal morphology in a manner correlating with increased endosomal escape.[17]

1.3 Extracellular Vesicles

EVs are micro- and nano-scale, membrane-bound particles produced naturally by all cell types and present in all bodily fluids.[18] EVs were first identified via electron microscopy and originally termed "platelet dust" in 1967 by Wolf *et al*.[19] In the following years, a consensus was reached that these particles were composed of the standard cellular material and they did not display any virus-like activity, perhaps existing as inconsequential cellular debris. It wasn't until 1981 that Trams *et al.* realized a given sample of EVs contained at least two subpopulations: larger EVs (500 – 1,000 nm) that were observed to form from the outward budding of the cellular membrane, and smaller EVs (40 nm) that displayed distinct properties, more similar in composition to MVBs within the endosomal network.[20]

In recent years, much work has gone into identifying and characterizing new subpopulations of EVs. Amongst the subpopulations, several features of EVs have been identified that reflect their ability to convey information and molecular cargo throughout circulation. EVs have been implicated as metastatic drivers in certain cancers, modulators of immune activity, and systemic homeostatic regulators. As these findings accumulate, the interest in EVs as both a diagnostic modality and a drug delivery vehicle have expanded.

1.3.1 Biogenesis of Extracellular Vesicles

Throughout the literature on EVs, there exist several classification approaches to describe the subpopulations of EVs. Defining characteristics between subpopulations can include density, the presence of various protein markers, tissue or cell type of origin, and biogenesis pathway. In this thesis, a biogenesis-derived classification is used on two grounds: (1) At the time of writing, this is the most commonly implemented classification system, and (2) it adequately accommodates the descriptions of EV heterogeneity and EV-engineering approaches in a complimentary manner to paper I in this thesis.

Through this approach, EVs can be subdivided into exosomes that originate within the endocytic network, and microvesicles (MVs) that are produced at the cell membrane. This is a general and imperfect description, as it has also been observed that exosome biogenesis can directly occur at the plasma membrane.[21] Further, cells undergoing apoptosis release a subpopulation of EVs known as apoptotic bodies. Exosomes, MVs, and apoptotic bodies are collectively referred to as EVs.

Identifying EVs by biogenesis is difficult since both biogenesis pathways overlap to some degree, and several factors can influence this overlap, as the work in this thesis demonstrates. Generally, particle size can be used to differentiate EVs. Apoptotic bodies are largest of these three groups (< 2,000 nm), followed by MVs (1,000 – 50 nm), and exosomes are generally the smallest (120 – 30 nm).[22] However, the overlap between particle size distributions prevents this from being a complete method to identify EVs. To better identify EVs, researchers have investigated the various EV biogenesis pathways to determine if unique protein markers exist between EV subpopulations. Herein, we touch on key proteins involved in exosome and MV biogenesis.

1.3.1.1 Exosome Biogenesis

Exosomes are created within the endosomal network. As endocytic vesicles progress and mature throughout the pathway, they are constantly moving throughout the cell, fusing and budding with other endosomes, and undergoing extensive membrane protein restructuring and lipid remodeling. The endosomal membrane is a highly dynamic component, and there is not a single type of endosomal body that is uniquely destined to form exosomes. Rather, both early endosomes and late endosomes, amongst other vesicles, have been observed to transition into MVBs.[23] This transition is characterized by the invagination and inward budding of the endosomal membrane, resulting in the formation of a smaller, luminal vesicle that exists within the endosome, termed intra-luminal vesicles (ILVs). ILVs housed within the MVBs have three possible fates: (1) they may back-fuse with the MVB membrane, depositing their cargo back into the cell cytosol, (2) they may be trafficked further into the endosomal network and fuse with a lysosome so that the ILVs are degraded, or (3) the MVB is directed to the cell periphery, where it merges with the plasma membrane and releases the ILVs to the extracellular environment as exosomes.

There exist several manners by which an endosome may initiate its transition to an MVB. One of the most highly characterized pathways is reliant on the formation of a series of complexes called the endosomal sorting complex required for transport (ESCRT)-0, ESCRT-I, -II, and -III. The activity of the ESCRT complexes is regulated by accessory proteins, including ALG-2-interacting protein X (ALIX), amongst others.[24] ESCRT-dependent pathways of exosome biogenesis are triggered first by the ubiquitination of proteins on the endosomal membrane, which are recognized by hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs). Hrs then recruits the formation of ESCRT-0 at the endosomal membrane. In a series of events, ESCRT-1 is recruited, followed by ESCRT-2, which ultimately drives the invagination of the endosomal membrane. ESCRT-III is the final component which acts to sever the stalk of the invagination, producing the ILV.[25]

Apart from this canonical ESCRT-dependent pathway for ILV formation, there exist other ESCRT-dependent pathways by which ALIX can trigger membrane invagination without ESCRT-0, -I, or -II. In this case, ALIX may bind to various other complexes, including syndecan-syntenin complexes, to initiate membrane invagination. However, ESCRT-III is still required for the final membrane scission step.

There also exist ESCRT-independent pathways for MVB/ILV formation. This was demonstrated in 2009, wherein a study showed that depleting cells of all 4 ESCRT complexes did not abolish MVB formation.[26] One such pathway is dependent on sphingomyelinase, which converts sphingomyelin to ceramide in the endosomal membrane. This conversion seems to be correlated to the initiation of membrane budding at both the endosomal membrane and the plasma membrane.[27] Other ESCRT-independent pathways utilize tetraspanin proteins, such as CD63. Clustering of CD63 in microdomains at the endosomal membrane has also been shown sufficient to induce ILV formation.[28] The coexistence of these complex, highly intertwined endosomal pathways highlight the difficulties that arise when attempting to identify EV subpopulations based on biogenesis.

1.3.1.2 Microvesicle Biogenesis

The biogenesis of MVs is less characterized than exosomes, however certain key processes have been observed. To maintain simplicity, EV shedding at the plasma membrane is generally described as a "budding" mechanism. Considering that the plasma membrane is a highly dynamic, fluid, and flexible structure, MVs can form from a number of mechanistically unique events.[29] Similar to exosomes, all cell types can produce MVs and the MVs retain characteristics of their parent cells.

MV biogenesis begins with membrane blebbing, which is characterized by distinct changes in the plasma membrane composition and morphology. Lipids and proteins are trafficked to the site of MV formation, and the proteins which relocate to the membrane site play a determining role in the biogenesis pathway of the MV. One of the better-characterized pathways of MV biogenesis involves arrestin domain-containing protein-1 (ARRDC1), which gives rise to a subpopulation of MVs termed ARRDC1-mediated microvesicles (ARMMs). In this, ARRDC1 recruits the ESCRT-associated proteins tumor susceptibility gene 101 (TSG101) and vacuolar protein sorting-associated protein 4 (VPS4), that translocate to the plasma membrane from the endosomal membrane. At the plasma membrane, TSG101 interacts with a ARRDC1, driving vesicle shedding from the membrane.[30] The resultant ARMMs contain enriched levels of ARRDC1 and TSG101.[31]

In addition to the membrane remodeling that occurs during MV biogenesis, there exists a cytoskeletal regulatory component. All protrusions that occur at the cellular membrane are the result of actin polymerization, and there has been a strong recent focus on determining the role of these protrusions in MV formation.[29] Thus, it stands to reason that actin polymerization is a complimentary process driving MV formation.[32] Corroborating this, actin accessory proteins have been identified in EV samples.[33] The complete annotation of MV biogenesis remains elusive, much for similar reasons as that of exosomes – the biogenesis pathways are highly complex and intertwined.

1.4 Small RNA Therapeutics

Small RNA therapeutics, herein referred to as ONs, are generally 15-30 base pairs in length and can be chemically modified to alter their pharmacokinetic characteristics. Antisense ONs (ASOs) function via Watson-Crick base pairing with a complementary pre-mRNA or mRNA strand. ONs can theoretically be designed to bind to any RNA sequence depending on the nucleotide sequence, and can thus post-transcriptionally regulate protein synthesis, alter splicing variation, inhibit protein translation, or initiate mRNA degradation.

With traditional small molecule drugs, the target specificity cannot be altered without effecting the pharmacokinetic characteristics of the molecule, and vice versa. Essentially, structure and activity cannot be separated in small molecules. For ONs, however, specificity is determined by the nucleobase sequence, while the pharmacokinetic profile is separately determined through the molecule's chemical characteristics.[34] These two characteristics can be individually optimized in ONs.

There are several classes of oligonucleotides based on their mechanism of action, described comprehensively in our recent review.[3] Two of the most commonly utilized ASOs are gapmers and splice-switching ONs (SSOs). Gapmers bind a complimentary target mRNA strand and subsequently recruit Ribonuclease H (RNAse H) to degrade the complimentary strand, resulting in the knockdown of the targeted mRNA.[35] SSOs bind to the complimentary sequences of pre-mRNA and disrupt the normal splicing activity of the transcript by interfering with the assembly of the splicing machinery. This approach can have several outcomes depending on the splicing patterns that were affected. A mis-spliced protein can be functionally restored or pathological splice variants can be abolished.[3,36]

Due to the complementarity between an ASO sequence and its target RNA strand, off-target toxicities are considerably less common with ASOs than with other genetic drugs. For example, microRNA (miRNA), do not require full complementarity for the entire molecule. Increasing the number of nucleotides comprising the ASO can increase specificity, however may result in a loss of functional activity.

1.5 Current Status of the Small RNA Therapeutics Field

RNA therapeutics exist within the larger realm of genetic drugs. While the majority of conventional drugs target proteins, whether intracellular or extracellular, genetic drugs such as RNA therapeutics work by regulating or modulating gene expression. This is accomplished by introducing exogenous nucleic acid sequences into cells. These exogenous sequences can vary in their length and chemical composition. At the time of writing, RNA therapeutics can be generally divided into three main therapeutic modalities: ONs including short inhibiting RNAs (siRNAs), mRNA-based vaccines, and mRNA-based therapeutics. At the time of writing, there are 14 Food and Drug Administration (FDA) -approved RNA-based therapeutics. The first 12 of these are ONs, and the other two, most recently approved therapeutics are the mRNA vaccines developed against the SARS-CoV-2 virus. In this thesis, we focus on addressing the issues related to ON therapeutics.

The persistent challenges in the RNA therapeutics field come at several levels:

- Chemical: the RNA molecule must have an adequate half-life and stability.
- Cellular: The molecules must be able to cross biological membranes to accumulate in their necessary cellular compartments.
- Immunological: The RNA molecules should be able to bypass immunorecognition and degradation and should not induce an undesired immune response.
- Tissue: The RNA molecules should be able to reach the targeted cells in high enough quantity and in a favorable kinetic profile to alter the disease state of the tissue.
- Clinical: The specificity of the drug should be high in the target tissues. Further, toxicity and off-target effects should be minimal. The therapeutic molecule's activity should be predictable across the patient population.[3]

The most infamous barrier to the field is the inability to achieve "endosomal escape" on the cellular level. In an ON context, this refers to the inability of ONs to effectively permeate the endosomal membrane, usually being sequestered within the lysosomal compartments. As most ONs target DNA or RNA sequences in the cytosol and/or nucleus, their sequestration is considered a non-functional outcome.

1.6 Drug Delivery Systems for Small RNA Therapeutics

The functionality of RNA-based therapeutics relies heavily on their abilities to avoid degradation by nucleases, localize adequately to the target tissues, and to reach the appropriate intracellular compartments within the target cells. Drug delivery systems for RNA, in this context, involve molecular engineering approaches that aim to increase cellular uptake and endosomal escape.

At the time of writing, there are four commonly-utilized approaches to improve the delivery of therapeutic RNAs: chemical modification of the RNA, direct bioconjugation of the RNA to a delivery-mediating ligand, the use of synthetic nanoparticles - predominantly lipid-based nanoparticles (LNPs), and the use of viral vectors.[37,38] The work of this thesis serves to expand this list by improving upon two lesser-used strategies that have recently grown in popularity: the use of biological nanoparticles such as EVs and the co-treatment of RNA with delivery-enhancing small molecule compounds (SMCs). Each delivery strategy has its own drawbacks and benefits, implying that optimal drug design should include a delivery strategy which is mechanistically synergistic with the molecular activity of the therapeutic RNA. The delivery strategies discussed herein are illustrated in figure 1.

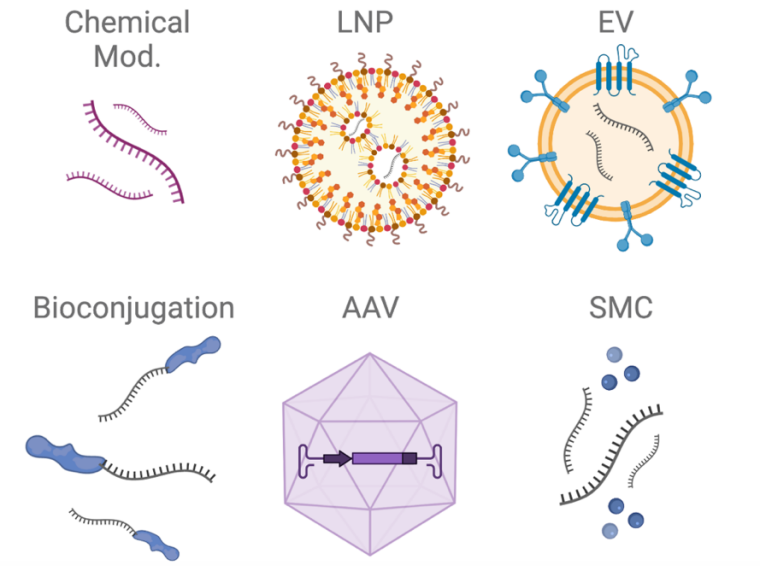

Figure 1. Delivery strategies for RNA therapeutics. Figure adapted from Bost *et al.* 2021 and created in Biorender.

1.6.1 Chemical Modification

Unmodified nucleic acids are inefficient as therapeutics, as they are easily degraded in serum and are not readily internalized by cells *in vitro* or *in vivo*. To address this, extensive work in recent decades has been conducted to alter the chemical characteristics of nucleic acids without disrupting their molecular action. Many of these chemical modifications are entirely synthetic, however, others are naturally occurring, such as in bacteria. Every nucleic acid has three main functional groups that can be altered chemically: the nucleobase, the carbohydrate group, and the backbone linkages.[3,39,40]

Chemical modifications to the nucleobase are often incorporated into the design of antisense RNA therapeutics such as gapmer ONs or SSOs. Modifications to the nucleobase can increase the binding affinity of an RNA or DNA to its complementary sequence in the targeted strand. This increased affinity enhances the thermal stability of the formed duplex, improving the ability of the modified ON to mask a splice site, inhibit translation machinery, or irreversibly target an RNA strand for degradation.[41] In many of the nucleobase modifications, an additional, small functional group is added to the 5-position of the pyrimidines in a strand. A common modification, known as 5-methyl-C, increases binding affinity by stacking methyl groups between nucleobases in the major groove of the formed nucleic acid duplex.[42] Importantly, increasing the binding affinity of an RNA strand can increase its off-target binding, implying the extent of modification should be optimized for RNA therapeutics with anti-sense activity.

The deoxyribose sugar group in DNA and the ribose sugar group in RNA play a role in the 3-D conformation of the nucleic acid strand, influencing both binding affinity and resistance to nuclease degradation.[43] These modifications can lengthen the therapeutic's half-life from days to several weeks. In RNA, the addition of an electron-withdrawing group to the 2'- O position of the ribose sugar causes the sugar to convert to a pucker conformation, which is a more favorable conformation for RNA-RNA binding to occur.[44] Additionally, carbohydrate modifications have been developed that serve to "lock" the nucleotide into a favorable conformation. In these locked nucleic acids (LNAs), a chemical linkage is formed between the 2'-O and the 4' position of RNA.[45,46]

The natural phosphorodiester (PO) linkages of DNA and RNA are susceptible to rapid degradation by nucleases *in vivo* and display low protein-binding affinity, which can decrease cellular association. At least partial modification of the backbone linkages is necessary for any antisense nucleic acid-based drug which is not encapsulated in a delivery vehicle, as evidenced by the structures of all FDA-approved antisense ONs.[47] The most widely used backbone modification is the phosphorothioate (PS) modification. In 1981, it was shown that by replacing the non-bridging oxygen with a sulfur atom, nucleases lose ability to recognize and cleave the nucleic acid strand, thereby increasing the resistance against degradation.[48] It was later found that the PS modification also increases the tissue and cellular uptake of ONs by increasing the non-specific protein binding between PS-ONs and serum proteins such as albumin.[49] This binding increases their time in circulation and helps evade blood clearance.

Therapeutic ONs can be heavily chemically modified, and several FDA-approved ONs do not require the use of additional delivery approaches. The first ON to receive FDA approval was

Vitravene (fomivirsen) in 1998 for the treatment of cytomegalovirus (CMV) retinitis in patients with acquired immunodeficiency syndrome (AIDS).[50] Fomivirsen is a fully PS-modified DNA ON. In a phase III trial, fomivirsen was able to delay the progression of CMV retinitis from 13 days to 71 days before ultimately losing demand due to improved AIDS treatments.[51,52]

1.6.2 Bioconjugation

While chemical modification can drastically improve the therapeutic characteristics of nucleic acid-based drugs, there exist further chemical approaches to increase the cellular targeting and activity of these drugs. Bioconjugation involves the direct covalent attachment of a functional ligand to the RNA. Various groups have shown improved RNA delivery by conjugating proteins, peptides, lipids, or carbohydrates to the therapeutic cargo.[53] This approach is commonly used in addition to previously discussed chemical modifications. In some instances, the protein that is conjugated to the RNA may be tens or hundreds-fold larger than the RNA itself. As this delivery approach does not involve encapsulating the RNA, it is best suited for ONs.

The most commonly utilized bioconjugation approach is N-Acetylgalactosamine (GalNAc) conjugation, which increases siRNA delivery to hepatocytes. In this approach, an siRNA molecule is covalently linked to 3 GalNAc molecules. The target for GalNAc, the asialoglycoprotein receptor (ASGPR), is well-conserved in its function and highly expressed specifically on hepatocytes across a range of species.[54] ASGPR specifically binds the terminal GalNAc terminal of carbohydrates and internalizes the receptor-bound cargo through receptor-mediated endocytosis. Once internalized, ASGPR is recycled to the cell membrane within minutes, which allows continual uptake of the GalNAc-conjugated RNA, ultimately resulting in high intracellular concentrations of the therapeutic. Recent work has shown that although GalNAc-conjugated siRNA may not immediately escape from their endosomal compartments, it can be internalized in high enough amounts that a slowly occurring endosomal escape is sufficient to impart a therapeutic effect.[55]

Givosiran is an siRNA developed by Alnylam Pharmaceuticals targeting 5'-aminolevulinate synthase 1 (ALAS1) for the treatment of acute hepatic porphyria.[56] Givosiran utilizes a GalNAc-bioconjugation approach to increase hepatic delivery and uptake of the siRNA after subcutaneous delivery. Downregulation of ALAS1 mRNA with givosiran has been able to reduce the number of porphyria attacks in patients. At the time of writing, there are over 20 additional GalNAc-conjugated therapeutics in clinical trials.[57]

Another approach utilizes the conjugation of lipids, which has also been explored for ON delivery in a range of tissues.[17] One of the most common lipid moieties to utilize is cholesterol, which comprises 15% - 30% of cellular membranes.[58] Cholesterol is able to intercalate in the cell membrane, essentially anchoring the conjugated siRNA to the cell until it is endocytosed. Additionally, cholesterol can associate with plasma lipoproteins *in vivo*, hitching a ride into the cell as these lipoproteins are internalized by the cell.[59] Cholesterol-siRNA cellular uptake occurs rapidly and is historically proven for local delivery *in vivo*, for example to the skin or the eye.[60,61] Recently, cholesterol-conjugated DNA-RNA heteroduplexes have been shown to cross the blood-brain barrier after systemic delivery.[62]

A third commonly explored class of conjugations is cell-penetrating peptides (CPPs).[63] These peptides are generally rich in cationic amino acids. CPPs can be covalently attached to ONs with neutral backbone chemistries, such as in phosphorodiamidate morpholino ONs (PMOs), to increase PMO uptake.[64] The direct conjugation of CPP to an ON is however difficult to achieve from a chemical approach. Therefore, CPPs are more commonly used as a complex-forming agents with negatively charged nucleic acids. CPPs are generally effective at encouraging cellular uptake and endosomal escape, but some CPPs suffer from a lack of specificity and high toxicity, leading to off-target effects.[65] Ongoing developments of CPPs are spurred on by findings that appropriate dosing *in vivo* can minimize their potential toxicity.[66]

1.6.3 Lipid Nanoparticles

The discovery and engineering of cationic ionizable lipids have fueled their use as a formulation component for several lipid-based nanoparticulate structures, including what we now call LNPs. In the early 1970s, initial formulations of lipid complexes could encapsulate proteins, forming liposomes, which were able to enhance intracellular delivery of those proteins.[67] These first-generation lipid carriers were mostly comprised of only phospholipids and cholesterol. As knowledge of lipid delivery systems expanded, in 1987 the cationic lipid DOTMA was used to form lipoplexes with nucleic acids for effective intracellular delivery.[68] This process, termed lipofection, remains a commonly used laboratory technique for the introduction of DNA and RNA into cells *in vitro*. Nowadays, there is a strong focus on further developing lipid nanoparticle formulations with added utilities, including improved encapsulation efficiency, maintaining neutral surface charge, evading the immune system, and biodegradability.[3] Current LNP formulations are comprised of four components: ionizable cationic lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-lipids.

The ionizable lipid component is defined by two main characteristics: its shape and its acid-dissociation constant. Early-generation lipids that are permanently cationic, including DOTMA, display unfavorable toxicity profiles, imparting cellular damage and decreasing translational kinetics. Conversely, new-generation lipids are designed to include a tertiary amine in the lipid headgroup, which is neutral in circulation but undergoes protonation, becoming cationic, when pH decreases.[69] The advantages here are twofold: a neutral particle surface in circulation decreases immunostimulation and toxicity while increasing the precision of the targeted delivery to intracellular, acidic organelles.[70] Breakthrough ionizable lipids include DLin-MC3-DMA, SM-102, and ALC-0315. Currently, DLin-MC3-DMA, the ionizable lipid utilized in the first FDA-approved LNP-based drug, is widely used in research settings for the delivery of RNA *in vitro* and *in vivo.*[71] SM-102 and ALC-0315 are two biodegradable ionizable lipids that have been used in the formulations of the groundbreaking Moderna and Pfizer/BioNTec COVID-19 vaccines, respectively.

At the time of writing, the development of biodegradable cationic lipids is what separates next-generation LNPs from their previous generations. Earlier attempts at incorporating biodegradability into the LNP utility resulted in a loss of LNP-mediated cargo delivery. In 2014, Whitehead *et al*. demonstrated efficient delivery of siRNA *in vivo* using LNPs formulated with a biodegradable cationic lipid.[72] This was accomplished by incorporating cleavable ester linkages into the lipid tail groups, which undergo hydrolysis by intracellular

esterases. The presence of these ester linkages results in enhanced liver clearance in non-human primates as compared to DLin-MC3-DMA.[73]

LNPs provide benefits to nucleic acid delivery on several fronts. Firstly, LNPs provide a protective compartment that houses the RNA molecules internally, preventing nucleases from being able to access and degrade the RNA. Depending on the formulation and the ratios of the component lipids, the size profile of the LNPs can be predictably altered. In the context of *in vivo* delivery, it is advantageous to control the particle size. For example, in hepatic delivery, particles with a < 80 nm diameter and a narrow size distribution can effectively traverse the fenestrated hepatic vasculature to reach high numbers of hepatocytes.[74] For tumor extravasation, a larger 50-100 nm nanoparticle can encapsulate more cargo while still penetrating into the tissue due to the vascular leakiness of tumors, also known as the enhanced permeability and retention (EPR) effect.[75]

To date, most LNP delivery systems have shown effective hepatic delivery of RNA upon intravenous administration. After systemic administration, apolipoprotein E (ApoE) adsorbs to the surface of the LNP. Low-density lipoprotein receptor, which is enriched on hepatocyte surfaces, binds the ApoE and drives the internalization of the LNPs.[76] Approximately 80% of intravenously-administered LNPs accumulate in the liver as a result of this mechanism.[77]

Onpattro (patisiran) is an example of an LNP-encapsulated siRNA that targets the liver. Onpattro is approved for the treatment of hereditary transthyretin amyloidosis (hATTR), a multi-system disease arising from the production of misfolded transthyretin (TTR) protein within the liver. Onpattro is administered intravenously every 3 weeks, resulting in an 80% reduction in misfolded TTR levels in serum within days after treatment.[78] This ultimately improves patient neuropathy and quality of life.

1.6.4 Viral Vectors

The first viral gene transfer was achieved by using a retrovirus to introduce a gene to tumor-infiltrating leukocytes *ex vivo* in 1990 by Rosenberg *et al*.[79] Retrovirus-mediated delivery, which involves genomic integration of the viral DNA, was chosen because it seemed intuitive that this genomic insertion would enable long-term expression of the therapeutic transcript. The efficacy of this treatment was debatable, and no adverse safety effects were noted in this study. The following years saw a rapid increase in the number of lenti- and retrovirus-based delivery studies. These were mostly unsuccessful, due to low clinical success and a few cases of extreme immunotoxicity.[80,81] The field of viral delivery was plagued by the realization that retrovirus-mediated nucleic acid delivery is not adequately precise, and insertional mutagenesis can give rise to the oncogenic transformation of cells.[82] These problems raised considerable doubts about the feasibility of viral delivery, however, they also ignited a major push to better understand vector biology. In recent years, the development of safe and effective mammalian viruses has been a key focus for viral nucleic acid delivery.

The massive problems that occurred from retrovirus treatment could have been lessened if an adeno-associated virus (AAV) vector was used instead. AAVs were studied as early as 1982, when an AAV genome was cloned into plasmid DNA, transfected to cells, and then lysate from the transfected cells was shown to infect further cells.[83] Nowadays, AAVs are

predominantly utilized for viral delivery. AAVs have several advantages when it comes to nucleic acid delivery. First, they have an evolutionarily-selected tropism for targeting a specific tissue or cell type. This is a differentiating factor between the 12 AAV serotypes and the hundreds of additional variants identified. Secondly, many viruses contain the necessary machinery to ensure that the genetic cargo is not only delivered intracellularly but specifically to the nucleus. In contrast, non-viral vectors depend on stochastic delivery events and nuclear accessibility which may only occur during cell division.[37]

There are currently several FDA and European Medicines Agency (EMA)-approved therapeutics which utilize viral delivery, mainly AAV-based delivery.[84] The majority of *in vivo* therapies target retinal disorders, liver disease, and central nervous system diseases and the most commonly used viral vector platform is the AAV vector. Wild-type AAV is a non-enveloped virus with a diameter of about 25 nm and can pack genes up to 5 kb in size. In most recombinant AAV vectors, the wild-type capsid and structural components are conserved while the therapeutic genes are introduced via expression cassettes. Tissue tropism is dependent on the serotype of the AAV, as the various serotypes display different capsid protein binding affinities with various cell receptors.[85,86] The viral targeting efficiency can be further modified through engineering the capsid proteins to display functional peptides.[87]

Modern AAVs also display a favorable safety profile compared to other viral vectors. AAVs cannot function on their own as they require a helper virus for replication. This renders them functionally non-pathogenic. Further, once AAVs reach the nucleus, their genetic cargo forms episomal structures, resulting in a very low frequency (< 0.1%) of viral DNA undergoing genomic integration into the host DNA. This also implies that AAVs are best suited for delivery to cells that either do not divide, or divide at a slow rate, as episomal genomes cannot be replicated and therefore the number of copies per cell halves with every cell division.[88]

1.6.5 Extracellular Vesicles

While LNP-based and viral vector-based therapeutics have already arrived to the clinical forefront, biological nanoparticles such as EVs are still under clinical development. At the time of writing, there are numerous clinical trials for EV-based therapeutics.[89,90]

The focus on developing EVs as a therapeutic moiety was spurred originally by the discovery that certain EVs display an innate therapeutic activity. As noted earlier, EVs have shown a natural tropism for uptake by various cell types, dependent on the cell type that the EV is produced from.[18] EVs isolated from mesenchymal stem cells (MSCs) have been observed to reduce pro-inflammatory signals and reduce oxidative stress and assist in tissue regeneration.[91] To date, the innate therapeutic repertoire of EVs has expanded to involve numerous anti-inflammatory, anti-tumor, and pro-regenerative functions.

It also stands true that EVs can propagate harmful events. However, these events occur in a manner synergistic to therapeutic engineering strategies. For example, tumor EVs have been implicated in cancer progression due to the fact that they can encapsulate and deliver telomerase reverse transcriptase (hTERT) mRNA to healthy fibroblasts.[92] Taken into an RNA-delivery context, these findings implicate an endogenous ability of EVs to deliver

functional mRNA *in vivo*. This ability can be greatly enhanced with modern biological engineering methods. By loading EVs with therapeutic mRNA and introducing additional abilities via recombinant protein engineering, an efficient RNA delivery vehicle can be created. Perhaps the most sought-after EV design concepts involve the ability to increase targeting specificity and the ability to load a desired cargo.

EV targeting refers to the ability of an EV to selectively initiate uptake by a certain cell type or tissue while avoiding internalization by undesired cells. The initial contact between the EV and the desired acceptor cell, and the subsequent internalization into that cell, are driven through both stochastic and receptor-mediated events. As EVs have a short half-life *in vivo* (levels of circulating EVs peak 5 minutes after intravenous injection), a targeting strategy should be robust enough to occur within minutes.[93] This is accomplished by overexpressing ligands, receptors, or signaling factors in the EV-producing cells, which are then displayed on the EV surface. This has been used to incorporate the rabies virus glycoprotein (RVG) peptide to the EV surface, which increases EV delivery to the central nervous system, traversing the blood-brain barrier after systemic administration.[94,95] Similarly, targeting can be enhanced by improving the evasion of non-functional outcomes. Expression of CD47 on the EV surface decreases macrophage and monocyte detection, increasing the time in circulation.[96] By utilizing appropriate targeting strategies, EVs are able to specifically deliver their therapeutic cargo with minimal off-target effects.

The ability to load EVs with desired therapeutic cargo has been proven for loading proteins and RNAs.[97,98] Luminal encapsulation can be achieved through exogenous or endogenous means (figure 2). In short, exogenous RNA loading occurs after EVs have been produced. A batch of harvested EVs is subjected to physical or chemical disruption, allowing soluble RNA to enter the EVs through pores that momentarily form in the EV membrane. Conversely, endogenous RNA loading occurs during EV biogenesis. This is generally accomplished by introducing RNA overexpression constructs to the EV-producing cells (passive endogenous loading), or the RNA overexpression can be coupled with expression of an EV-sorting protein containing an RNA-binding domain (RBD) (active endogenous loading).[99,100]

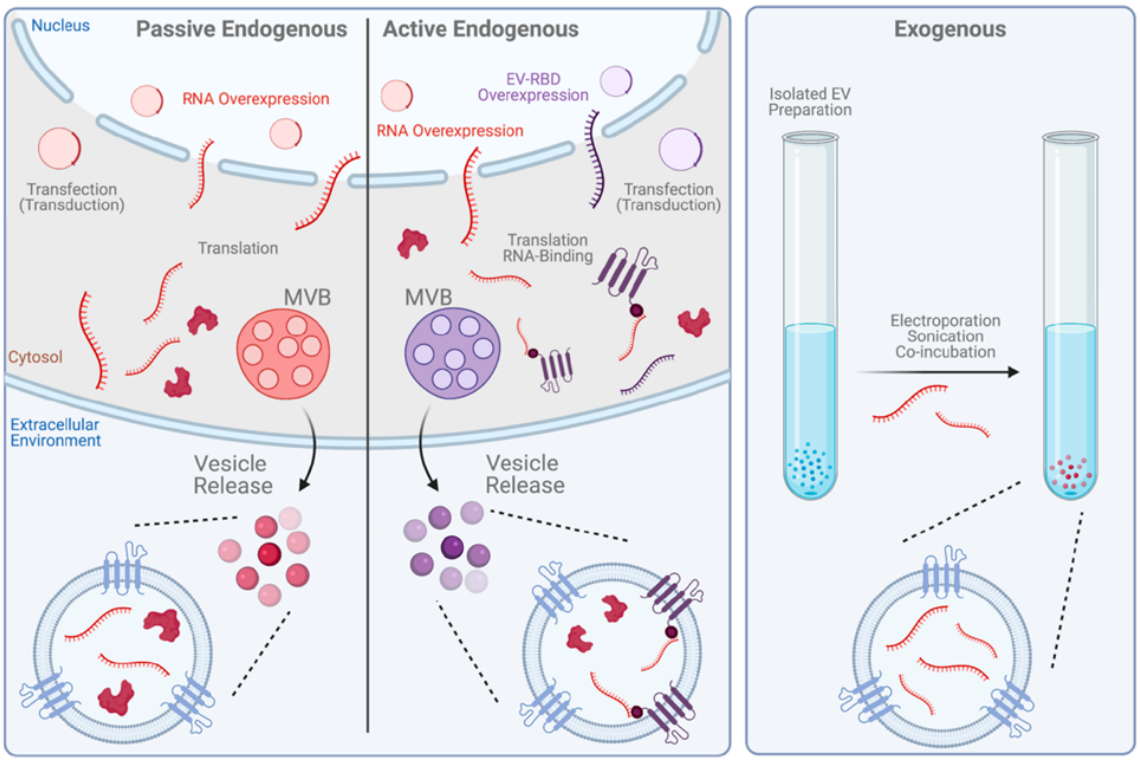

Figure 2. EV loading approaches for RNA. Figure adapted from Bost *et al.* 2021 and created in Biorender.

1.6.6 Activity-Enhancing Small Molecule Compounds

Recent work has shown that the route of intracellular trafficking for an ON can influence its pharmacologic action.[101,102] This spurred the development of small molecule compounds which could synergistically bolster ON delivery, either through regulating endosomal trafficking or influencing endosomal membrane permeability. This may occur by either a simple co-treatment strategy of the compounds and the ONs or may be accomplished through directly conjugating the compound to the ON molecule. Early candidates, such as Retro-1, were known to influence intracellular trafficking of bacterial and plant toxins, and were subsequently identified as capable of enhancing ON delivery.[103] However, the early endosomolytic compounds displayed unfavorable properties such as low potency and insolubility in water.[104]

One group of activity-enhancing small molecule compounds are cationic amphiphilic drugs (CADs). CADs are capable of reversibly buffering the endosomal lumen during acidification, ultimately destabilizing the endosomal membrane.[105]A commonly used CAD is chloroquine, which displays potency as an ON-enhancing compound *in vitro*.[106] However, chloroquine requires a high micromolar concentration (40-100 μM) to be used effectively as a CAD, making it a suboptimal candidate for use in a molecular conjugation approach.[107] Recently, Juliano *et al*. has identified several newer compounds capable of inducing ON escape from late endosomes at concentrations of 5 – 30 μM.[108] To date, there has been limited success in identifying new compounds that effectively promote the therapeutic activity of co-treated

cargo, in part due to the lack of high-resolution characterization techniques for investigating therapeutic-compound combinations.

1.7 Current Shortcomings in the Delivery of Small RNA Therapeutics

Chemical modifications are most heavily relied upon to increase the therapeutic activity of antisense ONs (ASOs). This is in part due to the relatively small size of ASOs (compared to other genetic drugs), which enables them to distribute favorably in tissues, given that they are administered through appropriate routes. For example, Nusinersen is an FDA-approved ON for the treatment of spinal muscular atrophy (SMA) that is capable of increasing survival and motor development in children with SMA. However, Nusinersen must be administered directly into the cerebrospinal fluid via lumbar puncture, a generally unpleasant procedure that can only be done at a healthcare site, which can limit patient accessibility. Similarly, Macugen (pegaptanib) is an FDA-approved ASO for the treatment of age-related macular degeneration.[109] Macugen is administered via intravitreal injection (direct injection into the vitreal chamber of the eye) and requires frequent re-administration.[110] This uncomfortable administration resulted in low patient adherence to treatment until Macugen ultimately lost its market to a more efficacious, protein-based immunotherapy. Further improvements in the ability of these delivery systems are needed to improve the real-world outcomes of these therapeutics.

Additionally, immunogenicity must be considered when designing a delivery strategy. Viral-mediated delivery is hampered by the inherent fact that humans have evolved immorecognition to several viral species. Other lipid-based delivery systems can also be recognized by the immune system, leading to adverse immunological effects.[111] For example, Onpattro (patisiran) is an siRNA that utilizes an LNP delivery system. In a phase III trial, a pre-treatment steroid regime had to be implemented to suppress immune-related side effects due to the immunogenicity of the LNPs.[112]

While these shortcomings persist, the aim of the work in this thesis is to improve the RNA delivery landscape by expanding our understanding of the endosomal pathway. Additionally, we seek to expand the toolkit of delivery strategies by focusing on engineered EVs and SMCs that can enhance endosomal escape of ONs.

1 RESEARCH AIMS

Research into the origin, function, and potential exploitability of the endocytic network has become central to the field of medical biology in the past few decades. The ability of cells to internalize and secrete bioactive molecules is widely implicated in both homeostatic and pathologic processes. Inspired by this, this thesis first aims to characterize the process by which cells package exogenous cargo into EVs, thereby bolstering the EV production and, secondly, to exploit the same cellular pathway to increase the intracellular delivery of nucleic-acid based therapeutics. The individual aims and objectives within these research projects include:

2.1 PAPER I

- To utilize a bioluminescence-based EV quantitation system to compare EV output from several EV-engineering constructs in various *in vitro* cell culture conditions.
- To evalutate serum components in cell growth media that may optimize production of engineered EVs.
- To determine the underlying transcriptional drivers of the increased EV production which results from the optimal cell growth environment.

2.2 PAPER II

- To identify a novel family of endosomolytic compounds which exhibit favorable characteristics compared to current ON transfection techniques.
- To use state-of-the-art microscopic techniques to characterize the responsible mechanism of the identified compounds which induces an increase of functional ON delivery within the treated cells.

2.3 PAPER III

- To continue the goals of research article II by identifying novel chemical analogues that exhibit endosomolytic activty through analysis of compound structure-activity relationships.
- To expand the utility of the identified class of compounds by demonstrating efficient delivery of additional cargo.
- To expand the mechanistic characterization of the family of endoomolytic compounds on a transcriptional level.

3. MATERIALS AND METHODS

The endosomal network is a highly dynamic and convoluted network, which adds a layer of complexity to characterization and engineering approaches. There is a strong focus in developing new biological models and high-resolution analytical techniques. However, even with the substantial progress in recent years, there remains gaps in the methodologies. The techniques that have been utilized in this work are discussed here in their technical context and their drawbacks.

3.1 PAPER I

3.1.1 Cell Culture

In research article I, HEK293T cell lines expressing CD9-Luc, CD63-Luc, or CD81-Luc were generated via stable transduction with constructs encoding ThermoLuc (TLuc) luciferase recombinantly fused to codon-optimized CD9, CD63, or CD81. Construct expression can be variable between cell lines due to differential transduction efficiency. To address this, we utilized a strong constitutive promoter. The trade-off here is that we attain comparable expression between the cell lines, however by inducing such high levels of overexpression, we lose biological relevance to the endogenous roles of the tetraspanins.

3.1.2 EV Quantitation

For research article I, cells were seeded and cultured for 24 or 48 hrs. After this period, the growth media was changed to EV-production media for an additional 48 hr, unless noted otherwise. Conditioned EV-production media (CM) was collected and subjected to centrifugation to remove cellular debris. Detergent was then added to the CM to lyse EV membranes, and then the lysed CM was subjected to a luciferase assay using a luminometer. EV luciferase signal was either reported in absolute terms or normalized to cell quantity or total cell protein.

Control experiments were performed to ensure that all luciferase-signal was originating from protein present in EV lumen. We took care to correlate the EV quantitation values from our luciferase readout with quantitation values from traditional methods such as western blotting against EV-marker proteins and electron microscopy to examine EV morphology.

Quantitative characterization of EV-protein fingerprint was performed via a bead-based multiplex EV analysis utilizing flow cytometry. In brief, a final volume of 60 µL of undiluted CM was loaded onto wells of a pre-wet and drained filter plate before 10 µL of MACSPlex exosome capture beads were added to each well. Filter plates were incubated overnight at 450 rpm. Beads were washed and the filter plate was put on a vacuum manifold until all wells were drained. EVs bound by capture beads were counterstained with detection antibodies. This was followed by another washing step before transferring samples to a flow-cytometry-compatible plate. Flow cytometric analysis was performed and fluorescence intensities for all 39 capture bead subsets were background-corrected.

Additionally, nanoparticle tracking analysis (NTA) was applied to determine the concentration of all samples. All samples were characterized with an NTA instrument equipped with analytical software and a 488 nm laser. At least five 30 s videos were recorded per sample in light scatter mode with visually optimized acquisition settings. Software settings for analysis were kept constant for all measurements. All samples were diluted in filtered PBS to an appropriate concentration before analysis.

Herein, it is argued that the combination of these characterization approaches adequately validate our EV-engineering approach. Further details into EV quantitation can be found in paper I.

3.1.3 Challenging EV Production

To identify media components that influenced EV production, we introduced various biological reagents into EV-producing cell media. The production of luciferase-engineered EVs was thereby systematically challenged. The reagents added included various signaling factors and high molecular-weight protein species. Additionally, we challenged EV production with either Hek wt EVs or MC3-LNPs. For the experiments in which additional reagents were tested, these reagents were introduced to the EV-production media during the media change step, ensuring that EV-producing cells had equivalent cell culture conditions up to this point. Added reagents included insulin, transferrin, and selenium (ITS) solution, GlutaMAX®, fibronectin, globulin, and albumin. In all cases, luciferase signal was analyzed after 48 hr of EV production.

Hek wt EVs had been previously collected and stored as described by our group's earlier works into efficient EV isolation and storage.[113] EVs were thawed at RT and numbers were counted using NTA before diluting in EV-production media to the desired concentrations. Similarly, MC3-LNPs were diluted in EV-production media to the desired concentrations before being added to cells during the media-change step.

3.1.4 Fluorescent Microscopy

Fluorescent microscopy was employed to visualize the cellular location of the testraspanins used in our EV-loading constructs. We expected to see differential tetraspanin localization due to the use of overexpression constructs in our engineered cells. All luciferase-expressing cells were seeded in glass-bottom chamber slides. Cells were allowed to adhere for 24 hr, washed with PBS, and then fixed for 10 min at RT. Following fixation, cells were permeabilized and blocking was performed. APC-conjugated primary anti-CD9, anti-CD63, and anti-CD81 antibodies were diluted in a blocking solution and incubated for 2 hr at RT. The stained cells were then washed with PBS and imaged with an Olympus fluorescence inverted microscope.

3.1.5 RNA Sequencing and Gene Ontology

Next, we sought to examine the transcriptional differences between cells cultured in various EV-producing medias. The ultimate aim was to identify possible EV-production pathways

responsible for the increase in EV output. In both paper I and paper III, Bulk RNA was extracted from cells with a TRI Reagent-based protocol. Detailed descriptions of RNA extraction can be found in paper I. RNA concentration was measured using a fluorometric analysis.

2 ng of RNA was used as input to the RNA-sequencing protocol and 50 bp single ends were sequenced. Reads were mapped to the human transcriptome to generate the read count matrix. Hierarchical clustering was performed on sample-to-sample Pearson correlations based on the most variable genes in the data set expressed above RPKM 4. Differential expression analysis was used to compare triplicate samples. Up and downregulated genes with an adjusted p-value < 0.001 were then analyzed with a complete set of all genes passing this threshold used as a control group. Gene ontology term fold-enrichments for each group were then divided by the enrichment in the control group to arrive at the Term Fold Enrichment displayed.

3.1.6 Ceramide-Dependent EV Biogenesis Inhibition

To validate the findings from the RNA sequencing, we utilized two inhibitory approaches targeting ceramide-dependent EV production. First, a sphingomyelinase inhibitor, GW4869 was treated to cells during the EV-production period. By inhibiting sphingomyelinase, the conversion of sphingomyelin to ceramide is prevented. The treatment concentration had previously been optimized to the highest concentration possible without observed cytotoxicity.

Additionally, siRNA smartpools consisting of four sequences targeting the genes ALIX and SMDP2 were designed by and ordered from Dharmacon. Cells were transfected with LF2000 at a final concentration of 100 nM siRNA for the 48 hr period before media was changed to production media. siRNA-mediated gene knockdown was confirmed in Hek wt cells with RT-PCR. Conditioned media was harvested and subjected to NTA as described above.

3.2 PAPER II & III

3.2.1 Cell Culture

In research articles II and III, HeLa- and HuH7-derived cell lines were used to screen and characterize compounds. Here, a plasmid carrying the luciferase coding sequence interrupted by an insertion of intron 2 from β-globin pre-mRNA carrying a cryptic splice site is stably transfected into cells. Unless the aberrant splice site is masked by SSO, the pre-mRNA of luciferase will be incorrectly processed. Thus, by using these cells, various SMCs can be evaluated by co-treating with SSOs and measuring luciferase activity.

Cells used for imaging in the GAL9 assays (described below) included HeLa- and HuH7-derived cells expressing a GAL9-fluorescent protein construct. Cells used in the endosomal colocalization assay included Huh7 cells (WT and in-house established cell lines

overexpressing RAB5-, RAB7-, or LAMP1-fluorescent protein constructs). The culturing conditions for each cell line are described in detail in the individual research articles.

3.2.2 Endosomolytic Compounds

The characterized compounds came from the AstraZeneca (AZ) internal small-molecule collection with the addition of one previously published compound, UNC2383 (herein referred to as CMP01), known to enhance endosomal escape. After screening, the lead compound candidate was subjected to mechanistic experiments to explore the structure-activity relationships, and subsequent chemical analogues were synthesized by AZ and shipped to our lab for testing. Detailed information on the synthesis of these compounds can be found in the Supplementary Methods of paper II.

3.2.3 Oligonucleotides

SSOs were purchased from Integrated DNA Technologies and synthesized with 2′-O-methylated (2′-OMe)-modified bases and phosphorothioate (PS)-saturated backbone linkages. For the non-fluorescent ONs, HPLC purification and Na+ salt exchange steps were included. SSOs were shipped and stored in IDTE buffer at a pH of 8.0 at 100 μM. The sequences of the ONs, including those with fluorescent modifications, are listed in Table 1 of paper II.

3.2.4 Splice-Switching Activity Assays

Cells were seeded in growth media with 1 μM or100 nM SSO. After 24 hr to allow adherence and SSO internalization, 10 μl of diluted SMC was added into each well at indicated concentrations. For positive control, SSOs were complexed with Lipofectamine 2000 (LF2000) and cells treated with a final concentration of 200 nM. ON concentrations above 200 nM would require LF2000 to be used in excess of the cells' toxicity threshold. In Paper II, after treatment, media was removed from the wells and replaced with fresh growth media for 4 h to allow translation of luciferase. In Paper III, media was not changed and cells were analysed for luciferase directly after 24 hr treatment.

For the detection of luciferase activity, 30 μL of cell lysate was transferred to white-walled plates. The luciferase intensity in each well was immediately measured via luminometer following auto-injection of 25 μL Luciferin substrate as per the Promega Firefly Luciferase Assay System. The timing parameters of photon measurements were optimized for this procedure.

3.2.5 Immunocytochemistry

HeLa and HuH7 cells were seeded on glass-bottom culture slides. Fluorescently conjugated SSO was added to the culture medium at 1 μM concentration upon seeding. Cells were let to attach for 24 h in the cell culture incubator, after which they were treated for 2 h with CMP05 endosomolytic compound, washed once with PBS and fixed. Following fixation, cells were permeabilized and blocking was performed. Primary antibody staining the lysosomal marker

LAMP1 was performed at 4 C overnight and secondary staining with donkey anti-rabbit IgG Alexa-Fluor 647 was performed at RT for 90 min. Finally, to fix the antibodies a post-fixation of the samples was conducted before STORM.

3.2.6 Stochastic Optical Reconstruction Microscopy (STORM)

Immediately before imaging, the samples were soaked in imaging buffer. The plate was sealed with parafilm to minimize oxygen entry. STORM was conducted with Nikon Ti Eclipse inverted microscope, housing cube filters and TIRF dichroic mirrors, equipped with a laser module used in this study. A 100 x oil objective (N.A. 1.49) was used, in combination with a 1.5 x lens, resulting in a final magnification of 150 x. The camera had a pixel size of 13 μm, giving a final pixel size of 87 nm with the 150 x magnification. For each STORM acquisition, a 256 x 256-pixel region of interest (ROI) was imaged. A widefield diffraction-limited image was taken from each ROI for reference before starting the STORM acquisition, for comparison. STORM acquisition was only started when the photoswitching of the fluorophores reached an optimal level, which was visually confirmed. In two-color STORM imaging, the channels were recorded sequentially, and the higher wavelength was recorded first to minimize the damage caused to the fluorophore in the other channel.

The images were reconstructed with the ThunderSTORM plugin in Fiji37 followed by drift correction using the ThunderSTORM cross-correlation algorithm. Moreover, images were filtered based on the sigma values (standard deviation of the Gaussian fit over the point spread function of each blink) to remove low-quality signals (e.g., noise and partially overlapping fluorophore signals). For the lysosome size quantification, additional intensity-based filtering was conducted to further remove background and noise and thus enable higher-quality feature detection. For a more detailed description of the reconstruction and post-processing parameters, see Methods and Supplementary Methods of paper II. All the images were visualized with the Normalized Gaussian visualization method using a magnification of 10 and an image-specific uncertainty constant.

3.2.7 GAL9-Recruitment Endosomal Rupture Assay

For screening and characterization experiments examining endosomal damage and GAL9 recruitment, HeLa or HuH7-derived cell lines expressing mCherry-GAL9 were seeded 16 hr before experimental usage. Dose–response curves of indicated compounds were dispensed by utilizing an automated acoustic dispenser into growth media. At experimental start points, media containing appropriate compounds and doses was transferred to plates using a liquid handling robot. At assay endpoints, cells were washed and fixed at RT. For nuclear visualization, Hoechst was added before imaging.

For time-lapse experiments, cells were imaged within a humidified environmental chamber that was maintained at 37 C and supplemented with 5% CO2. Plates were imaged using a spinning-disk confocal microscope with a 20 x objective (NA 0.75). Images were processed utilizing Columbus image-analysis software to identify and quantify cells and mCherry-GAL9 structures.

3.2.8 SSO-Endosome Live-Cell Colocalization Assay

HuH7-derived cells expressing red fluorescent protein (RFP) – fused to endosomal markers (RAB5, RAB7, and LAMP1) were plated 1 day before the experiment in glass-bottom quartering cell culture dishes. For studying intracellular trafficking of SSO, cells were washed with cell culture medium and pulse-incubated with 1 μM fluorescent SSO and either 2.5 μM CMP05 or corresponding concentration of DMSO in growth medium for 15 min. The cells were thereafter washed with cell culture medium and imaged immediately with continuous exposure to 2.5 μM CMP05 (or corresponding concentration of DMSO).

Confocal images were acquired on a Nikon C2 + confocal microscope using an oil-immersion 60 x objective. Pinhole size was set to 90 μm (corresponding to 3.3 AU) to detect particles throughout the volume of the cell, as well as to increase the signal and therefore particle detection. The sample was excited and detected with appropriate excitation laser lines and emission filters and the SSO and fluorophores were imaged sequentially.

The samples were imaged every 5 min with a field of view of 187 x 187 μm, containing in the range of at least 15 cells per frame. The images were analyzed using the ImageJ software with the spot colocalization plugin. Approximate particle size was set to four pixels, the threshold set to 15% for both channels, larger particles were segmented and maximum distance between colocalized spots was set to four pixels. The number of detected particles was normalized to the number of particles detected in the first frame (t = 0 min). Samples containing cells displaying CMP05-originating toxicity within the field of view were removed from the analysis.

3.2.9 RNA Sequencing and Gene Ontology

The RNA sequencing procedure for paper III was conducted as described above for paper I. The tSNE-nearest neighbor map was generated based on the most variable genes in the data set expressed above RPKM 4 in R. Differential expression analysis utilized the Deseq2 package to compare quadruplicate samples in R. Up and downregulated genes with an adjusted p-value < 0.05 were then analyzed using Panther Gene Ontology Analysis (panther.org), with Term Fold Enrichment and p-values displayed.

4. RESULTS & DISCUSSION

4.1 PAPER I

As EVs approach clinical relevance as a therapeutic drug delivery modality, there has been an extensive push to improve EV production. This has been attempted through two main approaches (1) genetically engineering the EV-producing cells towards a mechanistic increase in their EV-production, and (2) altering the extracellular environment to physically or metabolically bias the cells towards increased EV release. Historically, studies that seek to investigate EV output are limited by the quantitative methods available. The extracellular environment contains an abundance of nanoparticulate species which can be difficult to separate from EVs. These include protein aggregates, lipoprotein complexes, ribonucleoprotein complexes, and other cell debris. Therefore, our group has led an extensive effort in recent years to develop a quantitation method with high sensitivity and specificity.

In this study, we have built upon our previous work investigating components of the extracellular environment that impact *in vitro* EV production. We utilize a robust series of bioluminescent constructs which are capable of endogenously loading EVs with tetraspanin-luciferase fusion proteins. This enabled us to quantify the production of engineered EVs based on their luminal cargo with high specificity.

With this approach, we demonstrate that there is an interplay between cell media serum and EV production. Generally, the tetraspanins CD9, CD63, and CD81 display differential prevalence across a given sample of EVs, which reflects their roles as markers of various subpopulations of EVs. In our work, we were able to detect a considerable increase in engineered-EV production when cells were cultured in Opti-MEM without serum, regardless of which EV-marker was used to engineer the cells. Interestingly, cells cultured in Opti-MEM retained lower levels of these tetraspanins relative to their produced EVs, as compared to cells cultured in serum-containing growth media. This seemingly indicates that serum-free Opti-MEM alters the cellular equilibrium of exocytosed vs. retained tetraspanin protein. Given this, we sought to identify serum components that may be responsible for inhibiting EV output.

We individually tested several cell media components that affect cellular metabolism and growth signaling. We even hypothesized that large molecular serum components, such as protein aggregates, may act on a competitive level to inhibit EV production. We successfully identified two components that were able to affect EV production: exogenous EVs could downregulate EV production and fibronectin could bolster EV production. Fibronectin has previously been shown to enhance EV uptake in certain cells.[114] These findings imply that there may exist a means by which EV-producing cells can recognize EVs and EV-associated factors in their environment, and can modulate their own EV-production or release in response. Further, this recognition does not extend to other nanoparticulate species, such as MC3-LNPs, which had no significant effect on EV output. Taken together, we hypothesize

that serum components including EVs and EV associated factors can influence EV production via molecular interactions rather than physical competition.

We subsequently hypothesized that if a molecular mechanism exists, it could shift the transcriptome of the EV-producing cells. By employing RNA-sequencing, we were able to identify several EV-biogenesis-related cellular processes which had altered levels between cells cultured in serum-free Opti-MEM and serum-containing medias. Of key interest was the increase in ceramide and sphingolipid biogenesis in cells grown in serum-free Opti-MEM. These processes are implicated in both exosome and MV biogenesis by mobilizing patches of the plasma membrane and inducing the formation of ILVs within the MVBs.[115] By inhibiting sphingomyelinase with both pharmacological and siRNA treatments, we confirm these findings in regard to engineered-EV production. We therefore conclude that at least part of the substantial increase in EVs observed in serum-free Opti-MEM is the result of upregulated ceramide-dependent exosome biogenesis.

A theme throughout this project involved the challenge of fitting our data into the biogenesis-based classification system of EVs. Due to the role of ceramide in EV production at both the endosomal membrane and the plasma membrane, we cannot say whether the increase in EV production occurs at the endosomal membrane or at the plasma membrane. The EV characterization techniques, such as the multiplexed bead-based analysis (MACSPlex) and western blotting, support an increase in exosomes over MVs. We observed an increase in exosome markers that corresponds to the increase in total EVs as measured by the NTA. However, we are unable to paint a complete picture of EV biogenesis with only the RNA sequencing and this EV characterization data. Therefore, we relied heavily on our luciferase-loading strategy to quantitate the effects of media components on the EV production.

It is important to clarify that our engineering approach has two limitations in this study. Firstly, the endogenous functions of the tetraspanins which our constructs are based on may not be retained in the EV-producing cells. This is evidenced by the translocation of the tetraspanins identified in the fluorescent microscopy and is likely due to the overexpression of these constructs far beyond endogenous levels. For example, we observe that our expression constructs cause CD63 to present at the plasma membrane in addition to its normal endosomal location. CD63 which has translocated to the plasma membrane may not retain its ability to recruit exosome-production machinery, as it would on the endosomal membrane. Further work is needed to resolve the extent of involvement of CD63 at these two locations in our engineered cell lines.

Secondly, the construct expression was not consistent across all EV-producing cells. This can also be observed in the fluorescent microscopy in paper I. Inconsistent construct expression can be a driver of heterogeneity in the EV populations collected from these cells. As EV populations are already notoriously heterogenous, this could exacerbate the apparent diversity between EVs produced from our engineered cells, compared to EVs engineered via other means. We have attempted to address EV heterogeneity in our samples by employing a wide range of EV characterization techniques to adequately correlate the luciferase activity to EV

population. The 2018 MISEV guidelines corroborate the need for robust EV-characterization tools, such as those we employed here including NTA, EM, Western blot, and the MACSPlex. Taken together, we can confirm that the luciferase signal measured in our experiments reliably quantitates EV production on a sample-wide level. In conclusion, paper I lends insight into the mechanisms driving increased engineered-EV production in optimized EV-production culture conditions.

4.2 PAPERS II & III

One of the largest hindrances to the widespread adoption of nucleic-acid based therapeutics is the low efficacy they exhibit without an appropriate delivery strategy. Historically, ONs have been chemically modified to exhibit favorable characteristics, increasing their functional delivery. However, there is still much room for optimizing the molecular accessibility of ONs to their targets in the cell cytosol and nucleus.

Taken together, papers II & III comprise a project which aims to expand the toolkit for enhancing macromolecular delivery. Recent work has shown that proper dosing of chemically-modified ONs can increase their internalization *in vitro.* However, the majority of these internalized ONs are still sequestered to non-functional cellular compartments, such as endosomes and lysosomes. Therefore, we sought to seek a novel small molecule compound (SMC) capable of enhancing the functional delivery of ONs by inducing their escape from the endocytic pathway. An ideal candidate compound should be able to increase the activity of the co-administered ON in a low micromolar concentration (ideally, equimolar to the ON concentration), should interfere with cellular trafficking as minimally as possible to achieve ON escape, and should exhibit minimal toxicity. While there exist other delivery strategies that show promise, SMCs offer the advantage that they can be utilized efficiently within a screening context by a simple co-treatment approach with a therapeutic cargo. The ability of a potent endosomolytic SMC to bolster ON activity does not influence the ONs molecular activity, as may be the case with other delivery strategies such as bioconjugation.

Through a collaborative effort with AZ, we initiated a compound screen in which over 30 compounds in total were tested for their ability to increase the activity of a SSO by inducing endosomal rupture. Two assays were used to screen the compounds: an ON-activity assay, and an endosomal rupture assay. In the first, an SSO was treated alongside the SMCs to cells expressing a dysfunctional luciferase mutant. The SSO, when functionally delivered, masks a splice site within the faulty luciferase pre-mRNA, yielding a splice variant with bioluminescent activity. Therefore, the functional delivery of the SSO could be effectively quantitated by means of bioluminescent assay. In the second screen, a cellular marker of endosomal rupture, GAL9, was recombinantly fused with a fluorescent protein. Upon treatment with the SMCs, the fluorescent GAL9 could be visually observed to translocate into distinct puncta at the damaged endosomes. These puncta could then be quantified with image analysis software.

In our initial screens, we compared 6 molecularly distinct compounds to a previously published compound, UNC2383. We identified several compounds that displayed ON-enhancing activity, but only one compound was able to increase ON activity to higher levels, and at a lower treatment concentration, than UNC2383. In parallel, the endosomal rupture assay confirmed that the increases in ON activity correlated to the incidence of endosomal rupture events. These results indirectly hinted that our lead candidate compound, CMP05, was able to enhance ON activity by inducing endosomal leakage. Importantly, other compounds were observed to induce endosomal rupture, but no corresponding increase in ON activity was observed.

To characterize CMP05, we employed a range of next-generation microscopy techniques. The first, STORM, is capable of visualizing cellular structures at a higher resolution than possible with traditional fluorescent microscopy. Intriguingly, STORM images revealed that after compound treatment, late endosomes in the treated cells had a swollen morphology, as identified by late endosome membrane-associated protein 1 (LAMP1). Further, the swollen endosomal morphology correlated to visual observations of endosomal escape. Fluorescently-conjugated ON colocalized with LAMP1 to a high extent in untreated cells. However, upon compound treatment, the fluorescent ON was observed throughout the cell cytosol. These findings from STORM were confirmed with live-cell, confocal GAL9 imaging of cells treated with fluorescent ON. ON-containing endosomes decreased in number simultaneously as GAL9 puncta (damaged endosomes) increased in number. These finding are taken together to support the hypothesis that CMP05 induces endosomal swelling and rupture, which correlates with ON leakage from endosomes, and further correlates with increased ON activity as measured by our functional assay.

Across paper II & paper III, over 25 chemical analogues were generated. Of these 25, several exhibited the ability to increase ON activity to varying degrees. In paper III, a new lead candidate was identified, named CMP05-16. Importantly, this compound was able to enhance ON delivery in a nearly-equimolar concentration (2 μM ON and 2.5 μM CMP05-16) and without necessitating a media-change step to maintain cell viability. The functional increase in ON activity corresponded again to the quantitated extent of endosomal rupture in the GAL9 assay. Seven cell lines expressing fluorescent GAL9, from a variety of different origin tissues, were treated with all 19 compounds in Paper III. CMP05-16 induced endosomal rupture in all cell lines at 2.5 μM concentration. Additionally, several other compounds were able to induce endosomal rupture as observed by GAL9 recruitment. This involved both compounds identified in the ON-activity assay, but also compound which were not identified as increasing ON activity. This seemingly implies the existence of compounds which are capable of disrupting the endosomal membrane, but not in such a manner that it translates to increased functional ON delivery. We therefore continued our mechanistic characterization experiments with CMP05-16.

We first sought to ensure that CMP05-16 is capable of increasing the activity of ONs with (1) a different mechanism of action from SSOs and (2) a different chemical structure. We treated

cells with a gapmer ON targeting endogenous metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). At a concentration of 2 μM, CMP05-16 was able to effectively deliver the MALAT1-targeting ON, resulting in a 60% decrease in MALAT1 mRNA transcripts. Through this, we confirm that the compound-mediated ON delivery is not specific to ONs of a specific molecular mechanism, and further, that ON delivery to endogenous genetic targets can be achieved to effectively bolster RNA knockdown.

At concentrations above 2.5 μM, cells appeared less confluent in their wells. This occurred in the absence of acute cytotoxicity markers such as lactate dehydrogenase (LDH). We therefore employed RNA sequencing on cells that had been treated with an excessive concentration of CMP05-16 to ascertain the mechanism of toxicity. Interestingly, several cellular processes related to mitochondrial-regulated cell death were upregulated. Mitochondrial function relies on the formation of a pH gradient between the mitochondrial membranes. One hypothesized mechanism of toxicity involves promiscuous CMP05-16 buffering against the formation of the necessary mitochondrial proton gradient. In order to confirm this hypothesized action, more work needs to be done investigating the role of mitochondrial-initiated autophagy and apoptosis.

Ongoing work is continuing to (1) expand this class of compounds based off the observed structure-activity relationships, (2) enhance delivery of therapeutic species other than ONs, (3) identify an efficient *in vivo* indication accessible through these compounds, and (4) characterizing the toxicity mechanisms in more detail. New types of therapeutic cargo can be tested with these compounds, including protein therapeutics, ribonuclear complexes, and nanoparticulate species. Theoretically, these compounds may be able to function as an additive component to an existing drug delivery strategy, such as a in a bioconjugation strategy or a lipid nanoparticle formulation.

In conclusion, the work from papers II and III have identified a novel class of compounds which exhibit favorable properties over previously utilized endosomolytic small molecules. These compounds are able to bolster the activity of co-treated ONs by inducing endosomal membrane rupture in a manner dependent on endosomal acidification during trafficking.

5. FUTURE PERSPECTIVES

The work in this thesis involves two projects seemingly unique from each other at first. However, the common thread that ties them together is the same thread that has creeped into numerous research fields in the past decades. The endocytic network is proving itself as a highly dynamic, complex puzzle that will require an enormous, coordinated effort to solve. As mentioned, ongoing work is conducted by physicists who aim to unravel the mechanisms of lipid membrane disruption at various pHs, by chemists to discover new ionizable moieties capable of bolstering drug delivery, and by biologists that seek novel applications for the ever-growing drug delivery toolkit.

On the EV front, the future seems bright. The obvious goal for the EV field is to develop a new macromolecular drug delivery platform, as discussed herein. In order for this goal to be realized, improved protocols are needed including EV engineering with reliable homogeneity, scalable manufacturing, and characterization methods for both fundamental science and for quality control.

In less obvious terms, there exists a crucial need for better technical approaches. For one, research is currently hampered by the lack of a single, universal classification system which adequately accounts for the heterogeneity between EV subpopulations. Further, the field is currently lacking an understanding of the endogenous drivers of differential EV uptake, including *why* EVs from a certain cell type are preferentially up taken by another specific cell type.

In RNA therapeutics, the future is equally promising. Rapid growth in the field has been due to the numerous advancements in stability, delivery, and immunogenicity of the therapeutic RNA. Within this, ONs have become an important therapeutic modality. Not only are therapeutic targets being identified almost weekly, but new chemical modifications and delivery strategies are being developed to bolster ON efficacy.

We are likely entering a new period of ON drug design. The previous generation of ONs are delivered through relatively simple drug delivery approaches, such as local administration of a naked ON or by means of a single targeting strategy. Future generations of ON therapeutics will likely be delivered through complex, combined delivery systems with dramatically enhanced pharmacokinetic properties and tissue specificity. With these advances, the number of small RNA therapeutics that reach clinics – and more importantly, patients – meaningfully grows.

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