Detection and quantification of lipid-mediated siRNA delivery to the cytosol

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DOCTORAL DISSERTATION

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Abstract:

Cancer is one of the leading causes of death globally. Novel therapeutics is urgently needed to advance treatment, especially for generalized disease where current treatment options have a poor prognosis. In particular, targeted therapy that can address genetic changes would be of great value.

RNA interference is an evolutionary conserved gene regulatory mechanism that can be used by introducing exogenous synthetic double-stranded RNAs, so called small interfering RNA (siRNA). siRNAs are sequence-specific inhibitors that are easily designed and could in theory target any gene of interest, making siRNA a promising modality for targeted therapy. However, a key challenge in translating siRNA into the clinic is the inefficacy to deliver siRNA across the plasma membrane, but most importantly, to escape the endosomal system and reach the cytosol where they can interact with the RNA interference machinery. Multiple delivery strategies have been proposed to improve delivery to the cytosol, but because of a lack of methods to accurately quantify this step, the efficacy of current delivery strategies is unknown and the scope for improvement is thus unclear.

The aim of this thesis was to develop novel methods to study the process of endosomal escape and cytosolic delivery of RNA. In particular, advanced high resolution microscopy techniques have been used to in detail characterize and determine the efficacy of lipid mediated delivery of RNA. With these methods, single-cell knockdown kinetics of cytosol delivered siRNA is determined, and the dose-response correlation between knockdown and intracellular siRNA concentration is elucidated. In a second study, endosomal structures damaged by membrane-destabilizing substances are characterized and their potential improvements on cholesterol conjugated siRNA delivery. Lastly, several mechanistic barriers limiting the lipid nanoparticle delivery of siRNA and mRNA are identified.

This thesis advances our understanding on the limiting step of endosomal escape and cytosolic entry of RNA during lipid-based delivery. The tools and knowledge presented in this work will contribute to the development of future delivery strategies aimed at enhancing the effectiveness of siRNA and other nucleic acid-based therapeutics.

Key words: RNA interference; RNA therapeutics; endosomal escape; lipid mediated delivery; live-cell imaging

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“IT’S A TRAP!” – Admiral Ackbar

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Abstract

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Populärvetenskaplig sammanfattning

Cancer är en ledande dödsorsak globalt och vid generaliserad sjukdom saknas idag ofta effektiva och tolerabla behandlingar. Nya behandlingsstrategier, särskilt målinriktade behandlingar med möjlighet att adressera specifika genetiska förändringar vore således av stort värde.

RNA interferens (RNAi) är en evolutionärt konserverad cellulär regleringsmekanism där endogent kodade korta dubbelsträngade RNA molekyler, s.k. microRNA (miRNA) kan nedreglera och finjustera uttrycksnivån av komplementära gener. Inom preklinisk forskning har RNAi kunnat utnyttjas som ett verktyg för att stänga av specifika gener genom att introducera syntetiska korta dubbelsträngade RNA molekyler, s.k. small interfering RNA (siRNA). siRNA är sekvensspecifika inhibitorer som är enkla att designa och kan praktiskt taget nedreglera vilken gen som helst (s.k. knockdown). Ur ett terapeutiskt perspektiv gör detta siRNA till idealiska målinriktade cancerläkemedel.

Sedan upptäckten av RNAi har omfattande forskning fokuserat på att omsätta denna mekanism för terapeutiska ändamål. Kemiska modifikationer har utformats för att förstärka siRNA mot kroppens försvarsmekanismer och avancerade leveransstrategier har utvecklats för att kunna leverera siRNA in i målceller. Detta har lett till fem godkända siRNA läkemedel som behandlar olika sjukdomar i levern. Trots stora framsteg inom fältet kvarstår två betydande hinder – leverans till andra vävnader än levern samt förbättrad intracellulär leverans till målcellerna, ut i cytosolen där RNAi verkar. Endast en ytterst liten fraktion av det siRNA som tas upp av målcellerna når cytosolen medan det resterande läkemedlet begränsas av cellens endosomala system. Utrymmet för effektivisering är således stort.

I den här avhandlingen har metoder utvecklats för att karakterisera och bestämma effektiviteten av lipidbaserade RNA-leveransstrategier med målet att skapa ökad förståelse för lipidleverans av RNA och således förbättra förutsättningarna för att utveckla effektivare leveransstrategier till cytosolen . Dessa metoder har framförallt baserats på avancerad fluorescens-mikroskopi där individuella celler filmas över tid, s.k. live-cell imaging, vilket möjliggör detaljerad undersökning av det endosomala utträdet av RNA samt cytosolär leverans av RNA.

Med dessa metoder har vi bl.a. kunnat bestämma singelcellkinetik vid siRNA medierad knockdown och särskilt kunnat bestämma dos-responskorrelationen mellan biologisk aktivitet av siRNA (knockdown) och intracellulär siRNA koncentration, som tidigare varit oklart. Vi har även kunnat karakterisera endosmala strukturer som destabiliseras av membrandestabliserande substanser m.h.a. fluoroscerande markörer och membranskademarkörer, samt studera dess effekt på siRNA leverans med kolesterol. Slutligen har vi kunnat identifiera flertalet mekanistiska barriärer som begränsar lipidnanopartikel-leverans av siRNA och mRNA.

List of original papers

The thesis is based on the following papers and referred by their roman numerals indicated below:

I. **Imaging small molecule-induced endosomal escape of siRNA**
H. Du Rietz, **H. Hedlund**, S. Wilhelmson, P. Nordenfelt & A. Wittrup
Nature Communications 2020 Vol. 11 Issue 1 Pages 1809

II. **Single-cell quantification and dose-response of cytosolic siRNA delivery**
H. Hedlund, H. Du Rietz, J. M. Johansson, H. C. Eriksson, W. Zedan, L. Huang, J. Wallin & A. Wittrup
Nature Communications 2023 Vol. 14 Issue 1 Pages 1075

III. **Live-cell imaging of cytosolic RNA delivery with lipid nanoparticles reveals cellular and biophysical barriers**
J. M. Johansson*, H. Du Rietz*, **H. Hedlund***, H. C. Eriksson, E. Oude Blenke, A. Pote, S. Harun, P. Nordenfelt, L. Lindfors & A. Wittrup.
Shared first authorship*
Manuscript unpublished

IV. **Intracellular dose-response determination of siRNA by two-tailed RT-PCR of electroporated cells**
H. Hedlund, J. M. Johansson, H. Du Rietz & A. Wittrup
Manuscript unpublished

Abbreviations

2’-F	2’-deoxy-2’-fluoro
2’-OH	2’-hydroxyl
2’-OMe	2’-O-methyl
Anti-miRs	ASOs that inhibit miRNA function
Ago	Argonaute
Apo	Apolipoprotein
ASGPR	Asialoglycoprotein receptor
ASO	Antisense oligonucleotide
AU-content	Adenine uracil content
bp	Base pair
cDNA	Complementary DNA
CD63	Cluster of differentiation 63
Chol-siRNA	Cholesterol-siRNA confocal laser scanning microscopy
CLSM	Confocal laser scanning microscopy
COX	Cyclooxygenase enzyme
CRISPR	Clustered regularly interspaced short palindromic repeat
DGCR8	DiGeorge syndrome critical region gene 8
ds	Double-stranded
dsRBP	dsRNA-binding protein
dsRNA	Double-stranded RNA
EEA1	Early endosome 1
eGFP	Enhanced green fluorescent protein
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle fluorescence correlation spectroscopy
FCS	Fluorescence correlation spectroscopy
FOV	Field of view
GalNAc	*N*-acetylgalactosamine
GaAsP	Gallium arsenide phosphide

GC-content	Guanine cytosine content
hATTR	Hereditary transthyretin-mediated amyloidosis
IC_{50}	Half-maximal inhibitory concentration 50
IFN-α	Interferon-α
IL-6	Interleukin-6
KRAS	Kirsten rat sarcoma viral oncogene
LAMP1	Lysosomal associated membrane protein 1
LDLR	Low-density lipoprotein receptor
LNP	Lipid nanoparticle
ncRNA	Non-coding RNA
nt	Nucleotide
NSAID	Nonsteroidal anti-inflammatory drugs
MC3	Dlin-MC3-DMA
MID	Middle domain
MIP	Maximum intensity projection
miRNA	microRNA
mRNA	Messenger RNA
MYC	Myelocytomatosis oncogene
PAZ	Piwi-Argonaute-Zwille domain
PEG	Lipid-anchored polyethylene glycol construct
pri-miRNA	Primary miRNA
PS	Phosphorothioate linkage
PSF	Point spread function
Rab	Ras-related in brain
RES	Reticuloendothelial system
RISC	RNA induced silencing complex
RLC	RISC-loading complex
RNA	Ribonucleic acid

RNAi	RNA interference
RNase	Ribonuclease
ROI	Region of interest
SARS-CoV-2	Ribonuclease servere acute respiratory syndrome coronavirus 2
siRNA	Small interfering RNA
SMA	Spinal muscular atrophi
SMN	Survival motor neuron
ss	Single stranded
TIE	Translation inhibitory element
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRBP	TAR RNA-binding protein
TTR	Transthyretin gene
uORF	Upstream open reading frame
UTR	Untranslated region
VEGF	Vascular endothelial growth factor yellow fluorescent protein
YFP	Yellow fluorescent protein

Introduction

Cancer is globally one of the most lethal diseases today and accounts for almost ten million deaths yearly. It is generally described as uncontrolled growth and spread of abnormal cells to nearby tissues. If not contained or treated, these cells could potentially reach other parts of the body in a process called metastasis. Upon cell division, the genetic information is replicated and incorrections are normally restored by DNA repair pathways. However, in rare occasions such incorrections could slip through the system and result in mutated daughter cells. If such mutations occur in genes that are responsible for *e.g.* cell growth, proliferation or survival, there is a risk that these cells become unresponsive to natural signals and regulatory mechanisms; making them abnormal or cancerous[1].

Cancer is more accurately described as a group of diseases and include possibly thousands of different types, each with their own characteristics. Thus, there is no universal cure for a disease with such heterogeneity. However, many of the available treatments have successfully cured a wide range of malignancies, and some of which had previously been considered fatal have a significantly improved prognosis today. For instance, Hodgkin lymphoma was once a life-threatening disease, but because of effective chemotherapy regimens and radiation therapy, many patients have good long-term survival prospects[2].

Today, first line treatment is usually a combination of surgery, chemotherapy and radiation therapy. These options are generally effective for early-stage cancers, but for generalized disease they usually result in poor curative outcome. Surgery can be used for palliative treatment but it is often not possible to remove all metastatic sites. Chemotherapy and radiation therapy may have some success in shrinking the tumors and possibly extend survival, but their lack in specificity limits their therapeutic potential. Targeted therapies such as tyrosine kinase inhibitors or immunotherapies have shown great promise[3, 4], but additional avenues should be investigated for highly specific and personalized treatments.

RNA-based therapy is a research field that have received a lot of attention the last couple of years. Since the COVID-19 pandemic with worldwide repercussions, the first ever approved RNA-based vaccine was developed and administered to billions of people[5]. Just a couple of months after isolating the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike trimer that would become the translated antigen-product of the messenger RNA (mRNA), the RNA vaccine was

established and clinical studies were initiated. While the success of the RNA vaccine concept may have seemed straightforward at the time, it was made possible, in part, by two decades of prior research dedicated to optimizing the conditions for utilizing the RNA molecule as a therapeutic modality[6]. RNA is in its 'naked' state, a sensitive macromolecule that has poor pharmacological characteristics, *i.e.* prone to nuclease degradation, activation of the immune system, and unable to passively cross the cell membrane. Therefore, substantial efforts have been devoted to reinforcing the macromolecule to withstand the harsh *in vivo* environment and reach the intended target within the cell. Much prior research activity had previously been pursued for short RNAs that had received a lot of attention for highly specific gene silencing. As Fire and Mello (and others) discovered the endogenous gene regulatory pathway called RNA interference (RNAi) that could accurately downregulate any gene by sequence specific knockdown utilizing small interfering RNAs (siRNAs)[7], there has been a great interest in exploiting this Nobel prize awarded mechanism for therapeutic purposes. Indeed, such targeted therapy could be an ideal cancer therapy with specific knockdown of cancer driving genes.

However, reaching the correct tissue and gaining entry into the cytosol of the target cell remains challenging for siRNA therapeutics. Multiple delivery strategies have been proposed but even the most effective delivery strategies are believed to deliver less than 1% of the endocytosed siRNA to the cytosol[8], while the non-delivered material remains contained by the enclosing endosome and is eventually degraded in lysosomes. An important factor that limits the further development of siRNA delivery strategies is the lack of methods that can study endosomal release and cytosolic delivery of siRNA. Indeed, such methods would be of great value for assessing the total efficiency for a potential delivery strategy and identify delivery bottlenecks, and thus aid in the development of novel delivery strategies. Given this, the aim of this PhD thesis has been to investigate lipid mediated delivery of siRNA, by developing novel methods to detect and quantify cytosolic delivery of the macromolecular siRNA. The aim is to advance our toolkit to study RNA delivery in general and some of the methods presented here can be applied to multiple types of RNA therapeutics. Hence, the thesis summary will touch upon other RNA based therapies to some extent.

RNA interference

Gene regulation is a highly complex process encompassing a vast number of pathways that facilitate regulation at various stages of gene expression. The process is important for cell differentiation and development, maintaining homeostasis, response to environmental changes etc. Among these is a pathway called RNA interference (RNAi) that plays a significant role in post-transcriptional gene regulation. The pathway is also believed to have been crucial in the cellular response to viral infections before animals and plants diverged. RNAi is an evolutionary conserved mechanism found in cells across species, that utilizes short RNA molecules for downregulation of specific genes through translational inhibition of mRNA. These short RNA molecules derive from longer noncoding RNAs that can be separated into a number of classes. Among them are exogenous siRNAs that are short double-stranded RNAs (~22-nucleotides long) and endogenous microRNAs (miRNA) that are transcribed as single-stranded RNAs and processed into short double-stranded mature miRNAs[9].

Beginning in the early 90s, Ambros and coworkers discovered an endogenous regulator in *Caenorhabditis elegans* that could control developmental timing. The regulator came to be known as lin-4 and was the first miRNA to be recognized[10]. A few years later, groundbreaking work by Fire and Mello reported specific gene silencing in *C. elegans* by introducing exogenous double-stranded RNA[7], which they were later awarded the Nobel prize in Physiology or Medicine for in 2006. The first demonstration of the conversion of long dsRNAs into 21-23 nt siRNAs as well as triggering of the RNAi pathway by synthetic siRNAs was later reported by Tuschl and coworkers[11]. The two classes of small RNAs were initially thought to be separate from each other, however it was later revealed that miRNA and siRNA depend on the same family of proteins, namely Dicer and Argonaute (Ago). Dicer enzymes are responsible for identifying longer double-stranded RNAs (dsRNAs) and subsequently cleave them into shorter pre-miRNAs/siRNAs[12]. Ago is the catalytically active RNase that facilitates translational repression. However, while there is a mechanistic overlap for miRNA and siRNA, their origin and potential outcome diverge to some extent.

The RNAi pathway

miRNA pre-processing in the nucleus

While the precursors of miRNAs originate from the genome, siRNAs are either administered exogenously to the cytoplasm or in some species, other exogenous sources such as viral infections could potentially introduce it[13]. Thus, pre-processing in the nucleus is only performed on miRNA precursors (Figure 1). In the initial phase, miRNAs are single-stranded RNAs transcribed by RNA polymerase II as primary hairpin-containing transcripts or primary miRNA (pri-miRNA). Pri-miRNAs typically feature some mismatches and contain multiple stem-loops and terminal loops, and usually contain at least 1000 nucleotides (nts). The maturation is then initiated as the nuclear RNase III called Drosha crops one of the stem-loops in the junction of the stem of the pri-miRNA, approximately 11 base pairs (bps) from the opening of the hairpin-loop. This is delicately orchestrated together with a cofactor called DiGeorge syndrome critical region gene 8 (DGCR8), which together with Drosha forms the microprocessor complex[14]. After cleavage, the resulting ~70 nucleotide pre-miRNA is transported to the cytoplasm by Exportin-5 and RanGTP[15].

RISC assembly in the cytoplasm

Reaching the cytoplasm, the RNAi pathways between miRNA and siRNA converge and the precursors typically share similar fates. Dicer is initially recruited and the precursor RNAs are trimmed down to smaller RNA duplexes of 21-25 nt fit for loading onto Ago. For miRNA, the terminal loop is specifically removed[16]. The trimmed duplex additionally results in 2 nt overhangs at the 3'-ends and phosphate groups on the 5-ends[17], which are important motifs for loading. For some siRNAs, this step is surpassed by introducing such overhangs synthetically. Dicer then subsequently perform Ago loading (Ago2 for siRNA), which is assisted by dsRNA-binding proteins (dsRBP), usually the TAR RNA-binding protein (TRBP), and together they form the RISC-loading complex (RLC)[18]. One of the strands of the dsRNA is then selected as guide strand, as the Piwi-Argonaut-Zwille (PAZ) and middle (MID) domain of Dicer binds to the 3'-overhang and the phosphate group on the 5'-end, respectively, to generate the effector complex called RISC. The other strand, *i.e.* the passenger strand, is discarded[19].

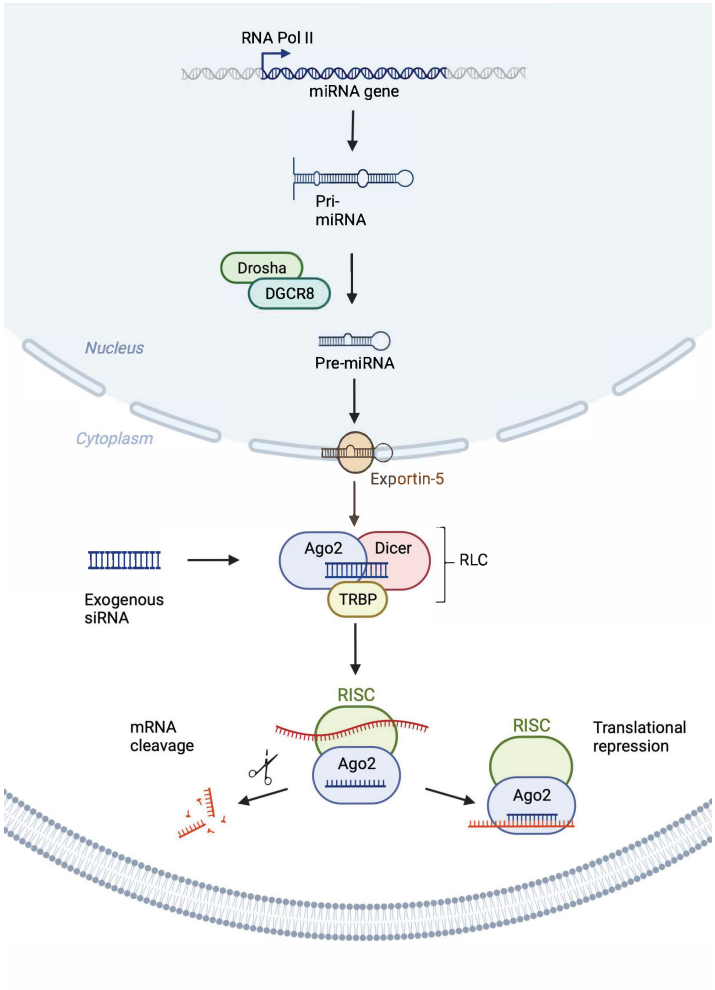

Figure 1. The RNA interference pathway.
Pri-miRNA is transcribed in the nucleus by RNA polymerase II and processed to pre-miRNA by the microprocessor complex (Drosha and DGCR8). Pre-miRNA is transported to the cytoplasm by Exportin-5 and RanGTP. siRNA or miRNA interacts with Dicer and TRBP for RLC assembly and subsequent Ago2 loading. Guide strand is selected to form RISC and mRNA target is either repressed from translation if incomplete complementarity or cleaved by Ago2 and degraded if perfect complementarity. Created with BioRender.com.

RNA interference by RISC

As RISC formation is completed and the guide strand of either miRNA or siRNA has been loaded into Ago, the effector complex searches the cytoplasm for potential mRNA targets sharing sequence complementarity with the guide strand. When an mRNA is found, the seed sequence that constitute 2-8 nt of the guide strand,

initialize binding to the target mRNA. Subsequent silencing is then proceeded by two mechanisms. If perfect complementarity is attained between the seed sequence and the mRNA sequence, Ago proceed by cleaving the mRNA, resulting in subsequent degradation. For incomplete complementarity, the RISC induces non-endonucleolytic translational repression[20].

While this canonical pathway describes the general concept of RNA interference, there is an important distinction between miRNA and siRNA, which is their respective modes of target recognition and downregulatory outcome. miRNAs generally have different degrees of complementarity with multiple RNA targets and can therefore alter gene expression for a plethora of targets with a single miRNA sequence. siRNAs on the other hand, are generally designed to share almost perfect complementarity to its target RNA and are thus highly specific against single RNA targets[21]. Thus, for scientific applications and as therapeutics, siRNA-type designs have typically been the more compelling option due to its straightforward rationale, although there are potential avenues for miRNA as well.

RNA-based therapy

It is well known that the druggable targets for small molecule therapies is limited to a small fraction of the expressed proteins. Approximately 10-14% of the proteins can be targeted because of apparent active sites or allosteric sites, *i.e.,* mainly enzymes, transporters, ion channels, and receptors. Other proteins that lack obvious binding pockets such as scaffolding proteins, structural proteins, transcription factors, chaperones or assembly/disassembly factors pose a significant challenge for conventional drug development due to their multifaceted functions and unclear target sites[22]. The "undruggable" nature becomes even more evident when only ~1.5% of the human genome encode for proteins, while the majority are transcribed as non-coding RNAs (ncRNAs)[23, 24], indicating the need for other therapies with a larger repertoire of druggable targets.

RNA-based therapeutics is a steadily growing field that have the potential to address many of the current limitations faced by today's therapies. Since the discovery of the mRNA in 1961[25] which was awarded the Nobel prize in 1962, the field of RNA molecules has yielded an additional three Nobel prizes for the scientific discoveries, *i.e.,* RNA splicing[26] in 1993, RNAi[7] in 2006, and CRISPR gene-editing technology[27] in 2020. These scientific achievements signifies the excessive potential that the field has and have paved the way towards using the molecule as a therapeutic modality.

The therapeutic platform of RNA-based drugs can theoretically function in each macromolecule that constitutes the central dogma of molecular biology, namely DNAs, RNAs, and proteins. Translatory interference of mRNA and inhibition of ncRNAs can be achieved with siRNAs, antisense oligonucleotides (ASOs), and miRNAs through traditional Watson-Crick base-pairing. Protein replacement treatments and immunization can be achieved with *in vitro* produced mRNAs that is subsequently delivered to the target cell. For genome editing, target RNA sequences in clustered regularly interspaced short palindromic repeat (CRISPR)-based drugs can be modified to treat a certain genetic disorder. Finally, RNA aptamers can act similar to small-molecule inhibitors and antibodies and block specific protein activity.

An important aspect for RNA-based drugs is the flexibility and the precision that could potentially be achieved. RNAs are in principle 2-dimensional structures in the sense that they are sequences of nucleotides. And, the therapeutic mechanism for the majority of the proposed RNA-based drugs is, albeit simplified, Watson and

Crick base pairing or codons that translates to amino acids. Such design makes RNA-based drugs extremely flexible as it ultimately boils down to a sequence of code. Thus, small genetic changes that could give rise to disease can accurately be addressed with minimal effort[28], introducing a new potential paradigm for personalized medicine.

While promising, there are multiple physiological barriers for RNA therapeutics. RNA is a highly sensitive macromolecule in its naked state and require rigorous chemical modifications and sophisticated delivery strategies to become therapeutically applicable. This will be discussed in greater detail in the chapter 'Barriers for RNA therapeutics and strategies to overcome them'.

Introduced below are some of the main RNA therapeutics that are generally highlighted for their therapeutic significance (Figure 2), with particular emphasis on siRNA due to the aim of the thesis.

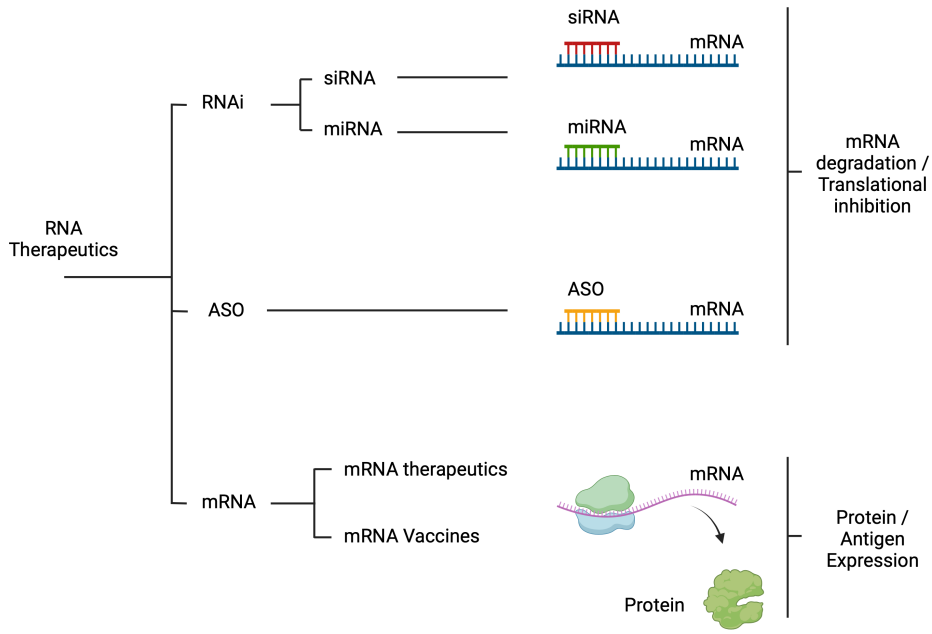

Figure 2. Schematic of the main RNA therapeutics.
RNAi can be subdivided into siRNA and miRNA therapeutics. Both function to inhibit gene expression, with siRNAs selectively targeting specific mRNAs leading to translational inhibition or mRNA degradation. miRNAs target multiple mRNAs and can only induce translational inhibition. ASO is another class of inhibitory therapeutics that target specific mRNAs and can cause translation inhibition or mRNA degradation. mRNA drugs can be subdivided into mRNA therapeutics and mRNA vaccines, acting as *e.g.,* protein replacement therapies or as antigens to cause an immunological response, respectively. Created with BioRender.com.

Antisense oligonucleotides (ASOs)

ASOs are oligonucleotides that are single stranded (ss) and target specific RNA through Watson-Crick base-pairing. They are generally 12-30 nucleotides of length and the binding specificity to the target RNA is determined by the degree of complementarity as well as binding energy modulation by specific modified bases usually incorporated in the design. Protein expression can be reduced, restored, or modified with ASOs and they are typically divided into two types of mechanisms: occupancy-mediated degradation and occupancy-only models with various post-hybridization mechanisms[29, 30].

Occupancy-mediated degradation

ASOs that mediate occupancy-mediated degradation cleave the target RNA at the site of ASO binding, resulting in specific gene downregulation. Because the pathway uses specific enzymes, it is sometimes also referred to as enzymatic RNA degradation[31]. The most well-defined mechanism involves the RNase H1 enzyme. It is highly selective and ubiquitously distributed throughout the cell, and explicitly cleave RNA-DNA heteroduplexes in both the cytoplasm and the nucleus[32]. Another occupancy-mediated degradation pathway are so-called ribozymes[33]. They fold into hairpin or hammerhead structures and subsequently facilitate cleavage of the target RNA[34]. Ribozymes can also be modified so that their substrate recognition domains can promote either cis or trans-site cleavage. There are also some initial studies on a mechanism called nonsense-mediated mRNA decay in which the ASO trigger alternative splicing of the pre-mRNA resulting in an mRNA containing premature termination codons[35]. Or the ASO act on mature mRNA to trigger a “no-go decay” mechanism which mRNAs with multiple stalled ribosomes are degraded[36].

Occupancy-only mechanism

The second variant of ASO mediated mechanisms is referred to as the occupancy-only mechanism. As the name entails, the ASO binds to the specific target but do not involve any catalytic enzymes, but instead act as a steric blockade. Therefore, the pathway has a wider utility and can be used for both down- and up-regulatory purposes. In the case of downregulation, three mechanisms are presently described. The ASO can block translation by sterically hinder ribosomal recruitment to the target mRNA[37, 38]. A phase I study for the treatment of prostate cancer evaluated such translational-blocking ASOs for the purpose of downregulating the transcription factor myelocytomatosis oncogene (MYC)[39]. The 5’cap can be cleaved by ASOs that guides catalytic enzymes responsible for cap structure disassembly[40]. Thirdly, polyadenylation sites can be targeted on pre-mRNA sequences to alter its stability, and thus gene expression levels[41].

For upregulation, there is an additional subpopulation of mechanisms that ASOs may be used for. Splice altering ASOs have so far been the most explored mechanisms of action, with nusinersen being the first clinically approved antisense drug exploiting this mechanism[42-44]. Nusinersen treats spinal muscular atrophi (SMA), a motor neuron disease that is caused by the loss of function of the survival motor neuron 1 (*SMN1)* gene that normally encodes for the key protein SMN that is responsible of forming and maintaining neuromuscular junctions. There is, however, a second gene (*SMN2*) that encodes for limited amounts of a more unstable SMN protein. This instability is caused by a mutated splicing enhancer sequence that excludes one of the exons. The active ASO in nusinersen corrects for this mutation by binding to the *SMN2* pre-mRNA and facilitate inclusion of the exon, thus resulting in generation of a stabilized SMN protein[44].

Other mechanisms of ASO steric blockades that can facilitate upregulation of protein expression is by target miRNAs responsible for downregulating the gene of interest. Known as anti-miRs, they specifically bind to the miRNA and suppress it from binding to its target mRNA, thus increasing the expression of the corresponding protein. Some initial studies have further shown that ASOs may be used as a masking agent for miRNA-binding sites on mRNA, also leading to increased protein levels[45, 46]. Finally, upstream open reading frames (uORFs) and translation inhibitory elements (TIEs) can be targeted for increased protein translation, inhibiting the binding of suppressive structural elements[29, 30].

Small interfering RNAs (siRNAs)

siRNAs are short double-stranded (ds) RNAs, typically 21-23 nucleotides, that mediate target specific knockdown of cytoplasmic RNAs utilizing the RNAi pathway[11, 47], as described in the chapter 'RNA interference'. siRNAs have also been suggested as agents for chromatin remodelling and histone modification in the nucleus[48, 49]. However, presently there is no consensus on the nuclear functions of siRNAs. For cytoplasmic RNA targeting, the sequences are typically designed to have perfect complementarity with the targeted RNA, making siRNA therapeutics an ideal therapy for specific targeting. However, as the therapeutic field has grown more "rules" have emerged to design a potent sequence. Therefore, there are a number of considerations that will play an important role for the pharmacodynamics of the designed siRNA sequence, which will ultimately influence target specificity and off-target activity.

Trigger motifs and sequence selection

RNA duplexes that are larger than 21 bp interact with Dicer for cleavage and subsequent loading into the RISC via the RLC[50]. Smaller siRNAs tend to bypass Dicer cleavage and is loaded into RISC by interacting with the TRBP or by Dicer[51, 52]. Dicer typically recognize 2 nucleotide overhangs on the 3'-ends, usually with two uracil or two thymine nucleotides. These overhangs are expected to form on endogenous RNA duplexes produced in the Dicer cleavage reaction[53], which could be an explanation for its necessity in siRNA synthesis. Generally, siRNAs are designed to bypass Dicer cleavage for direct strand selection and interaction with RISC. The choice allows for a more extensive use of chemical modifications that is generally needed for metabolic stability and improved performance[54] (discussed in more detail in the chapter 'Barriers for RNA therapeutics and strategies to overcome them'). However, Dicer cleavage have been shown to enhance RNAi activity because of a more consistent selection of the antisense strands[50], which could pose a potential advantage for some therapeutic applications.

Since siRNAs are double-stranded, both strands can theoretically be incorporated into RISC[52, 55]. Thus, guide strand selection by Dicer between the sense strand (incorrect) and antisense strand (correct) will have a pronounced effect on the resulting RNAi-activity, with lower potency and potential off-target effects if the wrong strand is loaded[56]. Stability and binding efficiency between the strands can be balanced by guanine-cytosine content (GC-content), and it is preferable to have at least 30-50% of GC-content to influence RISC incorporation[56]. The strand with its 5'-end at the end of the less thermodynamically stable part of the siRNA has also been shown to be selected as guide strand when loaded onto Ago2, as elegantly shown by Czech *et al.* and Khvorova *et al.*[51, 52]. Thus, ideally 5'-end of the antisense strand should contain a higher adenine-uracil content (AU-content). Other means of facilitating guide strand selection is by designing 'asymmetric' siRNAs with a 2 nt 3'-overhang on one side and a blunt end on the other, where the strand with the 3'-overhang tends to be selected as guide strand[55].

After the guide strand has been incorporated into the RISC, the RNA-RISC complex subsequent action involves binding to the target mRNA. This step is partly assisted by the Ago proteins that improve binding kinetics by reshaping the guide strand bases to enhance potential hybridization with the mRNA target and facilitate search of mRNA target sites[57, 58]. Still, transiting ribosomes and mRNA-bound proteins can impede access[59] and complicating binding. There are available prediction software[60], but they are presently not reliable for evaluating such complex interactions. Another complicating factor is the seed-region which constitute the primary binding region between the guide strand and mRNA. It is generally no more than 8 bases and could potentially bind to a wide range of potential off-targets. Fortunately, Ago2 require almost perfect binding, and thus partially bound off-target effects will not be targeted for degradation, but that particular mRNA might

become translatory inhibited which is less detrimental. Additionally, there are multiple available tools for screening sequences for potential off-target risks[61].

As a therapeutic

Utilizing siRNA-mediated silencing for therapeutic purposes could open new doors for currently undruggable diseases. For instance, many oncogenic targets fall into the category of "undruggable" because of *e.g.* the lack of active binding sites[62], but could potentially be targeted by siRNA therapeutics. Indeed, initial studies have attempted to silence Kirsten rat sarcoma viral oncogene (KRAS) or MYC in mouse models[63, 64], however, more substantial studies are needed. Currently, there are five approved siRNA drugs, all targeting different mRNA transcripts in the liver[65-69]. The first clinically used siRNA drug, called patisiran, was approved by the FDA in 2018. Patisiran targets a mutated transthyretin gene (TTR) in the hepatocytes that gives rise to hereditary transthyretin-mediated amyloidosis (hATTR). The mutated gene cause amyloid deposits that accumulate in the peripheral nervous system due to misfolding of the resulting protein, leading to polyneuropathy. With patisiran, the mRNA of the mutated TTR is instead degraded by RNAi, resulting in a decrease in TTR production and limiting disease progression[65]. To reach beyond the liver, further improvements concerning delivery and endosomal escape of siRNA is needed (read chapter 'Barriers for RNA therapeutics and strategies to overcome them')

MicroRNAs (miRNAs)

miRNAs are powerful genetic regulators that can regulate a wide range of target genes with a single miRNA, utilizing the RNAi pathway. From a therapeutic standpoint such characteristics can be extremely attractive, as multiple therapeutic targets can be altered with one fairly simple molecular design[70]. For instance, a particular study showed that a single miRNA could redirect an entire cellular pathway that were involved in T cell regulation[71]. The addressable spectrum further increases as several miRNAs typically regulate the same gene or pathway, adding to the complexity of altering such regulatory network. Another fortunate characteristic with miRNAs as potential therapeutics is that they are often entirely conserved across several vertebrate species. Same miRNA compound could therefore theoretically be used in preclinical studies as well as in prospective clinical studies[72]. Albeit powerful, the pleiotropic function will also increase the likelihood of downregulating off-targets that would consequently cause unwanted side effects. Indeed, such scenario was observed in a first clinical trial with a tumor-suppressive miRNA mimic[73]. Thus. rigorous mapping of the miRNA target will be necessary to avoid the potential risk for unwanted side-effects.

miRNA therapeutics generally aim to modify, or reverse, changes in miRNA expression that give rise to disease. Such alterations are either achieved by enhancement or restoration of endogenous miRNAs that suppress pathological development or by blocking damaged miRNA pathways that drive disease progression. The current repertoire of routes for miRNA enhancement/targeting include synthetic miRNAs (*i.e.* mimics of endogenous miRNAs), recombinant vectors carrying miRNA encoding sequences, and anti-miRs[72] that have already been introduced in the ASOs section. There are currently a handful of miRNA therapeutics in clinical trials. Besides anti-miRs, that constitutes a significant portion, there are a few synthetic miRNA drug candidates. As a cancer therapy, a promising synthetic miRNA is the mimic of miR-34a. The mir-34 family has been identified as regulators of the p53 tumor suppressor protein[74], and is frequently inactivated for various cancers but could potentially repress tumor growth if reconstituted. Indeed, studies in cancer mouse models revealed notable suppression of tumor growth and metastasis when miR-34 is either re-expressed or delivered to the tumor[75]. Similarly, preclinical trials of let-7, a miRNA frequently downregulated or lost in lung cancer, revealed notable reduction of tumor size after administration in mice[76].

Messenger RNAs (mRNAs)

The introduction of exogenous mRNAs to recipient cells could be very effective in many therapeutic contexts, and it was first demonstrated *in vivo* after injecting *in vitro* transcribed mRNA in mouse skeletal muscle cells which resulted in a detectable protein expression[77]. Being the messenger between genetic information and protein production, mRNA comes with many useful pharmacological qualities. For instance, the mRNA generally has a short half-life for regulatory reasons. In the context of vaccines, such transient effects are important to limit the immunological response but generate a sufficient reaction to confer immunity without causing prolonged side effects. Introducing foreign mRNA to the cytoplasm also stimulate a number of immunological responses, such as the activation of toll-like receptors (TLRs), providing mRNA with inherent adjuvanticity[78]. Additionally, compared to protein-based vaccines where the protein usually derives from bacteria, the antigen produced by mRNA vaccines will be translated by the host machinery. Therefore, the protein structure will closely resemble the protein translated from the viral genome[79]. Indeed, these beneficial traits comes as no surprise after the remarkable success of the mRNA-based vaccine against the SARS-CoV-2, which brought significant attention to novel modality[5]. Other ongoing studies focus on mRNA vaccines as a potential cancer therapy. An mRNA construct containing predicted neoepitopes and driver gene mutations where synthesized and vaccinated in patients with metastatic gastrointestinal cancer. The vaccine demonstrated T cell specific

responses against the same predicted neoepitopes and receptors targeting the included mutated driver gene[80]. Similar studies are also being carried out for melanoma, glioblastoma, acute myeloid leukemia, and renal cell carcinoma[81-84].

mRNA-based therapy can also be used in the context of protein replacement[85], where the disease is a result of a protein deficiency. For extended duration of effect, however, the mRNA would require extensive chemical modifications to prevent degradation. Substantial efforts have therefore been devoted to chemically stabilize the 5'cap, the 5' and 3' untranslated region (UTR), and the pol(A) tail, prolonging their initial duration of effect from a few minutes to more than a week[86]. Site-specific chemical modifications that also improve stability and is extensively used in short RNA therapeutics, would additionally prolong the lifespan of the mRNA. But, because of the length of mRNAs, they can only be *in vitro* transcribed (not chemically synthesized as short RNAs), which makes base modifications currently unavailable[87]. Currently, there is only a handful of protein replacement therapies in clinical trial, mainly focusing on cardiac diseases utilizing mRNA transcripts encoding the vascular endothelial growth factor (VEGF)[88]. But several preclinical studies for cancer[89], lung diseases[90], and other diseases are also ongoing[91].

Barriers for RNA therapeutics and strategies to overcome them

The field of RNA-based therapy face a multitude of challenges to reach its full potential, and there are several physiological barriers to overcome[92]. The barriers depend on the route of administration, and compared to systemic administration, local administration is generally unaffected by barriers associated with systemic circulation. But in the case of having a broad therapeutic effect, *e.g.,* reaching metastatic sites in a number of organs, systemic administration is preferred. The potential barriers introduced here will therefore be based on systemic administration.

Barriers

The physiological environment for 'naked' RNA is extremely hostile, with several extracellular and intracellular barriers (Figure 3). RNAs have poor stability and pharmacokinetic characteristics. For instance, the phosphodiester bonds that make up the backbone of the RNA is very sensitive to catalytic ribonucleases (RNases) and phosphatases. RNA that is systemically administrated into circulation will thus rapidly be degraded into fragments by circulating endo and exonucleases, not being able to reach the intended tissue and have a therapeutic effect[93]. For oligonucleotides such as siRNAs and ASOs, the pores of the glomerulus, the filtering unit of the kidney, are large enough to clear the circulating RNA. As a result, small RNAs will have a short half-life and unrealistic dosing of a potential therapeutic would therefore be necessary to achieve the intended dose in the target tissue. Once the RNA reaches the target organ, it hast to exit the circulation and be transported in the tissue interstitium to reach the target cells. Here it must become internalized via endocytosis, which generally require an internalization ligand or structure. Because siRNAs and ASOs are large hydrophilic polyanions (~14 kDa), they cannot naturally diffuse across the cell membrane like small molecule drugs. Larger mRNAs are obviously even less prone for such transitions. Other barriers that interfere with RNA therapies are the scavenge macrophages within the reticuloendothelial system (RES) or innate-immune responses that are activated upon endocytosis through recognition by TLRs that resides within endosomal

structures[93]. Finally, as the RNA has been endocytosed it has to escape from the enclosing endosome to reach the cytosol to perform its biological function, *e.g.,* to interact with RISC. This step is considered to be highly inefficient, and one of the major bottlenecks for RNA therapeutics.

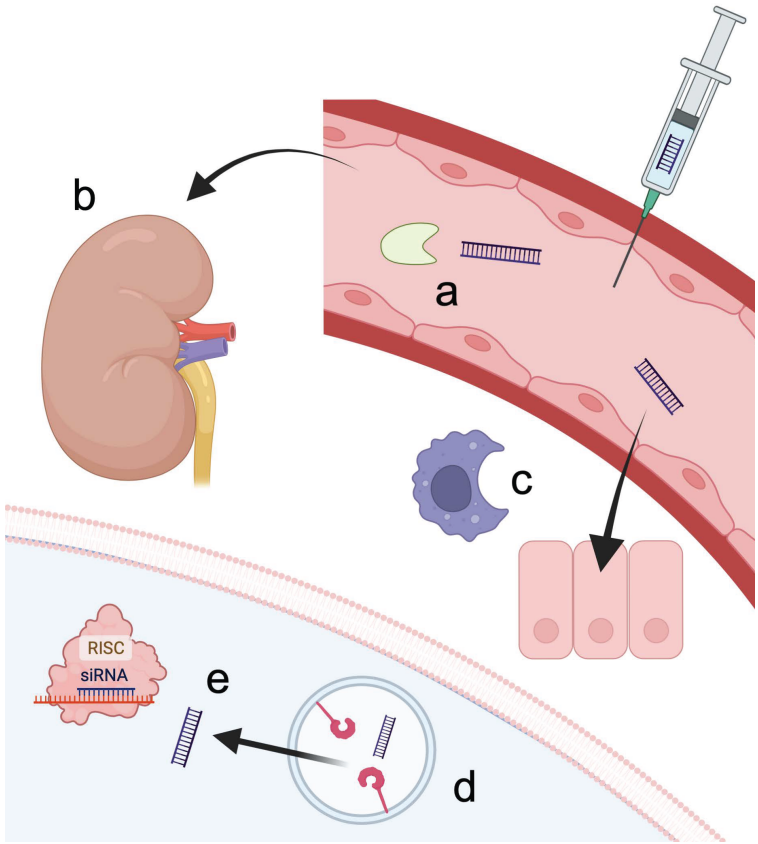

Figure 3. Barriers to RNA therapeutics.
Systemically administered therapeutic RNA face a number of barriers to reach the target tissue and into the target cell. (a) Circulating RNases will rapidly degrade any present RNA that reaches the bloodstream. (b) The kidneys will clear smaller RNA that fit through the pores of the glomerolus. (c) In the reticuloendothelial system (RES), scavenge macrophages resides and will clear the RNA by phagocytosis. (d) Toll-like receptors within the endosomes will elicit an immunological response as the RNA enters through endocytosis. (e) The enclosing membrane of the endosome will prevent the RNA from reaching the cytosol. Created with BioRender.com.

To overcome these barriers, RNA-based therapeutics require sophisticated chemical modifications and delivery strategies to minimize the risks of degradation, renal clearance, and immunological responses and facilitate productive delivery to target tissues and, importantly, into the cytosol of the target cell through endosomal escape. This chapter will mainly focus on improvement strategies for oligonucleotide delivery, but some improvements are also applicable for other RNA

therapeutics. Current bottlenecks that hamper RNA delivery will also be highlighted.

Chemical modifications

Chemical modifications of oligonucleotides are an important part of improving pharmacokinetics, pharmacodynamics and biodistribution of the macromolecule when systemically delivered. The main types of modifications are backbone modifications, ribose sugar modifications, and base modifications, but there are also alternative chemistries depending on the specific purpose[94]. Due to the wide range of possible modifications that can be applied to each nucleotide, numerous sequence combinations or permutations have also been suggested to further improve potency and reduce potential toxicities[6].

Backbone modification

There are many kinds of backbone modifications, but one of the most widely used modification for therapeutic oligonucleotides is the incorporation of phosphorothioate (PS) linkages in the inter-nucleotide phosphate group. The non-bridging oxygen atoms is replaced with a sulfur atom, which provides the oligonucleotides with a number of improved characteristics. The replacement significantly decreases the interaction with nucleases. PS is a bulkier group than oxygen and sterically impede nuclease access to their recognition sites[95]. Plasma proteins, such as albumin, will also have increased affinity to the oligonucleotides[96, 97], which may result in a longer circulation time as it can no longer pass through the 'filters' of the glomerulus[98]. The accumulation of plasma proteins might also facilitate increased cellular uptake through increased hydrophobicity[99]. However, too high protein binding has showed an increased risk for *in vivo* toxicity[100]. ASOs can generally have a fully modified PS backbone, and still recruit RNase H for mRNA cleavage. siRNAs, on the other hand, can only be partially modified with PS as heavily modified sequences will considerably weaken its interaction with its target sequence[6]. The insertion of PS modifications can vary but it is generally introduced at the ends of both strands for siRNAs, providing sufficient resistance and acceptable half-life. Despite increased stability that could potentially result in a more widespread distribution, siRNAs and other oligonucleotides that are PS modified are still primarily accumulated in the clearance organs, with some exceptions[101, 102].

Ribose modification

The ribose sugar is a frequently modified component on therapeutic oligonucleotides, especially the 2'-hydroxyl group (2'-OH). Due to its nucleophilic characteristic the hydroxyl group can participate in a number of chemical reactions, and it is generally required for hydrolysis of the phosphodiester linkage on the RNA backbone. Substitution of the 2'-OH group to a less reactive group is therefore common practice to stabilize the RNA. The 2'-O-methyl (2'-OMe) is one of the more typical substitutions[103]. Similar to backbone modifications, 2'-OMe enhance molecular stability by impeding attacking ribonucleases[104]. The introduction of 2'-OMe additionally reduces immune stimulation by acting as an antagonist against TLR7, reducing the induction of both interferon-α (IFN-α) and interleukin-6 (IL-6)[105]. Studies have also shown that 2'-OMe incorporation mediate increased affinity for mRNA target binding[106], improving pharmacokinetic properties of the oligonucleotides. There is a wide range of other substitutions and it is not uncommon to alternate between *e.g.* 2'-OMe and 2'-deoxy-2'-fluoro (2'-F) to improve the pharmacological properties even further.

Base modification

Base modifications are still in an early stage of research but have been proven beneficial for RNA therapeutics. Exchanging native bases with artificial bases, such as using pseudouridine instead of uridine, can be a potential avenue for bypassing innate immune recognition and stabilize them from degradation by nucleases[107, 108]. For example, through computational modelling and subsequent experimental evaluation, synthetic alterations of the adenosine base of an siRNA revealed effective reduction in tumour necrosis factor alpha (TNFα) as a response of disrupted nucleotide/TLR8 interaction[109], while maintaining functionality. It has also been shown that very precise base modifications can promote insertion of the correct siRNA strand into RISC, providing significant reduction in off-target effects[110]. However, there are safety concerns regarding the unknown metabolic fate of these modifications, and pharmaceutical companies are therefore being careful before such pathways have been clarified[111].

Delivery strategies

Beside chemical modifications that improve stability, immunogenicity, and efficiency of RNAs, reaching the desired tissue and into the cytosol of a cell by endosomal escape, generally requires a dedicated delivery strategy. While there are modified ASOs that have been clinically approved that do not rely on a highly specific delivery strategy but is instead introduced into cells by gymnotic ('naked')

uptake[112-116], short double stranded RNAs such as siRNAs and larger mRNAs are unable to do so[103]. The most accessible organ for siRNA drugs by systemic delivery is currently the liver, and to some extent the kidneys. However, there are currently only siRNA drugs targeting the liver that have been approved by the FDA[112-116]. There are, however, clinical trials for diseases in eye, skin, lungs, and brain, although these organs are primarily accessed by local, topical, intranasal, or cerebrospinal injection[117]. Thus, in the case of systemic delivery, reaching beyond the liver remains a challenge. Numerous strategies have been proposed using a variety of materials that facilitate endocytic uptake, but for efficient targeting to specific organs there is still a lot of work left to be done.

Delivery

Tissue targeting and cell uptake

Delivery strategies can be separated into active and passive tissue targeting[87]. Passive tissue targeting is essentially when the delivery strategy relies on interactions with serum proteins and other trafficking molecules within the systemic circulation, to reach its target tissue. A typical example is lipid nanoparticle (LNP) mediated RNA delivery which "passively" targets the hepatocytes in the liver. LNPs take advantage of the circulating apolipoprotein (Apo), which are lipid-binding proteins that plays an important role in the transport of cholesterol. For LNPs, ApoE is especially adsorbed on the LNP surface, and is subsequently transported for endocytic interaction with low-density lipoprotein receptors (LDLR) that are expressed on the hepatocytes[118].

In the case of active tissue targeting, ligands that target a specific biomolecule that is present on target cell, such as a highly expressed receptor, is conjugated directly onto the RNA itself or is incorporated as a part of a delivery strategy. A prime example is the ligand called *N*-acetylgalactosamine (GalNAc) that significantly improved RNA delivery to the liver. GalNAc binds to the highly expressed receptor on the hepatocytes called asialoglycoprotein receptor (ASGPR) and mediate endocytosis of the RNA payload. Less than a percent of the RNA is believed to subsequently escape the endosomes, but because of the enormous uptake facilitated by the large number of ASGPR, the total release is more than sufficient for complete RNAi mediated knockdown[119]. The ideal scenario would be to find equally high expressed receptors for other tissues and organs, especially where disease is the driving force for receptor upregulation. For instance, tumor cells have the ability of upregulating specific receptors due to a higher demand of nutrients[120]. These receptors could be potential targets for ligand associated delivery of RNA drugs.

However, not all cancers have highly unique receptors to be targeted, and for other diseases we may not be as fortunate as with hepatocytes and their ASGPR. Indeed, it should be recognized that the success of the FDA approved siRNA drugs is in part

due to the liver being a metabolically active organ that serves a central role in protein synthesis, detoxification, and production of necessary biochemicals. Consequently, drugs naturally accumulate in the liver to be metabolized, making the organ highly amenable as a drug target. The high cell uptake of RNA therapeutics will therefore to some extent counteract the effects of another major bottleneck, *i.e.,* having insignificant endosomal escape of RNA. Thus, for other tissues with only moderate uptake, the therapeutic will not make the cut, as most of the RNA payload will be limited by the enclosing endosomes while therapeutically insignificant amounts will reach the cytosol[121]. Unsafe dose levels, with high risk of toxicity would therefore be necessary to have a therapeutic effect[122]. This unbalance must be addressed from within the cell - namely improving endosomal escape of RNA into the cytosol[123].

Endosomal escape and cytosol entry

Following endocytosis (Figure 4), the RNA therapeutic is initially trapped in early endosomes, which are weakly acidic vesicles with a pH of ~6.5. As the early endosomes mature into late endosomes the acidity increases to a pH of ~5.5, thus a higher concentration of hydrogen ions will be present in the vesicle. The late endosomal compartment eventually fuses with the lysosomal compartment and the content within the late endosome is degraded by lysosomal enzymes[124]. There are thus two alternative pathways for RNA therapeutics. Either the RNA is trapped within the endosomal membrane and degraded in the lysosomes, collectively referred to as non-productive uptake. Alternatively, the RNA is able to escape the enclosing endosome and reach the cytosol of the cell to execute its biological effect, referred to as productive uptake or endosomal escape. Often, the fate of the RNA therapeutics ends in the former pathway[121]. Importantly, the RNA that succeeds to escape the endosomal compartment is still only believed to be less than a percent, even with the most efficient delivery strategies[8]. Thus, the potential for improvement is immense. But due to a lack of tools that can accurately detect the release of therapeutic RNA into the cytosol, conceivable bottlenecks cannot be identified.

Intracellular responses to damaged endosomes

In the case of successful endosomal damage and potential endosomal escape by the RNA therapeutics, there are additional intracellular responses that immediately react to the ruptured endosome. A subset of proteins from the family called galectins is known to accumulate on damaged vesicles and target them for autophagy. They reside in the cytosol and immediately respond to β-galactoside-containing glycans within the endosomal membrane that become exposed upon damage (Figure 4)[125]. However, inhibiting autophagy have not been shown to increase cytosolic release of siRNA[126]. In **Paper I** we evaluate four members of the galectin family as a potential marker for endosomal damage. We could conclude that galectin-9 was most rapid and abundantly recruited to endocytic vesicles damaged by transfection lipids. Cells additionally possess mechanisms that repair damaged endosomal

membranes. A relatively recent explored example is the Endosomal Sorting Complex Required for Transport (ESCRT), which have shown to promote repair on injured vesicles[127]. Very recently, so called stress granules have also demonstrated to rapidly accumulate on the site of membrane damage and stabilize it by acting as a plug. These granules are believed to act both in a regime with ESCRT and independently[128].

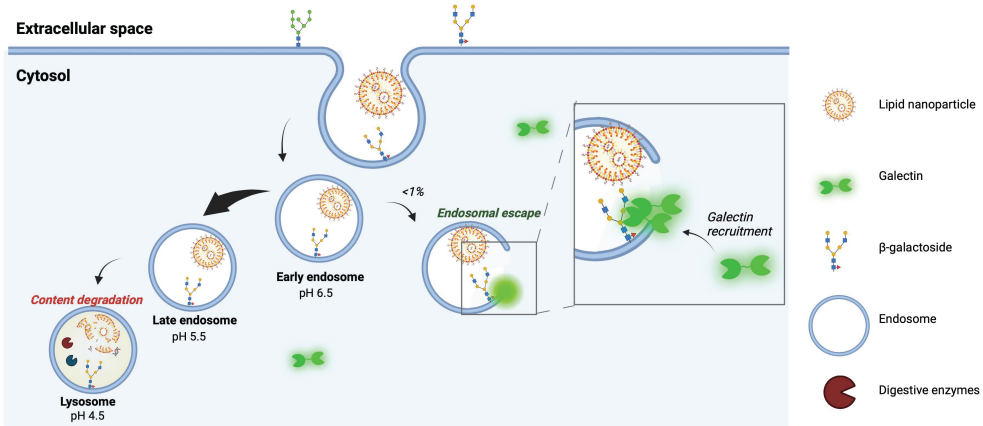

Figure 4. Schematic representation showing the intracellular fate of RNA therapeutics. The RNA therapeutics, exemplified by an LNP, are taken up by cells via endocytosis and initially reside in early endosomes that is weakly acidic. The LNP then has two alternative pathways: it can either remain trapped by the maturing endosome and eventually become degraded in lysosomes by digestive enzymes, referred to as non-productive uptake. Or the LNP will damage the endosomal membrane and release the RNA payload into cytosol to excert its therapeutic effects. The latter alternative is believed to be highly inefficient. In the case of endosomal escape, the β-galactoside containing glycans within the endosomal membrane will become exposed upon damage and cytosolic galectins will rapidly be recruited and target the vesicle for autophagy. Created with BioRender.com.

Lipid nanoparticles

As touched upon briefly, LNPs are a class of macromolecular drug delivery strategies that are exploited for the delivery of the FDA approved siRNA drug called patisiran, for the treatment of TTR mediated amyloidosis, and for the mRNA vaccines against SARS-CoV-2[79, 112]. They are about 50-100 nm in diameter and are typically synthesized using four types of lipids: ionizable lipid, cholesterol, lipid-anchored poly(ethylene glycol) (PEG) constructs, and helper lipids (Figure 5).

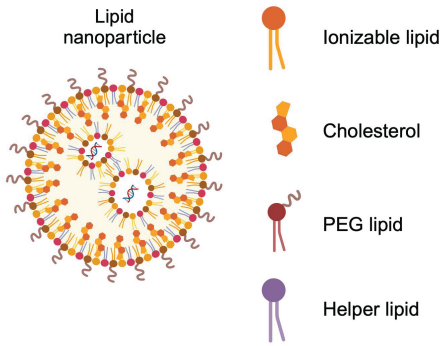

Figure 5. Lipid nanoparticle structure and composition.
The lipid nanoparticle is composed of several lipid layers and microdomains that contain the RNA payload. synthesized using four type of lipids that have different functions. The ionizable lipid neutralizes the anionic charge of the RNA and cause endosomal membrane destabilization to promote endosomal escape of RNA payload. Cholesterol provide rigidity and intergrity to the lipid nanoparticle. The PEG-lipid improve RNA encapsulation and influence the size of the particle. Helper lipids promote stability and encapsulation, and destablization once within an endosome. Created with BioRender.com.

Lipid components

The ionizable lipid is responsible for neutralizing the anionic charge of the RNA, making the RNA-lipid complex uncharged at physiological pH in circulation and facilitate improved cellular uptake[129]. The lipid additionally stabilizes the RNA against nuclease degradation[130]. When endocytosed, the ionizable lipid will become protonated as pH is decreasing during endosomal maturation, and the lipids typically have a pK_a similar to the pH of early endosomes. The protonation is in turn believed to facilitate interaction between the LNP and the negatively charged endosomal membrane and cause membrane destabilization to promote endosomal escape of the RNA payload[126, 131]. In the case of patisiran, they used the ionizable lipid called Dlin-MC3-DMA (MC3), but other ionizable lipids have also been proposed[87].

Cholesterol stabilizes the LNPs by altering the rigidity and integrity of the particle. It has also been shown that delivery efficacy can be affected by using cholesterol analogues. Indeed, Patel *et al.*, showed that the incorporation of the cholesterol analogue C-24 alkyl phytosterol into the LNP could increase intracellular delivery of mRNA[132]. Other alterations, such as using oxidized cholesterol could drive delivery towards other cell types. Here, Kupffer cells and liver endothelial cells were targeted rather than hepatocytes[133].

PEG-lipids are responsible for a number of important key properties in LNPs. The size of the LNP particle is influenced by the amount of PEG-lipid that is used in the synthesis process, with increased amount resulting in smaller particle size[134]. Typically ~1.5 mol% is used[134]. The PEG-lipid balance is crucial for preventing

abundant serum protein opsonization and to minimize reticuloendothelial clearance, which benefit biodistribution of the LNPs[135, 136]. Other factors that are improved by including PEG-lipids is the efficiency of encapsulating RNA and mask the LNP from immune responses[137, 138]. Finally, helper lipids are usually used to promote stability and encapsulation and subsequent destabilization once within the endosomal compartment[139].

Using a microfluidic system, these lipids are combined with the RNA to synthesize the LNP in a controlled manner, balancing the ratios of the lipids to achieve the intended properties of particles[140].

LNP mediated RNA delivery

As mentioned previously, LNPs are delivered via passive tissue targeting, *i.e.,* ApoE adsorbs on the LNP surface and mediate cell uptake by interacting with LDLR on the cell membrane. While LNPs represent the most advanced delivery strategy for therapeutic RNA, it is still unknown how endosomal escape of LNP mediated RNA delivery proceed, and why only a limited fraction of the payload reaches the cytoplasm. The protonation of the ionizable lipid is proposed to be driving the interaction with the negatively charged endosomal membrane, and it is believed to proceed by a phase shift to an hexagonal membrane lipid phase that promote endosomal escape[141, 142]. Indeed, it has been shown that LNPs create a tight interaction with a synthetic membrane when pH is decreased, which could be the initiator behind endosomal escape of RNA[131]. Using fluorescent or gold-labeled siRNA molecules, Gilleron *et al.* visualized MC3-LNP mediated release of siRNA using confocal and electron microscopy. They found that only 1-2% of the endosome containing siRNA escaped and that the damaged compartment was primarily a hybrid between early and late endosomes[143]. Cytosolic release of siRNA delivered by LNPs have also been correlated with recruitment of a member from the galectin family that interacts with β-galactoside-containing glycans within the endosomal membrane[125, 144]. Using a confocal live-cell imaging approach in combination with galectin-8, Wittrup *et al.* again observed very limited siRNA release (3.5%) from LNPs prepared with the ionizable lipid L319, which was mainly restricted to the early endosome compartments[126]. Others have claimed that endosomal escape of siRNA primarily occurs from late endosomes[145].

For LNP mediated delivery of mRNA, one study characterized endocytic compartments for a number of cancer cell lines, investigating luminal pH, morphology and location of endosomal compartments. They subsequently treated these cell lines with MC3-LNPs containing mRNA and found that the cell lines that exhibited fast endosomal maturation and lower vesicular pH had a positive influence on mRNA translation, indicative of improved release for cell lines with a highly active endolysosomal system[146]. Another study using MC3-LNP with mRNA payload recovered approximately 1% of the delivered mRNA from the cytosol, when treated to epithelial cells, suggesting very poor delivery of mRNA payload.

Additionally, they found that mRNA associated to endosomal compartments could be re-packaged into extracellular vesicles (EVs) that were functional both *in vitro* and *in vivo*[147]. One study further investigated the subcellular fate of LNP delivered mRNA by super-resolution microscopy, using a number of different ionizable lipids[148]. They concluded that the limited delivery of mRNA is due to impaired endosomal acidification because of prolonged uptake. This causes the mRNA to become trapped in unproductive defect endosomal compartments. Furthermore, they suggest early endocytic/recycling compartments to be the main escape route for mRNA from statistical correlations.

In **Paper III**, we address multiple mechanistically different barriers of MC3-LNP mediated delivery of siRNA and mRNA and investigate the sorting, integrity, and endosomal escape by single vesicle analysis. Using live cell imaging, we found that only a subset of internalized LNPs carrying siRNA/mRNA triggered endosomal membrane damage marked by galectin recruitment, while the vast majority remained unaffected by such damages. Additionally, some of these damages where in endosomes containing no RNA payload. Knocking down components of the ESCRT furthermore triggered more damages associated with galectin-9, indicative of a potential route for improved LNP RNA delivery. Using super-resolution microscopy we could also visualize disintegrated LNPs within the endosomal lumen and LNP remnants localized in close proximity to membrane damage marker accumulations.

Bioconjugates

As touched upon earlier, another means of delivering RNA to recipient cells is by conjugating molecules that act as delivery vehicles. These include polymers, peptides, antibodies, aptamers, small molecules, or other endogenous ligands[149]. For the relevance of this thesis, cholesterol conjugation will mainly be discussed.

An advantage with bioconjugates is that they consists of a single molecule component, meaning that the balance between an siRNA molecule and the bioconjugate will be a fixed molar ratio (usually 1:1). Compared to LNPs which comprise of multiple lipids that are carefully combined with the siRNA using a microfluidics system, bioconjugation synthesis is thus relatively simple. Their biodistribution profiles are also believed to be favourable, reaching tissues beyond fenestrated or discontinuous endothelia[150].

The first bioconjugate that showed functional knockdown *in vivo* was a cholesterol that was conjugated to an siRNA sequence targeting ApoB. After systemic administration of the cholesterol-siRNA (chol-siRNA) in mice the plasma levels of ApoB was significantly reduced, indicating successful downregulation[151]. Recently, a chol-siRNA was also able to knockdown the gene myostatin in murine skeletal muscle, which has previously been a difficult organ to reach[152]. As 15-30% of the

cellular membrane consists of cholesterol, the cholesterol conjugation spontaneously intercalate into the membrane upon interaction with the cells, as a supporting component in the membrane structure[153, 154]. The chol-siRNA is subsequently internalized by endocytosis. Alternatively, it is internalized by interactions with lipoprotein receptors[155]. The uptake is generally rapid, and within seconds, the chol-siRNA can be readily detected in early endosomes in a number of cell types[156]. However, despite effective uptake, high doses are generally required to produce a meaningful knockdown, indicative of poor endosomal escape of siRNA. Small molecule drugs with membrane destabilizing properties have therefore been an interesting avenue to improve endosomal release[157], but it has not been clear to what degree these compounds can improve endosomal escape of siRNA. Of these small-molecule drugs, chloroquine has been the prototypical molecule. It diffuses through the cell membrane and into the endosomes where it becomes protonated by the endosomal maturation. Chloroquine is then thought to act as a hydrophobic agent in the lipid bilayer and at ta critical concentration it lyse the endosome[158]. In **Paper I** we take advantage of galectin-9, utilizing it as a marker for lipid membrane damage to visualize endosomal release of chol-siRNA, triggered by membrane destabilizing compounds such as chloroquine. We could conclude that some certain small-molecule compounds, especially chloroquine, significantly improved endosomal escape of chol-siRNA, while also damaging endosomal compartments that where empty.

Another highly relevant bioconjugate is the previously discussed GalNAc ligand which binds to the ASGPR on hepatocytes. At any one time approximately 100,000 ASGPR are present of the cell surface of hepatocytes. Upon GalNAc binding on diffuse monomeric ASGPR receptors, rapid re-localization of other ligand bound receptors are recruited to form aggregates that results in clathrin coated pit formation and subsequent endocytosis. As the pH drops within the endosomal structure, the GalNAc is released from the ASGPR and the receptor is recycled back to the cell membrane. Subsequently the linker between GalNAc and the conjugated siRNA is degraded and less than 1% of the siRNA is subsequently believed to escape from the endosome and reach the cytosol[123].

In summary, RNA therapeutics face a wide range of barriers to have a therapeutic effect. Chemical advancements in the prevention of nuclease degradation, immunological responses and renal clearance have improved the field significantly, but delivery to extrahepatic tissues and into the cell cytosol remains a challenge. LNPs are so far the most optimized delivery strategy. However, it is likely that bioconjugates, such as aptamers or ligand conjugates, will surpass LNPs eventually. They are a promising class of delivery vehicles with efficient routes of internalization that have the capability to in theory address any organ, knowing what receptor to target. But, to an even higher degree than LNPs, these delivery strategies also suffer from poor endosomal escape of RNA[159]. There is additionally a gap of knowledge on how the RNA payload escapes the endosome, and clarification of this

mechanism will likely enhance future bioconjugation delivery systems. The methods in **Paper I** and **Paper III** could be potential avenues to address these challenges.

Dose-response of RNA therapeutics

In broad terms, the dose-response relationship is defined as the amount of exposure of a given substance and the subsequent effect on an organism. In pharmacology the substance can be translated into the amount of a particular drug used while the resulting effect can be a measurement from various biological responses. Typically, such responses are biological effect, toxicity, or lethality caused by the given drug dose. The biological effect could for example be the downregulatory response of a drug acting as an antagonist towards a specific enzyme or receptor, as the inhibitory interaction by nonsteroidal anti-inflammatory drugs (NSAIDs) with the cyclooxygenase enzyme (COX) that results in pain relief. In this case, pain relief would be the response which would be translatable to the given dose NSAID, with increased dose typically resulting in increased pain relief[160]. Identifying the response of multiple doses can in turn generate a dose-response model. Such models are crucial for *e.g.,* determining safe and effective doses while excluding inefficient or saturated doses that can be potentially harmful. A typical measurement is the half-maximal inhibitory concentration (IC_{50}), which represent the concentration of a substance needed to inhibit a particular biological response, or enzyme activity by half. Low IC_{50} values would thus indicate a highly potent drug and vice versa (Figure 6).

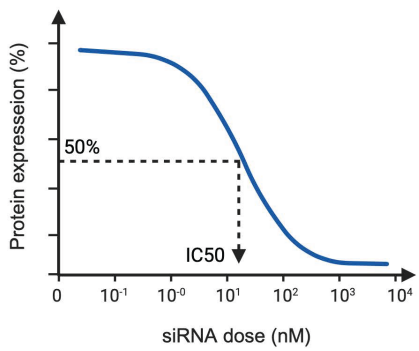

Figure 6. The dose-response relationship.
A dose-response curve showing the response in protein expression by different doses of siRNA. Low doses are insufficient to generate a response, while higher doses generate partial and maximal response (or knockdown). The dashed line indicate the IC_{50} for the used siRNA sequence. Created with BioRender.com.

Thus, for RNA therapeutics (as for any therapeutic), dose-response relationships can provide a lot of useful information, and there is a wide range of methods that can be applicable for the establishment of such relationships. Functional studies that address the knockdown effect of a particular siRNA sequence can be quantified by measuring the mRNA content by RT-PCR or the expressed protein by flow cytometry or western blot[126, 160]. Such studies are crucial for drug development to determine the biological effect of an RNA therapeutic, and combines all the aspects of the molecule's efficacy, *i.e.,* target delivery, uptake, and knockdown effect. Given the simplicity of such assays, high-throughput assays can also be used which is extremely valuable when the interest is to screen a vast number of drug candidates.

While informative, typical activity assays will not capture other critical information that would be desirable for evaluating an RNA therapeutic. In the case of a certain sequence or compound having weak activity for instance, the plausible reasons are plentiful. It could be because of insufficient cell uptake, poor endosomal escape, trafficking to the lysosome, off-target interactions and other non-specific interactions, low target affinity etc. . Thus, other methods for addressing these concerns are required.

There are several methods that are typically used for measuring cellular uptake[161]. Commonly, they take advantage of fluorescently labeling the RNA therapeutic. Cells are treated *in vitro* or tissue from *in vivo* experiments are harvested. Typically, cells are then analyzed by flow cytometry of fluorescence microscopy to measure the amount of fluorescent RNA that is detected within the cell. Obviously, resolution will be better with microscopy and will be able to distinguish if fluorescent signal is within or outside of the cell and determine the RNAs subcellular localization. But even with advanced confocal microscopy it will be difficult to distinguish between endosomal or cytosol localized fluorescent RNA, especially given the fact that only a small fraction of the RNA is released into the cytosol[126]. Thus, such methods are useful for total cellular uptake of RNA therapeutics but will not be suitable for measuring cytosol released RNA or productive uptake. The variability and inefficiency of cellular internalization between various tissues complicate the correlation between siRNA uptake and biological effect, therefore such association will not be able to distinguish between delivery efficiency and potency of a particular siRNA sequence. For instance, high cellular uptake and poor biological effect could either be due to limited delivery or an inefficient siRNA sequence.

Thus, being able to quantify successful cytosol delivered siRNA would be of great value for assessing delivery efficiencies and identify potential improvements for delivery strategies. Indeed, establishing the intracellular dose-response of RNA therapeutics would be able to address such concerns. Ideally, label-free high-throughput methods would be of great utility to evaluate the intrinsic properties of various siRNA sequences and conjugation strategies[121]. A number of methods for quantifying cytosol-delivered siRNA has been proposed, but they suffer from various limitations. One study suggested a quantitative strategy that measured

fluorescent signal from siRNA that became fluorescent when interacting with proteins expressed in the cytosol[162, 163]. However, from a quantitative standpoint, such enzymatic reactions are difficult to interpret given the unclear kinetics and efficiency to convert the siRNA into a fluorescent state. Other studies delivering ASOs or proteins have relied on fluorescence correlation spectroscopy (FCS) and mass-spectrometry-based nanoSIMS for quantifying cytosolic delivery[164-166]. Such techniques are quantitative and highly sensitive which is important for detecting low concentrations. But they are also limited by low throughput, relying on subjective region-of-interest selection within the cytosol. Finally, microinjection experiments have also been devised, injecting doses of siRNA into live cells. However, while an exciting experimental approach, the dose-response relationship has shown highly contradictory results, with IC_{50} values ranging from a dozen to several hundred siRNA molecules[167, 168]. In **Paper II** and **Paper IV** we investigated the dose-response relationship of cytosol delivered siRNA and the knockdown of a target gene, using a fluorescence confocal live-cell imaging-based method and an RT-qPCR based method, respectively for the two papers. Using the same siRNA sequence with clinically relevant potency and same fluorescently labeled target protein, we could conclude comparable intracellular IC_{50} values using two technically very different methods.

Present investigation

Aim

The aim of this thesis was to gain a better understanding of lipid-mediated delivery of RNA by developing methods to detect and quantify endosomal release and cytosolic delivery of siRNA. In the end, the hope is that such techniques will support the development of more effective delivery strategies to improve the therapeutic potential of RNA-based drugs. The four papers within this thesis address multiple methods to investigate the functionality of lipid mediated RNA delivery strategies, to identify bottlenecks and probe potential avenues for improvement. The specific aims of the four papers (**I-IV**) are:

I. To investigate the effects on endosomal release of cholesterol conjugated siRNA by membrane destabilizing drugs.

II. To elucidate the dose-response relationship between cytosol delivered siRNA and knockdown of a reporter gene using a confocal-based imaging method.

III. To investigate and characterize the endosomal escape mechanism behind lipid nanoparticle delivered RNA.

IV. To elucidate the dose-response relationship between cytosol delivered siRNA and knockdown of a reporter gene using a RT-qPCR-based method.

Methods

The main methods used in this thesis are described below. For further details, please see the Methods section in **Paper I-IV**.

Throughout **Paper I-IV**, the cervical cancer cell line HeLa was used. In **Paper I** the breast cancer cell line MCF-7 was also used in some experiments. Cells were cultured in DMEM with high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin at 37 °C and 5% CO_2.

All experiments conducted have been *in vitro,* and for the most part cell culture models were grown as 2D monolayers, but 3D spheroids were also formed for penetration assays.

Fluorescent compounds

In this thesis, fluorescent compounds have served as the main analytical component and have been thoroughly used in all studies. Fluorescent compounds are fluorophores or fluorophore-bound substances that upon excitation with a certain wavelength of light emits light with a longer wavelength that can be detected by a detector. There is a wide range of fluorophores that emit light of different wavelengths and multiple fluorophores can therefore be used in an experiment to study multiple fluorescent compounds simultaneously. The studies outlined in this thesis primarily used widefield fluorescence microscopy that excites the samples with non-coherent light (here LED), or confocal laser scanning microscopy (CLSM) that rapidly scans the samples with a coherent laser beam. Using either platform, labeled compounds can be visualized and localized in the imaged cells for subsequent analysis. Below are some of the main compounds that were used in the studies.

RNA – Payload to be quantified

In almost all studies in this thesis, the RNA has been labeled with a fluorophore. This provides the possibility to study the uptake and distribution of RNA within different compartments of the cell. It also allows for visualization of dynamic cellular processes, offering real-time insight into the intracellular fate of RNA. Most importantly, the fluorescent signal can be quantified, enabling a precise assessment of endosomal escape and cytosolic release of RNA.

eGFP – Target in knockdown assays

For knockdown experiments, a monoclonal HeLa cell line stably expressing a destabilized enhanced green fluorescent protein (eGFP) was used to study knockdown kinetics following siRNA treatment. The destabilized property (conjugation of a proteasomal targeting sequence to the protein) decreases the half-life of the protein and in **Paper II** the half-life was confirmed to be ~1 h for the established HeLa cell line, which makes it useful for rapid knockdown readouts that is desired for live-cell imaging studies.

Galectins – Markers of endosomal damage

For studies involving endosomal release, a monoclonal HeLa cell line stably expressing a yellow fluorescent protein (YFP) labeled galectin-9 protein was primarily used to study damages vesicles. As previously discussed, a subset of proteins from the family of galectins interacts with β-galactoside-containing glycans

within the endosomal membrane that becomes exposed upon endosomal damage. Fluorescently labeled galectins can thus be used as a marker of endosomal membrane damage. In **Paper I**, galectin-9 was determined to be the most abundant and rapidly recruited marker to damaged endosomes when investigating over expression of galectin-1, -3, -8, and -9.

Compartment markers – Identifying the compartment of release

For endosomal compartment studies, a range of compartment markers were used. At different stages of endosomal maturation, distinct proteins accumulate on the endosomes and can be used to probe compartment characterization. In the most relevant studies performed, early endosome 1 (EEA1) and Ras-related in brain 5 (Rab5) was used as early endosome markers, Rab7 and cluster of differentiation 63 (CD63) were used as a late endosomal marker, and lysosomal associated membrane protein 1 (LAMP1) was used as a lysosomal marker.

The study of endosomal release using widefield imaging

Image acquisition

In **Paper I** and **Paper III**, endosomal release was studied using live-cell imaging with widefield fluorescence microscopy (Figure 7). HeLa cells stably expressing a fluorescently labeled galectin-9 was primarily used to probe endosomal damage, but other transiently expressed markers were also used to some extent. To investigate compartment of release, plasmids encoding for the specific compartment marker were electroporated prior to image acquisition. To capture the fast movement of intracellular vesicles and the sudden moment of endosomal damage marked by fluorescently labeled galectin recruitment, high-temporal resolution imaging was used. As vesicles can be present in virtually the entire spatial extent of the cytoplasm of the cell and move in all directions, *z*-stacks encompassing a large part of the cell was captured. Typically, each frame consisted of 20-30 planes acquired as a *z*-stack with 300-500-nm intervals. Each frame was captured at a rate of one *z*-stack every few seconds (generally 2-3 s)over an acquisition period of ~10-20 min. The intensity of the LED lights was kept at a minimum and constant between the experiments.

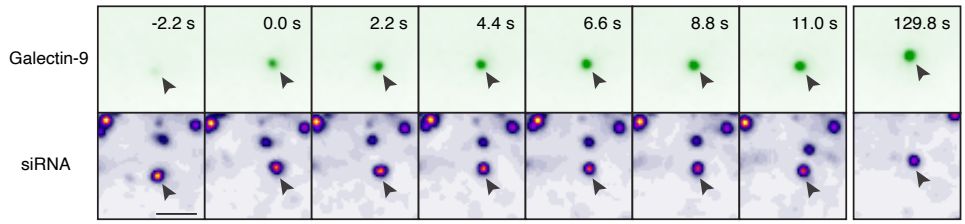

Figure 7. Galectin-9 recruitment to damaged siRNA-LNP containing vesicle using live-cell imaging.
HeLa cells stably expressing YFP-galectin-9 were incubated with fluorescently labeled siRNA-LNPs during live-cell imaging with widefield fluorescence microscopy. Images were acquired every 2.2 s for 10 min. Image sequence shows *de novo* recruitment of galectin-9 to a siRNA-LNP containing vesicle, indicated by arrowheads. Time = 0 s indicate first frame with detectable galectin recruitment. Scale bar is 2 µm.

For high-temporal resolution imaging, mechanical filters cannot be used due to their limited speed of movement. A beam splitter system was therefore used for studies were the fluorophores used could cause cross-excitation, *i.e.,* when the excitation and emission spectra of two or more fluorophores overlap, causing unintended fluorophore excitation resulting in image and quantification artifacts. In brief, the beam splitter separates low, medium, and high wavelengths of light to defined regions of the camera detector, making it possible to obtain fluorescent signal devoid of so-called cross-excitation and bleed-through artifacts at a cost of a reduced field of view (FOV). Typically, the beam splitter was used for compartment studies when three fluorescent compounds were used: galectin, compartment marker, and RNA.

For long-term acquisition, *e.g,* the study of LNP internalization and galectin-9 response in **Paper III**, similar acquisition settings were used but with a considerably lower frame-rate.

Image processing and analysis

After image acquisition, the acquired image sequence was processed for subsequent analysis and quantification of single endosomal release, which involved the use of a custom-made processing and analysis application (referred to as App) created in Matlab. For full documentation on the procedure, please see the "code availability" section in the online version of **Paper I**. In brief, each image *z*-stack in the resulting image sequence was deconvoluted to enhance the resolution and contrast of each frame and remove unwanted blur and out-of-focus light caused by the widefield microscope. Deconvolution is a computational process that uses a point spread function (PSF) which is a mathematical function of how a point source of light of a given wavelength spreads in the optical axis of the microscope. Thus, for each fluorophore used in the image, the computational process uses the captured point spread in the image *z*-stack and the PSF for the given fluorophore to iteratively

reduce blur and obtain a high-resolution image by calculating the point of origin of the detected light in the image.

To investigate damaged endosomes, maximum intensity projections (MIP) of the deconvolved *z*-stacks was first generated, which essentially creates a 2-dimensional image of the 3-dimensional *z*-stack. Each cell was then manually investigated for endosomal perturbations and *de novo* recruitment of fluorescent galectin was flagged in Fiji (an open source image processing software). Using the App, region-of-interest crops (ROIs) were generated for each flagged vesicle damage for inspection and single vesicle tracking in accordance with the purpose of the given study, *e.g.,* endosomal release kinetics or compartment of release. To probe endosomal escape, vesicles containing *e.g.,* RNA payload was first traced from the timepoint of appearance and traced for as many frames as possible. In brief, the App then identifies the t_0 and the x, y, and z position for the appearing galectin foci, and an object mask is fitted to the object in the plane of maximum intensity within the volume for subsequent quantification of fluorescent signal from the compound of interest.

The study of single-cell dose-response using confocal imaging

Image acquisition

In **Paper II**, single-cell dose-response of cytosolic siRNA delivery was studied using live-cell imaging with a CLSM 710 with an Airyscan detector unit (Zeiss). One of the main advantages with confocal imaging is its ability to produce high-contrast images of the focal plane while removing out-of-focus light. For the purpose of measuring fluorescent signal within a cell, as in this study, such technical traits are favourable to perform accurate and comparable fluorescent quantifications. Additionally, to capture weak signal from the limited release of fluorescently labeled siRNA, we took advantage of a GaAsP (gallium arsenide phosphide) array-confocal detector called Airyscan, which is normally used for super resolution imaging. This detector has a higher dynamic range than conventional detectors which means that it is able to capture very weak fluorescent signals despite the presence of very strong signals in the other parts of the frame. In this study it was used a conventional detector (non-super resolution), maximizing the FOV beyond recommended settings. This caused notable vignetting in the image with cells in the periphery appearing less fluorescent. This issue was corrected using a post-acquisition processing script in which a *z*-stack of fluorescent siRNA capturing the phenomena was used as a reference sample.

HeLa cells stably expressing eGFP was used for all dose-response quantifications. Upon image acquisition, cells were treated with lipoplexes of fluorescent siRNA that were formed with transfection lipids. These lipoplexes are usually 1 µm or larger and are easily distinguished as fluorescent aggregates in the sample. Upon

interaction with the cell membrane, these lipoplexes are endocytosed. At some point, the lipids subsequently interacts with the endosomal membrane and deliver a rapid bolus-like release of the fluorescent siRNA to the cytosol (Figure 8), filling the cytosol with a homogenous fluorescent signal emitting from the labeled siRNA[126]. To capture this sudden release of fluorescent siRNA, two *z*-plane images were acquired at 5 min intervals. One of the *z*-planes were acquired in the lower third of the cell to capture the largest area of the cell to maximize quantification of cytosolic siRNA fluorescence. The second *z*-plane was captured 4 µm above to mask hazy fluorescence from potential lipoplexes outside of the focal plane. A nuclear stain was additionally used to facilitate tracking of the cells and to quantify the knockdown of the eGFP protein. Typically, 4-6 positions of sparse and evenly distributed cells were selected per acquisition, with one position being untreated cells. Images were acquired for 12-32 h.

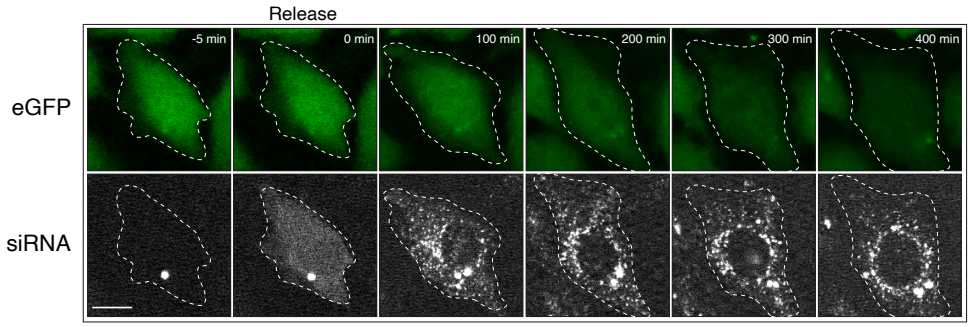

Figure 8. Cytosolic delivery of siRNA in eGFP expressing cell using confocal live-cell imaging. HeLa cells stably expressing eGFP were incubated with lipoplexes of fluorescently labeled siRNA during live-cell imaging with confocal microscopy. Images were acquired every 5 min for 20 h. Image sequence shows sudden endosomal release of siRNA filling the cytosol of the cell at t = 0 min and subsequent knockdown of the eGFP protein. Outline indicates cell boundary. Scale bar is 20 µm.

Image processing and analysis

After time-lapse image acquisition, a custom-made analysis pipeline was used for image processing and analysis. The pipeline consists of a denoise-step in Fiji, tracking and fluorescent quantification of single cells in Cellprofiler, and further processing and analysis in a custom-made application created in Matlab. For full documentation of the pipeline, please see the "code availability" section in the online version of **Paper II**. Below is a very brief summary of the main steps in the pipeline.

CellProfiler is an open source software for cell image analysis and were used to trace each cell and quantify the fluorescent signal in the lower *z*-plane. In brief, cells were traced using the nuclear signal as an object for tracking. The nuclear signal was also used as a mask to measure the eGFP fluorescence, providing a stable region to measure throughout the acquisition. The eGFP fluorescence was used to segment the cells, *i.e,* to obtain the cell boundary for subsequent cytosolic siRNA

fluorescence quantifications. To limit the siRNA fluorescence measurements to the cytosolic region, the cell segments were further masked from bright lipoplexes and the nucleus.

After single-cell quantification, automated detection of siRNA release events were performed by identifying sudden signal fluctuations in the fluorescent siRNA measurements. To discard false positives, a manual control step was performed. Using the custom-made application, identified release events were collated into individual ROIs and each detected release event was classified. The event could either be classified as a true positive or a false positive event, or the event was disqualified if a previous undetected event was suspected. After all events were classified, the magnitude of the siRNA signal was translated into siRNA concentration, using a 1000 nM siRNA reference measurement acquired before each experiment with time-lapse image acquisition.

The study of intracellular dose-response using RT-qPCR

In **Paper IV**, the dose-response relationship between cytosolic siRNA and target gene was studied using RT-qPCR. RT-qPCR is a very sensitive method and low amounts of target RNA can be quantified with high accuracy. The method is, however, restricted to quantifying RNA molecules longer than the PCR primers used, usually limiting the target RNAs to be no longer than 30-40 nucleotides. Therefore, siRNAs cannot be analysed with conventional primers. A key step in the method presented in **Paper IV** is adapted from a previous study that designed a two-tailed RT primer to synthesize complementary DNA (cDNA) of specific miRNA targets[169].

The two-tailed RT-primer is a ~50 nucleotides long RNA sequence that is folded into a hairpin structure. The hairpin structure has two hemiprobes at each end. The 3'- and 5'-hemiprobes consists of a few nucleotides that are designed to target the respective ends of the siRNA antisense strand, while the hairpin structure will impede other nonspecific interactions. When the hairpin structure and the siRNA antisense strand forms a complex, reverse transcriptase enzymes can initiate elongation to produce a cDNA product. The cDNA can then be quantified and analysed by RT-qPCR using two target specific primers against the cDNA sequence in combination with conventional SYBR-Green chemistry.

To deliver siRNA to recipient cells, electroporation mediated delivery was performed using a Neon Transfection System. This procedure bypass the inefficient step of endosomal escape and provides controlled dosing of siRNA to the cytosol. To block residual siRNA amounts from binding to the cell surface, cells were washed with PBS supplemented with dextran sulfate and salmon sperm DNA. Cells were then lysed and RNA content was purified for subsequent cDNA synthesis using the designed two-tailed RT primer targeting the siRNA sequence. Synthesized

cDNA product was analysed with a RT-qPCR system using specific primers to amplify the cDNA product.

Results

Paper I – Imaging small molecule-induced endosomal escape of siRNA

Introduction – Membrane destabilizing compounds could be a potential avenue to enhance release of entrapped siRNA within endosomal compartments. Three small-molecule drugs: chloroquine, siramesine and amitriptyline with membrane-destabilizing properties were thus investigated for enhanced delivery of ligand-conjugated siRNA. To probe endosomal escape, a novel live-cell imaging method was developed using cells overexpressing a fluorescently labeled membrane damage sensor (galectins).

Results – Galectin-9 was most abundant and rapid for recruitment of membrane damage after evaluating galectin-1, -3, -8, and -9 as potential membrane damage sensors, when investigated by fluorescent confocal imaging. Thus, galectin-9 was subsequently used as membrane damage sensor for live-cell imaging experiments. Chloroquine, siramesine, and amitriptyline enhanced chol-siGFP knockdown and the effect was most pronounced >24 h treatment. Chloroquine was most effective, lowering the chol-siGFP half-maximal inhibitory concentration (IC_{50}) ~17-fold, while inducing similar number of damages as siramesine (~4-fold improved IC_{50}). Similar chol-siGFP knockdown was achieved independent if amitriptyline, siramesine, or chloroquine was started 6-h before, 6-h after, or at the same time as chol-siGFP treatment, indicative of a broad window for improving target knockdown. Treatments on cells with internalized dextran further showed a higher degree of release when treated with chloroquine compared to siramesine, despite similar number of damages. When investigating dextran release from single vesicles, chloroquine damaged vesicles with overall higher dextran of dextran-containing vesicles, compared to siramesine. Investigating single-vesicle release of chol-siRNA showed efficient release in both siramesine and choloquine, but chloroquine generally targeted siRNA containing vesicles to a larger extent. Mapping damaged endosomal compartments revealed a broad range of endosomal compartments targeted by both chloroquine and siramesine, with 30-45% being late endosomal compartments. Taken together, the multiple endolysosomal compartments targeted by the small molecule drugs can explain the mis-match between the damaged compartment and the siRNA containing compartments (low hit-rate) for in particular siramesine and differences between cholorquine and siramesine knockdown enhancement. Small molecule induced release of chol-siGFP was further shown to be dependent on cell-type, when investigating their

effects in MCF7 cells compared to HeLa cells. Finally, knockdown experiments were performed in tumor cell spheroids after treatment with the three compounds, with chloroquine having the most pronounced effect and being most effective throughout the spheroid.

Paper II – Single-cell quantification and dose-response of cytosolic delivery

Introduction – Because of the lack of suitable methods to quantify cytosolic amounts of delivered siRNA, the dose-response relationship between cytosol delivered siRNA and knockdown of the target gene have been difficult to establish. Thus, current evaluation of potential delivery strategies has generally relied on techniques that cannot discriminate between delivery efficiency by the delivery strategy and siRNA sequence potency, making it difficult to identify conceivable improvements. The establishment of a method that can quantify cytosol amounts of siRNA and corresponding knockdown of a protein would therefore be of great value. Here, we developed a live-cell imaging-based strategy using confocal microscopy to quantify single-cell dose-response of cytosolic siRNA and subsequent knockdown of a destabilized eGFP protein.

Results – Cytosolic delivery of sub-nanomolar doses of siRNA delivered with lipofectamine 2000 were validated to be detectable with an Airyscan confocal imaging system and quantified with a custom-made pipeline for single cell analysis. Automated detection of sudden signal increases, indicative of cytosol siRNA release, were evaluated in terms of sensitivity and specificity, using galectin-9 co-localization with siRNA-lipoplexes as a reference, and estimated to be 95% and 97% respectively, when combined with a manual quality control step. To acquire reliable absolute siRNA release amounts and quantitation confidence, a mathematical model was applied that fits measured release events based on kinetics of the changes in the measured cytosolic siRNA signal after release. Investigating various siRNA-to-lipid ratios with two different siGFP sequences revealed similar release amounts between the sequences but the release magnitude was highly dependent on the ratio between siRNA and lipid. Time of release was relatively independent and adding more lipoplexes generally resulted in more release events occurring more rapidly. Typically, quantified release events of 1 nM or more were above a model fit of $R^2 = 0.75$ for both siGFP sequences, indicative of low estimated measurement uncertainties meaning data from individual cells could be used for downstream analysis. Collating highly reliable data from multiple experiments with varying doses revealed a clear dose-response relationship for both sequences. The cytosolic siRNA dose clearly affected knockdown induction kinetics, knockdown depth (nadir), and knockdown duration. At the time point when knockdown had reached nadir, the absolute IC_{50} for cytosolic siRNA was estimated to 0.31 nM (~1000 siRNA molecules) and 2.29 nM (~7000 siRNA molecules) for the potent

and the less potent siGFP sequence, respectively. Estimated IC_{50} was further highly analogous to a more advanced mathematical modelling based estimation of IC_{50} based on all time points, indicating the robustness of the experimentally derived IC_{50} values.

Paper III – Live-cell imaging of cytosolic RNA delivery with lipid nanoparticles reveals cellular and biophysical barriers

Introduction – LNPs is the clinically most advanced delivery platform for RNA therapeutics. However, while several RNA-LNP therapies are currently under clinical development and evaluation, limited capacity to facilitate cytosolic delivery of RNA payload restrict the potency of LNP based therapies, especially for extrahepatic tissues and tumors. This is, in part, due to the unclear mechanism behind the endosomal escape of LNP-delivered payload and a lack of understanding of why only a small fraction of RNA reach the cell cytoplasm. Thus, knowledge about the sorting, integrity and endosomal escape of LNPs carrying siRNA and mRNA could potentially clarify current bottlenecks and would be of great value for improving delivery of future LNP-based therapies.

Results – MC3-LNPs were initially investigated for induction of membrane damages. Using live-cell imaging with widefield microscopy, both MC3-LNPs carrying siRNA and mRNA triggered rapid cellular uptake and dose-dependent galectin-9, indicative of galectin-9^+ endosomal damages being a potential route to functional cytosolic delivery of LNP-formulated RNAs. Investigating single LNP damaged vesicles identified by galectin-9 with fast live-microscopy (method adapted from **Paper I**), revealed similar RNA release kinetics between siRNA- and mRNA-LNPs, with a higher release magnitude when individual release events were sub-grouped between productive and unproductive events. Release kinetics were dose-dependent for siRNA-LNPs, with lower doses having a larger siRNA release fraction. Differences in the degree of RNA payload content in the damaged vesicles were also observed between siRNA-LNPs (70% of damaged vesicles containing siRNA) and mRNA-LNPs (20%). These differences were clarified to be independent of specific experimental conditions and probably caused by segregation of the ionizable lipids and the RNA payload. Probing RNA release of endosomal damages recruiting the ESCRT machinery, showed that some vesicles recruit CHMP2A (ESCRT component) in absence of galectin-9, but no endosomal release of siRNA payload could be observed. However, downregulating components of ESCRT resulted in an increased number of galectin-9 foci forming, indicative of the plausible importance of ESCRT for vesicle integrity. Looking at a number of different endosomal markers in parallel with galectin-9 recruitment demonstrated that MC3-LNPs primarily damage $Rab5^+EEA1^{+/-}$early endosomes for both mRNA and siRNA payloads. Using super-resolution microscopy, the integrity of the RNA-LNPs were further visualized and disintegration of the particle could be appreciated

during endosomal maturation for both siRNA and mRNA, *i.e.,* being confined (as in intact particles) in $EEA1^+$ structures and gradually more disintegrated in later endosomal structures. Finally, assessing the sub-endosomal siRNA localization with CHMP2A or galectin-9 recruitment on fixed cells revealed an extremely close association between RNA payload (semi-intact particles) and the membrane damage sensor, suggesting nano-domain colocalization of ionizable lipid at the site of membrane damage on the endosomal membrane.

Paper IV – Intracellular dose-response determination of siRNA by two-tailed RT-PCR of electroporated cells

Introduction – Despite the rapid advancement of nucleic acid therapeutics entering clinical use, there is a high demand on delivery strategies that can deliver its payload to tissues beyond the liver. Thus, novel delivery platforms for other tissues are actively being proposed to broaden the range of treatable tissues. But, as described above, effective tools to determine the intracellular dose-response of an RNA compound is currently lacking and its thereby difficult to determine the efficiency of current delivery techniques and to estimate the amounts of cytosol delivered siRNA that is required for a desired pharmacological effect. Previous presented methods for such elucidation have either suffered from various limitation or been extremely demanding in terms of equipment, workload, and analysis (**Paper II**). Therefore, a widely applicable method for determining the dose-response of cytosolic siRNA would be of great value. Here, we have developed an RT-qPCR-based strategy using a previously described two-tailed RT primer (referred to as probe) designed to detect a specific siRNA for subsequent qPCR amplification and determination of the number of siRNA copies per cell after electroporation-mediated delivery. The biological outcome after electroporation could then be compared to the number of cytosolic molecules for dose-response determination.

Results – The probe was initially evaluated for specificity towards the target siRNA and was found to be highly specific, showing a strong positive readout using RT-qPCR (lower Ct values than control sample without target template). The Ct readout was unaffected when introducing non-target, demonstrating satisfactory specificity for the target siRNA. When investigating if electroporation mediated delivery could be used for specific delivery of siRNA to the cytosol, we found that this was indeed possible, with no obvious non-cytosolic signal. Fluorescently tagged siRNA could be detected within single cells when imaged with confocal microscopy, sharing similar characteristics as bolus-like releases of siRNA delivered to the cytosol described in **Paper II**. Electroporating various doses of siRNA into HeLa cells expressing the siRNA targeted eGFP protein, revealed dose-dependent knockdown when quantified by flow cytometry, demonstrating the possibility to establish cytosol specific IC_{50} for a particular siRNA sequence. Thus, HeLa cells were electroporated with siRNA doses within the dose-response interval and

subsequently quantified with RT-qPCR utilizing the probe, to elucidate the number of copies per cell. Using a reference curve, it could be determined that ~3×10^4 cytosolic siRNA copies resulted in close to maximal knockdown, while ~3×10^2 – 3×10^4 resulted in dose-dependent knockdown and cells with less cytosolic copies were virtually unaffected. With the measured concentrations the intracellular IC_{50} of the siRNA was estimated to be ~2800 cytosolic siRNA copies.

Conclusions and discussion

In this thesis, lipid mediated delivery of siRNA, but also mRNA, has been studied with the purpose of identifying novel avenues to detect and improve endosomal escape of RNA to the cytosol. Below, conclusions and implications of these efforts are briefly summarized.

Imaging small molecule-induced endosomal escape of siRNA

In **Paper I**, a novel method is presented to gain a better understanding of the endosomal escape bottleneck – one of the main limiting steps for delivery of RNA therapeutics. Using live-cell imaging in combination with the highly sensitive membrane damage sensor galectin-9, which is also identified in the paper as a marker for rapid recruitment to damaged endosomes, fundamental properties of endosomal escape can be probed. With the established method, drug-induced endosomal escape of chol-siRNA is assessed, using small molecule drugs with membrane destabilizing properties. Previous studies have investigated the use of such drugs and concluded improved knockdown of both chol-siRNA and LNP-siRNA delivery, but without being able to dissect the role of these small molecules[157, 170], *e.g.,* do they facilitate improved uptake or is endosomal escape improved? Visualizing endosomal escape allows for such investigations, and in **Paper I** the role of cholorquine, siramesine, (and amitriptyline to some extent) in chol-siRNA delivery is thoroughly addressed. By investigating ~2000 damage events, chloroquine and siramesine are shown to damage a wide range of vesicles in the endosomal system, mainly triggering damage on later endosomal and lysosomal structures. Importantly, the method reveals why there is a substantial knockdown enhancement using chloroquine but not to the same extent using siramesine. Indeed, investigating the hit-rate of chol-siRNA$^+$ vesicles, chloroquine is clearly superior over siramesine, damaging at least twice as many cargo containing vesicles. The analysis further reveals substantial mismatch between cargo containing vesicles and damaged vesicles, revealing the possibility to also identify mechanisms behand the observed inefficiencies using this method. This aspect is further pursued in **Paper III**.

To conclude, the presented method in **Paper I** is able to investigate the rate limiting step of endosomal escape with increased resolution, pinpointing efficiencies and limitations of RNA delivery strategies and potential inducers of endosomal escape. Thus, it will likely be an important contributor in the pursuit of identifying novel delivery strategies to improve delivery of RNA therapeutics.

Elucidating the dose-response relationship between cytosol siRNA and target gene

In **Paper II** and **Paper IV**, two novel methods are presented for elucidating the dose-response relationship between cytosolic siRNA and target gene – a previously not-well defined relationship that if attained could aid the development and characterization of novel delivery strategies. In **Paper II**, the method is based on a confocal live-cell imaging technique, while the method in **Paper IV** is based on conventional RT-qPCR. It is shown that the intracellular IC_{50} of an siRNA sequence with clinically relevant potency is approximately 2800 or 1000 siRNA molecules per cell, for **Paper II** and **Paper IV** respectively. Previous estimations investigating the cytosolic IC_{50} of another target gene using gold-labeled siRNA concluded between 2000-4000 siRNA molecules[143], which is in close agreement with our results. Other studies using Ago2-immuno precipitation concluded 10-110 RISC-loaded siRNA molecules[171], but with unknown RISC loading efficiencies. Furthermore, close to maximal knockdown induction with less accurate cytosolic siRNA estimations (~1.6 nM) from a previous study using the same siRNA sequence[126], is additionally in close relationship with that of in **Paper II** (~3 nM) and **Paper IV**.

In **Paper II**, knockdown induction is measured over several hours after a single siRNA bolus-like release has been delivered to the cytosol. This continuous measurement and cell-to-cell variability that is attained, revealed that IC_{50} is a dynamic concept that is dependent on time of siRNA release in addition to the potency of the siRNA sequence. Previous dose-response studies have relied on single time-point measurements, thus unable to distinguish such dynamics[172]. We could determine that knockdown induction, depth and duration are highly affected by dose, with higher doses having a more rapid induction, increased depth, and increased duration. Independent of dose, the knockdown nadir is reached 10-15 hours after siRNA release.

While it is not conceivable to acquire the same dynamic resolution in **Paper IV** as in **Paper II**, we show that with a fairly simple method that can be performed in most labs, it is possible to determine the dose-response relationship of an siRNA sequence with possibly similar accuracy as a highly advanced confocal imaging-based method, reducing workload significantly. The two studies reach similar IC_{50} values, despite the use of different delivery strategies (transfection lipids in **Paper II**, and electroporation in **Paper IV**), indicating that the two delivery strategies do not seem to fundamentally affect the cytosolic handling of siRNA. However, such claims needs to be further studied. Importantly, the method in **Paper IV** can determine the

IC_{50} of label-free siRNA sequences, which allows for accurate measurements of native (un-labeled) siRNA molecules, reduces experimental complexity and will be more cost-effective.

A key limitation with the method in **Paper II** is the size limit of the delivery strategy. In the paper we use cationic transfection lipids that are easily distinguished in the cells when in complex with siRNA, and they release detectable amounts of siRNA into the cytosol. But, for other delivery strategies such as LNPs or bioconjugates, the resolution and sensitivity of the microscope would be too low to distinguish if the fluorescent signal is derived from intact particles or of free cytosolic siRNA. Thus, for evaluating delivery strategies of a few hundred nm, a single-molecule detection strategy would probably be required. An important limitation to highlight in **Paper IV** is the inability to assess cell-to-cell variability as all measurements are performed on 500,000 cells. The fact that some cells additionally die upon electroporation and that killed or lysed cells might bind significant amounts of siRNA, further complicate reliable IC_{50} estimations. Such error sources must be further clarified.

Other limitations for both **Paper II** and **Paper IV** is the use of a single cell line, a single target gene, and two siRNAs. Thus, it would be interesting to investigate how the intracellular dose-response varies in other cells, tissues, and with other target genes and siRNA sequences. Indeed, there is literature that indicate that gene knockdown by siRNA can depend on various factors such as cell type-specific variability in mRNA susceptibility[173], mitotic activity[174], target mRNA abundance[175], and the expression of Ago2[176]. Such factors would be interesting to study from a dose-response perspective and is likely to be investigated in future studies using the now established methods in **Paper II** and **Paper IV**.

Cytosolic siRNA delivery with lipid nanoparticles reveals cellular and biophysical barriers

In **Paper III,** cytosolic delivery of clinically relevant MC3-LNPs carrying siRNA or mRNA is investigated. Using the established method in **Paper I** together with super-resolution microscopy, various aspects of LNP delivery is addressed.

Similar to previous observations investigating the release of RNA from LNPs[126, 143], only a small fraction of RNA payload carried by the LNPs is found to be released from disrupted vesicles, and is mainly released from $Rab5^{+}EEA1^{+/-}$early endosomes for both siRNA and mRNA payload. However, a substantial fraction of damaged endosomes did not carry any RNA, especially for mRNA payload, implying that only relying on the number of galectin-9 positive vesicles will not directly represent the capacity to successfully deliver RNA to the cytosol, for a studied delivery strategy. Additionally, the payload and the LNP formulation itself seems to have a profound effect on productive delivery, and one could imagine that multi-component RNA formulations used for *e.g,* CRISPR-system will be even more

challenging to achieve sufficient delivery for. The reason why we observe damaged endosomes with no RNA payload, and why there is differences in this phenomenon between siRNA payload and mRNA payload, can be discussed. There are limitations when it comes to the sensitivity of the microscopy assay, but as both mRNA-LNPs and siRNA-LNPs are detected as intact single particles, there is confidence that the lack of RNA signal in the damaged endosomes is indeed because of the absence of RNA payload. Using super-resolution microscopy, gradual disintegration of RNA-LNPs can be appreciated for the first time during endosomal trafficking. The separation of ionizable lipid and RNA payload into separate endosomal compartments can also be observed with this image resolution, supporting the concept that damaged endosomes not carrying any RNA could be the result of segregated ionizable lipids causing unproductive damage.

It is still not clear how the intralumenal RNA payload escapes into the cytosol after LNP induced endosomal membrane damage. However, the preceding phase which involve the protonation of the ionizable lipid and subsequent interaction with the endosomal membrane causing a hexagonal lipid phase transition, is believed to be the initial key step[141, 142]. As seen in **Paper I**, galectin-9 accumulates at endosomal damages during release of dextran (similar molecular weight as siRNA) when induced by small molecule drugs. The intraluminal binding of galectins to β-galactoside-containing glycans[125] additionally suggests that recruitment of galectin is a consequence of larger membrane damages, while the ESCRT machinery might sense smaller pertubations[127]. In **Paper III** we propose that the LNPs are partially disintegrated, and free intralumenal RNA is released through disruptions in the endosomal membrane, triggered as ionizable lipids are enriched locally in the lipid bilayer. This theory would support why there is only partial RNA release, as the remaining RNA would be trapped in the partially disintegrated LNP.

As previously mentioned, the ESCRT machinery detected membrane perturbations induced by RNA-LNPs that did not recruit any galectins, but those damages did not promote any cytosolic release of siRNA. It could be that the damages that recruit ESCRT are too small to release any RNA payload, or that the damage is rapidly repaired by the ESCRT membrane repair system. In **Paper III**, knockdown of key ESCRT-components resulted in increased galectin-9^+ foci, plausibly indicating that smaller damages that are not repaired eventually leads to larger damages. However. this effect is rather modest which could possibly be explained by substantial functional redundancy of the ESCRT system.

In conclusion, in **Paper III** we demonstrate a number of mechanistic barriers for LNP mediated delivery of RNA which limits effective delivery of siRNA and mRNA. The hope is that this work will provide a better understanding of LNP delivery systems in the pursuit to ultimately enable LNPs to efficiently deliver RNA therapeutics to extrahepatic targets.

Future perspectives

Intracellular delivery of RNA therapeutics is presently not well understood, and the need for tools to address this gap of knowledge is required to aid prospective RNA therapeutics to become more effective. In this thesis, novel methods to detect and quantify endosomal escape and cytosolic delivery of siRNA are presented.

The method in **Paper I** provides the means to address momentaneous release of RNA payload from the releasing endosome. Such tool will likely be a valuable asset to characterize delivery with other payloads or delivery strategies. Indeed, in both **Paper I** and **Paper III** two different delivery strategies are evaluated with the method, providing new insights into their respective characteristics. In **Paper II** and **Paper IV** we present two methods to quantify cytosolic delivery of siRNA and the subsequent knockdown of a target protein. With such measurements, the intracellular dose-response of RNA therapeutics can now be established to assess delivery efficiencies and identify potential bottlenecks for improvements.

In this thesis, endosomal release of lipid mediated siRNA and mRNA delivery have been explored, but it would be interesting to apply these methods to other delivery systems and perhaps with other forms of payload as well. Indeed, it is still unknown how bioconjugates such as the GalNAc conjugate is released from the endosome and if such release can be probed by the presented methods. It could be that they are released through other routes by mechanisms that do not trigger galectin recruitment or the ESCRT machinery, requiring other means of detection. The delivery of payloads such a CRISPR-Cas systems would also be interesting to address, as they are multicomponent systems that require all components to be released from the endosome to become therapeutically active. With the toolbox developed in this thesis, such delivery can now be explored. One strategy could be to exploit the time-resolved dose-response kinetics of cytosolic siRNA to obtain estimates of the cytosolic delivery during gymnotic siRNA delivery. Such estimates could then be used to further probe the mechanism of endosomal escape.

In the end, the hope is that the presented methods will provide new insights of the limitations of current delivery strategies and to guide improvements, to ultimately enhance RNA therapeutics delivery into target cells and currently unreachable tissues and organs.

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