THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Mechanisms of Lipid Nanoparticle-Mediated mRNA Transport Across Lipid Membranes

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Cover: Artistic representation of a lipid nanoparticle.

Abstract

After cellular uptake, RNA-containing lipid nanoparticles (LNPs) are typically located within endosomes, membrane-delimited intracellular organelles. To exert their therapeutic effect, their RNA cargo must translocate across the endosomal membrane to reach the cytosolic translational machinery. This critical step, known as endosomal escape, remains a major bottleneck for effective delivery.

The work presented in this thesis investigates the mechanisms underlying LNP-mediated endosomal escape using a simplified model system designed to mimic key features of the endosomal environment, including an anionic supported lipid bilayer (SLB) and acidification. Time-resolved fluorescence microscopy demonstrated that acidification triggers fusion of LNPs with the anionic SLB, suggesting a potential pathway for mRNA release and translocation across the endosomal membrane. However, translocation across the SLB was not observed to be the dominant mechanism for mRNA release at fusion sites.

Following administration, such as by injection, LNPs inevitably interact with serum proteins, resulting in the formation of a protein corona that alters their physicochemical properties. Comparative experiments with pristine and serum-preincubated LNPs revealed that serum exposure promotes fusion at less acidic conditions, indicating that the protein corona does not inhibit fusion with anionic membranes. Nonetheless, serum incubation also led to partial mRNA loss from LNPs, which may compromise delivery efficiency.

Together, these findings provide deeper insight into the physicochemical processes that govern LNP-mediated mRNA delivery and highlight factors that can influence endosomal escape. This may inform the rational design of more effective LNP formulations for RNA-based therapeutics.

Keywords: Lipid nanoparticle, endosomal escape, supported lipid bilayer, RNA delivery, protein corona.

List of Publications

This thesis is based on the following publications:

[A] N. Aliakbarinodehi §, **S. Niederkofler** §, G. Emilsson, P. Parkkila, E. Olsén, Y. Jing, M. Sjoberg, B. Agnarsson, L. Lindfors, F. Höök, F., "Time-Resolved Inspection of Ionizable Lipid-Facilitated Lipid Nanoparticle Disintegration and Cargo Release at an Early Endosomal Membrane Mimic". *ACS nano*, **2024**, 34, 22989-23000, DOI: 10.1021/acsnano.4c04519

Contribution: NA and SN contributed equally to this work (§). NA was responsible for conducting the experiments, while SN performed data analysis and contributed to manuscript preparation.

[B] **S. Niederkofler**, P. Parkkila, N. Sasanian, G. Emilsson, N. Aliakbarinodehi, Y. Jing, L. Lindfors, B. Agnarsson, F. Höök , "Effects of Serum Incubation on Lipid Nanoparticle mRNA Retention, PEG-Shedding, and Membrane Interactions".
Manuscript

Contribution: SN was responsible for designing and conducting experiments, data analysis, and manuscript preparation.

Other publications by the author, not included in this thesis, are:

[C] M. Sjöberg, E. Olsén, M. Mapar, P. Parkkila, **S. Niederkofler**, S. Mohammadi, Y. Jing, L. Lindfors, B. Agnarsson, F. Höök, F., "Multiparametric Quantification and Functional Characterization of Individual Lipid Nanoparticles Using Surface-Sensitive Light-Scattering Microscopy".
Submitted.

Abbreviations

LNP:	Lipid nanoparticle
DNA:	Deoxyribonucleic acid
RNA:	Ribonucleic acid
mRNA:	Messenger RNA
siRNA:	Small interfering RNA
ASO:	Antisense oligonucleotide
AAV:	Adeno-associated virus
PEG:	Polyethylene glycol
DOPE:	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTAP:	1,2-dioleoyl-3-trimethylammonium-propane
DOSPA:	2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate
DSPC:	1,2-distearoyl-sn-glycero-3-phosphocholine
FDA:	U.S. Food and Drug Administration
CPP:	Cell-penetrating peptides
ApoE:	Apolipoprotein E
PLGA:	Poly(lactic-co-glycolic acid)
PEI:	Poly(ethyleneimine)
PAMAM:	Poly(amidoamine)
GalNAc:	N-Acetylgalactosamine
ASGPR:	Asialoglycoprotein receptor
POPS:	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
BMP:	bis(monooleoylglycero)phosphate
DPPE:	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DOPC:	1,2-dioleoyl-sn-glycero-3-phosphocholine
PLL:	Poly-L-lysine
IgM:	Immunoglobulin M
IgG:	Immunoglobulin G

HDL:	High-density lipoprotein
TEOS:	Tetraethyl orthosilicate
NTA:	Nanoparticle tracking analysis
DLS:	Dynamic light scattering
CV:	Coefficient of variation
EE:	Encapsulation efficiency
MSD:	Mean square displacement
TIRF:	Total internal reflection fluorescence
RI:	Refractive index
SLB:	Supported lipid bilayer
TNS:	2-(p-toluidino)-6-naphthalene sulfonic acid

Contents

Part I

Overview

CHAPTER 1

Introduction

The therapeutic delivery of genetic material has emerged as a new strategy for the treatment of diseases, including those previously considered undruggable, and has facilitated the rapid development of vaccines. Notably, the successful deployment of messenger RNA (mRNA) vaccines against SARS-CoV-2 demonstrated the enormous potential of RNA-based therapeutics in addressing urgent global health challenges. The ability to rapidly design, produce, and adapt RNA-based drugs can offer advantages over traditional therapeutics, for instance in the fields of personalized medicine, oncology, and infectious diseases.[1]

Despite these advantages, the clinical application of RNA therapeutics faces challenges that are related to biological barriers. RNA molecules are inherently unstable in biological fluids, as they are rapidly degraded by extracellular ribonucleases (RNases).[2] Furthermore, their large size, negative charge, and hydrophilicity prevent them from passively crossing the hydrophobic core of cellular membranes. Therefore, the development of delivery systems capable of protecting RNA from degradation and enabling its delivery into the cytoplasm of target cells is essential for the clinical translation of RNA-based therapeutics.

Viruses have evolved to efficiently deliver their genetic material into host cells, which made modified viruses that are loaded with genetic material of choice, so-called viral vectors, an established delivery systems for nucleic acids. However, safety concerns related to immunogenicity, mutagenesis, and complex production

processes limit the widespread use of viral vectors in many therapeutic contexts. As a result, there is significant interest in developing synthetic, non-viral delivery systems that can offer comparable efficacy while maintaining scalability, safety, and versatility.[1]

Among synthetic delivery vehicles, lipid-based nanoparticles, such as lipid nanoparticles (LNPs) have emerged as efficacious carriers for nucleic acids. Although these delivery systems enable the cellular uptake of nucleic acids, one critical step known as endosomal escape remains a major bottleneck limiting their overall efficiency. Following cellular uptake, LNPs are typically trafficked into intracellular compartments called endosomes. However, to exert their therapeutic effect, the encapsulated nucleic acid cargo must be released into the cytoplasm. For LNPs, this release occurs with low efficiency, only a few percent at best, resulting in the majority of particles becoming trapped within the endosomal compartment. In contrast, viruses have evolved more effective mechanisms to overcome this barrier, achieving endosomal escape efficiencies estimated at 30–70%.[3] This stark contrast underscores the challenge of replicating viral delivery mechanisms using synthetic systems. Viruses rely on specialized proteins that undergo conformational changes in response to environmental cues, such as endosomal acidification, enabling membrane fusion or pore formation and facilitating efficient escape from the endosome.[4] In comparison, synthetic delivery vehicles primarily depend on physicochemical strategies, including electrostatic interactions between ionizable cationic lipids and anionic endosomal membranes, the incorporation of fusogenic or membrane-disruptive lipids, and osmotic destabilization of the endosomal compartment mediated by protonable groups.[5], [6]

Despite significant advancements in the design of synthetic delivery vehicles, enhancing endosomal escape remains a key challenge in improving the overall efficacy of RNA-based therapeutics. Specifically, for LNPs, the precise molecular mechanisms by which they facilitate endosomal escape remains, to the best of our knowledge, incompletely understood. Investigating these processes *in vivo* or using *in vitro* cell models is inherently challenging due to the complexity and dynamic nature of cells, as well as the limited availability of analytical methods capable of resolving events at the nanometer scale. One strategy to overcome such limitations is the use of simplified model systems that offer reduced biological complexity and increased experimental control. Such systems can enable investigation of specific mechanistic aspects of LNP-mediated RNA delivery, and form the basis of the work presented in this thesis, which focuses on elucidating the mechanisms underlying LNP-assisted endosomal escape. Understanding and improving the efficiency of endosomal escape could significantly enhance the therapeutic potential of RNA delivery systems. More efficient delivery could broaden the applicability of RNA-based therapeutics beyond current uses, enabling the treatment of a wider

range of diseases. These include conditions where traditional small molecule drugs are ineffective, such as genetic disorders requiring gene silencing or replacement, as well as personalized cancer immunotherapies and vaccines. Furthermore, it could facilitate the therapeutic use of a variety of RNA types, including mRNA, small interfering RNA (siRNA), and antisense oligonucleotides (ASOs), each offering distinct mechanisms of action and therapeutic opportunities.[7]

In this thesis, the background section provides an overview of the mechanisms that viruses employ to achieve endosomal escape. These mechanisms typically involve highly specialized protein machinery that facilitates membrane fusion or pore formation in response to endosomal cues, such as acidification. Following this, human-made gene delivery approaches are introduced, focusing on synthetic delivery vehicles. The current understanding of how these delivery vehicles achieve endosomal escape is presented, which generally relies on physicochemical interactions, including membrane destabilization through cationic lipids and pH-responsive components, rather than the intricate protein-mediated processes observed in viruses.[5] Lastly, the background section discusses the inevitable interaction between delivery vehicles and biological fluids following administration. This interaction leads to the adsorption of proteins onto the particle surface, resulting in the formation of the so-called protein corona. The presence of this corona can substantially alter the physicochemical properties of the particles, thereby influencing their biological identity and behavior *in vivo*.[8] Since protein coronation cannot be fully prevented, it represents an important consideration in the design and evaluation of delivery systems. Finally, brief summaries of the two papers included in this thesis outline the obtained insights. In paper A, a nanoporous silica-supported lipid bilayer is introduced as a model system that mimics key aspects of the endosomal membrane environment, including its anionic lipid composition and acidic conditions. This platform was used to investigate the mechanisms potentially contributing to endosomal escape, with a focus on pH-induced fusion events between LNPs and the anionic lipid bilayer, as well as subsequent cargo release. In paper B, the effect of protein coronation on LNP integrity and its impact on interactions with an endosomal membrane mimic are explored. Together, these studies aim to provide deeper insight into the physicochemical mechanisms governing LNP-mediated endosomal escape.

CHAPTER 2

Background

2.1 Viral Delivery of Genetic Material

Structure of Viruses

Viruses are submicron-sized biological entities that contain genetic material, typically DNA or RNA, and replicate by hijacking the cellular machinery of living organisms. Although host organisms have evolved defense mechanisms to protect against viral infections, certain viruses can overcome the defense, leading to infections that manifest in a range of pathogenic conditions, from mild illnesses such as the common cold to severe diseases like influenza and rabies.

The viral structure is generally composed of several types of proteins that serve as the fundamental building blocks of the virus particle (Fig 2.1a). These proteins can be broadly categorized into core proteins and capsid proteins. Core proteins are located in the interior of the virus, where they often play roles in the structural organization of the viral genome or act as catalysts in different stages of the viral life cycle. In contrast, capsid proteins assemble into a shell known as the capsid, which serves as a protective barrier for the viral genetic material. The macromolecular organization of core and capsid proteins typically displays a high degree of symmetry, resulting in distinct geometric shapes of the virus particle, such as the icosahedral morphology observed in adenoviruses.

In addition to these structural components, some viruses, referred to as enveloped viruses, possess an outer lipid membrane envelope that surrounds the capsid and core (Fig. 2.1b). This lipid membrane is typically derived from the host cell membrane, but also contains viral proteins that facilitate host cell recognition and entry, such as glycoproteins.[9]

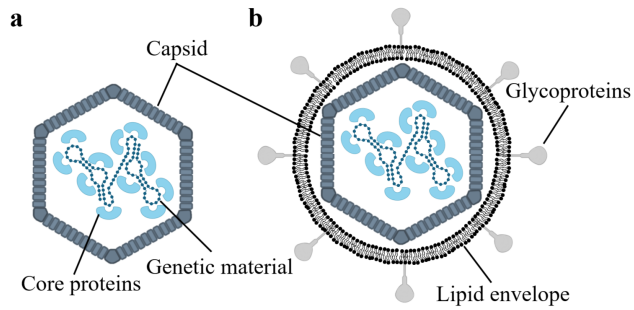

Figure 2.1 Simplistic representation of two common virus morphologies. **(a)** Non-enveloped, and **(b)** enveloped virus.

A Key Step in the Viral Life Cycle: Endosomal Escape

A simplified description of the viral life cycle includes four main stages: entry into the host cell, replication of the viral genome and production of viral proteins, assembly of new virus particles, and finally release from the host cell. Of specific interest for this thesis work are the processes related to the entry step, which enable the transport of genetic material from the extracellular matrix into the cellular interior, the cytosol. Cells are surrounded by a lipid membrane, which presents a barrier to large hydrophilic molecules such as DNA and RNA. To overcome this, viruses employ sophisticated mechanisms that enable them to deliver their genetic cargo across cellular membranes.[10]

The initial step in viral infection typically involves receptor-mediated binding to the host cell membrane. Viruses often display glycoproteins on their surface that recognize and bind to specific receptors on the cell membrane.[11] For many virus species, this binding event initiates endocytosis, a cellular uptake process in which the virus is internalized within a membrane-bound compartment known as an endosome. Although endocytosis brings the virus into the cell, it remains trapped within the endosomal compartment. To initiate replication, the viral genome must escape from the endosome into the cytosol.[4]

This critical process, often referred to as endosomal escape, plays a central role during viral entry. As the endosome matures, its internal environment becomes increasingly acidic, with the pH dropping from around 7.4 to approximately 5.0.[12] If the virus remains trapped, the endosome can fuse with a lysosome, leading to degradation of the viral particle. To avoid this fate, many viruses have evolved pH-responsive mechanisms that enable them to disrupt the endosomal membrane and release their genome into the cytosol before degradation occurs.

Viral Mechanisms for Endosomal Escape

Viruses have developed intricate molecular mechanisms to facilitate the delivery of their genomes into host cells. These strategies often involve a coordinated action of various viral proteins, located both on the virus surface and within its core. Typically, the viral proteins that facilitate the transfer of genetic material across cellular membranes undergo structural changes, which are triggered by cues that indicate host cell contact or endocytic uptake.

Most virus types are thought to follow an endocytic uptake pathway, during which the virus particle is processed within an endosome. Thus, changes of viral proteins are often triggered by factors that are specific to the endosomal maturation process, such as a decrease in pH, presence of endosomal proteases, or interactions with endosome-specific lipids. For instance, the hemagglutinin protein of Influenza A viruses is activated by acidification, the glycoprotein of Ebola viruses undergoes activation in the presence of endosomal cathepsin proteases, and the Vp5 capsid protein of Bluetongue viruses is activated depending on the levels of lysobisphosphatidic acid in the endosomal membrane.

The mechanisms by which these viral proteins facilitate cellular delivery vary and are not fully understood for all proteins. However, they can include processes such as destabilization of the virus particle, enabling release of its genetic material, as well as altered interactions between viral proteins and cellular lipid membranes or membrane proteins, leading to membrane fusion or disruption events.[4]

Fusion Proteins

Enveloped viruses feature membrane-associated glycoproteins on their surface, which mediate the fusion between the virus envelope and the endosomal membrane. While the structure of these fusion proteins can vary significantly, they share common mechanisms of action: a pH-triggered conformational change in the protein leads to the alignment and eventual merging of the viral lipid envelope with the endosomal membrane (Fig. 2.2a). This fusion process is typically facilitated by hydrophobic protein residues, which assist in embedding the protein into the lipid membranes,

and by residues that are responsible for initiating the conformational change. The mechanism of many viral fusion proteins is relatively well understood, largely due to structural studies that have resolved their conformations in both pre- and post-fusion states. For example, the hemagglutinin protein of the influenza A virus has been extensively characterized, providing key insights into the conformational changes that drive membrane fusion.[13]

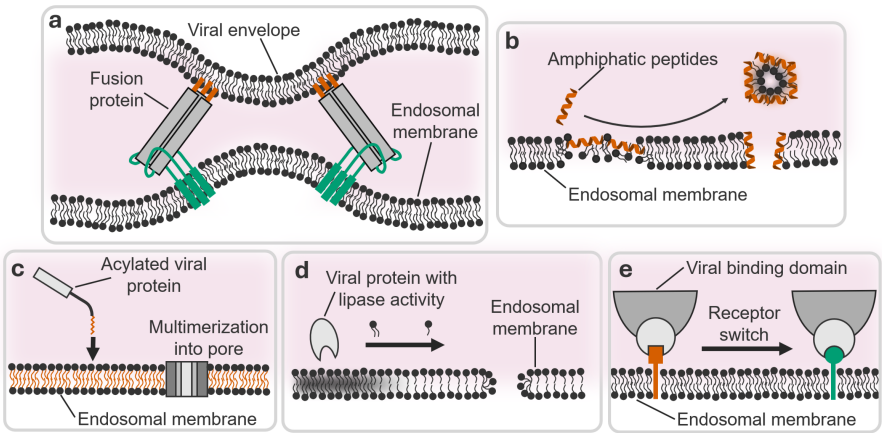

Figure 2.2 Schematic representation of the mechanisms employed by viruses to facilitate endosomal escape. The endosomal lumen is indicated by a purple background. **(a)** Fusion proteins, such as hemagglutinin, mediate the fusion of the viral envelope with the endosomal membrane. **(b)** Viral proteins containing amphipathic α-helical domains interact with the endosomal membrane, inducing disruption of the lipid bilayer. **(c)** Acyl-chain-modified viral proteins with membrane affinity can integrate into the membrane, leading to pore formation. **(d)** Viral proteins with enzymatic activity altering the lipid composition of the endosomal membrane, which can result in membrane curvature stress that facilitates escape. **(e)** Alterations in binding domains enable a receptor switch during endocytosis, which can enhance endosomal escape.

Membrane-disruptive Proteins

Non-enveloped viruses also rely on protein-based mechanisms to facilitate endosomal escape, enabling the delivery of their genetic material into the host cell cytosol. Unlike enveloped viruses, which typically employ membrane fusion proteins to merge their lipid envelope with host membranes, non-enveloped viruses lack a surround-

ing lipid bilayer and therefore use alternative strategies to breach the endosomal membrane barrier.

These strategies are primarily mediated by specialized viral proteins that interact directly with lipid membranes. Although they share some functional similarities with fusion proteins, such as activation by specific environmental cues like endosomal acidification, the presence of host proteases, or interactions with endosome-specific lipids, their mechanisms of action are often fundamentally different and, in many cases, remain less well understood [4]

One strategy employed by non-enveloped viruses involves proteins that possess an amphipathic α-helical domain capable of associating with lipid bilayers, leading to a disruption in membrane integrity without necessarily forming discrete pores (Fig. 2.2b). For instance, protein VI of adenoviruses contains an amphipathic α-helical domain that destabilizes the endosomal membrane, thereby enabling viral cargo to escape into the cytoplasm.[14]

Another common mechanism is based on the formation of membrane pores by pore-forming proteins (Fig. 2.2c). For example, polioviruses express the N-myristoylated VP4 protein, which becomes activated upon endosomal acidification. VP4 undergoes a conformational change, inserts into the endosomal membrane, and forms pores through which the viral cargo can be translocated into the cytoplasm.[15]

In addition to membrane pore formation, some viruses employ enzymatically active proteins that chemically modify cellular membranes (Fig. 2.2d). Parvoviruses provide an example of this strategy, as they encode VP1 proteins with phospholipase A2 activity. Upon activation, VP1 degrades specific phospholipids within the endosomal membrane, a process thought to induce membrane instability through curvature stress, thereby facilitating viral escape.[16]

Other Mechanisms

In addition to fusion proteins and membrane-disruptive proteins, other viral components contribute to endosomal escape mechanisms. For example, in the case of influenza A virus, endosomal acidification not only triggers the conformational change of the hemagglutinin fusion protein but also induces the dissociation of the viral M1 core protein from the viral genome. This step is thought to facilitate the release of the genome from the capsid and enable subsequent transport into the cytoplasm.

Another mechanism that supports endosomal escape is a cue-induced receptor switch following endocytosis. In certain viruses, acidification-driven conformational changes in viral proteins can alter binding specificity, leading to the release from one receptor and rebinding to another (Fig. 2.2d). This mechanism was observed for the Lassa virus, which initiates infection by binding to α-dystroglycan receptors

on the surface of the host cell. After internalization and exposure to the acidic environment of the endosome, the virus was shown to bind to lysosome-associated membrane protein 1 (LAMP1). This receptor switch is considered to lower the pH threshold required for membrane fusion, thereby facilitating efficient viral escape from the endosome.[17]

2.2 Therapeutic Delivery of Genetic Material

Undruggable Targets

The identity of living cells is primarily determined by the proteins they express, which perform a wide range of functions such as structural support, intracellular transport, membrane transport, signaling, and catalysis. Therefore, it is not surprising that protein malfunction can have detrimental effects on organisms, often manifesting as diseases. One therapeutic strategy to address such diseases involves the use of small molecules, which can bind to specific proteins to inhibit or modify their function. While small-molecule therapeutics have proven valuable for treating many conditions, they face limitations. Notably, only 10-14% of proteins possess known active binding sites for small molecules, and some diseases arise not from malfunctioning proteins, but from the absence or deficiency of specific proteins. Diseases that involve a deficiency of proteins, or proteins that lack known binding sites for small molecules, are considered 'undruggable' by conventional small-molecule treatments. In response to some of the limitations of small-molecule therapies, recombinant protein technology has enabled the development of therapeutics that replace or supplement deficient proteins.[1] Recombinant proteins are produced through genetic engineering techniques that enable large-scale synthesis of proteins, which can then be administered to patients to replace deficient or missing proteins. However, protein-based therapeutics face challenges, which are for instance related to complex production, limited stability and low bioavailablity.[18] Another approach for treating undruggable diseases is gene therapy, where genetic material is administered to patients.

Gene Therapy

Since cells contain the intrinsic translational machinery required for protein synthesis, the administration of genetic material encoding a missing or deficient protein represents a promising therapeutic strategy. This approach has the potential to overcome several limitations associated with small-molecule drugs and recombinant-protein therapeutics. However, gene therapies also present unique challenges, including issues related to the stability of genetic material, immunogenicity, and bioavail-

ability. Despite these challenges, the broad range of treatment possibilities offered by gene therapy has driven the development of numerous strategies aimed at overcoming these limitations.[1]

One well-established strategy involves the use of viral vectors, employing the natural ability of viruses to deliver genetic material into cells. In this approach, therapeutic genes are loaded into modified viruses, which are then administered to patients. Several gene therapies based on viral vectors have already received regulatory approval. Among these, adeno-associated viruses (AAVs) are commonly used due to their favorable safety profile and minimal risk of adverse effects.[19]

Nevertheless, the use of viral vectors in gene therapy raises certain concerns. Some virus types are capable of integrating their genetic material into the host cell genome, which can pose risks of insertional mutagenesis and unintended genetic alterations. While AAV-based therapies present a significantly lower risk of genome integration, the potential for such events remains a consideration in the development and application of viral gene therapies.[20]

To address these concerns, alternative strategies have been explored. These include the use of non-viral delivery vehicles that avoid reliance on viral machinery and the development of gene therapies that utilize RNA-based cargos rather than DNA. Unlike DNA, RNA typically exerts its function in the cytoplasm, which eliminates the need for nuclear delivery, and thereby reducing the risk of genome integration.[21] Furthermore, the inherent instability of RNA leads to its degradation, making effects transient and irreversible changes unlikely.

The ability to deliver various types of RNA, including mRNA, ASOs, and siRNA offers great potential for modulating gene expression and employing the cell's translational machinery in various therapeutic modalities. The success of RNA-based therapeutics has already been exemplified in clinical applications, and is likely to expand to a wide range of diseases and conditions.[22]

However, the inherent instability and susceptibility to degradation of RNA molecules pose significant delivery challenges. To overcome these obstacles, extensive efforts have been devoted to developing non-viral delivery systems designed to protect RNA during circulation and facilitate its efficient delivery into target cells. In the following sections, a brief overview of the current landscape of non-viral delivery vehicles for RNA-based therapies is given.

Non-viral Delivery Vehicles

Liposomes

Liposomes are supramolecular assemblies that form through self-assembly when amphiphilic molecules, such as lipids, are dispersed in an aqueous solution. These

spherical particles typically range from micron- to submicron-size, and are typically considered to have an aqueous core. However, depending on preparation type and lipids used, also more complex structures are commonly observed, such as stacked lipid bilayers.[23] Liposomes can be used for the delivery of low-molecular-weight drugs, such as Doxil, but are also effective for delivery of nucleic acids such as RNA. When used for the delivery of nucleic acids, liposomes are typically formulated with a fraction of the lipids being positively charged, which promotes electrostatic interaction with the negatively charged RNA molecules.[24]

Typical liposomes that are used for RNA delivery consist of a mixture of zwitterionic or uncharged lipids combined with cationic lipids. Commonly used zwitterionic or uncharged lipids include 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol, while cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or 2,3-dioleoyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propaniminium trifluoroacetate (DOSPA) are used to supply the cationic character. The electrostatic interactions between cationic lipids and RNA facilitate the formation of stable RNA-lipid complexes, which are commonly referred to as lipoplexes. These complexes have demonstrated enhanced gene delivery efficiency in *in vitro* cell cultures (transfection), outperforming the delivery of "naked" genes.[25]

Lipid Nanoparticles

Liposomal delivery systems have been successfully used in gene therapy for human patients, but they have notable limitations, which are, for instance, related to their limited capacity for RNA-loading, toxicity, and high size polydispersity.[26] A promising alternative to liposomes are so-called lipid nanoparticles (LNPs), which have shown superior performance in gene delivery applications. LNPs are also formed through self-assembly when lipids are dispersed in an aqueous solution, but unlike liposomes, they have a dense lipid-RNA core.[27]

LNPs typically include ionizable cationic lipids, which possess a pH-dependent charge. Use of such ionizable lipids is considered a key factor for the performance of LNPs, which enables efficient complexation with anionic RNA, but at the same time, the pH-sensitive charge also facilitates the release of the RNA in the appropriate cellular environment, enhancing the efficiency of delivery into the cytoplasm.[28] Along with the cationic lipid, LNPs typically contain phospholipids, cholesterol, and lipid-conjugated polyethylene glycol (PEG), which improves nanoparticle stability and reduces degradation, resulting in an extended circulation time.[29]

The potential of LNPs for gene therapy applications is highlighted by the approval of LNP-based therapeutics by medicinal regulatory agencies such as the U.S. Food and Drug Administration (FDA) and the European Medicines Agency. Approval

was granted for instance to the siRNA-therapeutic patisiran (brand name Onpattro, developed by Alnylam Pharmaceuticals) in 2018,[30] and the mRNA COVID-19 vaccines developed by Pfizer-BioNTech and Moderna in 2021.[31][32]

Polymer-based Nanoparticles

Polymers typically refer to synthetic compounds characterized by long molecular chains or branches with repeating motifs. Through polymer synthesis, a wide variety of polymers with distinct properties can be created. Among these, amphiphilic polymers have the ability to self-assemble into nanoparticles when dispersed in an aqueous solution, and they can further encapsulate RNA molecules, especially if the polymers posses cationic moieties. Some commonly used polymers used to make RNA nanocarriers include poly(lactic-co-glycolic acid) (PLGA), poly(ethyleneimine) (PEI), and poly(amidoamine) (PAMAM).

Polymeric nanocarriers have been successfully utilized for gene delivery in both *in vitro* and *in vivo* studies, showing promising results.[33][34] However, to date, they have not been approved as therapeutics by medicinal regulatory agencies for human use. One major challenge hindering their clinical application is biocompatibility issues, which may arise due to the synthetic polymers' intrinsic tendency to induce inflammation. Additionally, concerns about their degradation and clearance from the patient's system remain.[35]

To mitigate these biocompatibility issues, an emerging approach is the development of lipid-polymer hybrid nanocarriers. These carriers combine the advantageous biocompatibility of lipids with the structural stability and tunability offered by polymers. Lipid-polymer hybrid nanocarriers, which consist of a polymer core and a lipid shell, have shown success in gene delivery *in vivo*, providing a promising solution to the limitations of purely polymeric carriers.[36][37]

GalNAc Conjugates

An alternative approach for RNA delivery, which does not rely on nanoparticle structures, involves the direct conjugation of RNA with N-Acetylgalactosamine (GalNAc). GalNAc is a trivalent ligand that specifically binds to asialoglycoprotein receptors (ASGPRs) on hepatocytes, allowing for targeted delivery. Clinical studies have demonstrated that GalNAc-conjugated siRNAs are efficiently taken up by hepatocytes, showcasing the potential of this strategy.[7] This approach has gained considerable attention, with the FDA approval of the GalNAc-based siRNA therapeutic givosiran in 2019 (brand name Givlaari, developed by Alnylam Pharmaceuticals), emphasizing the promise of RNA delivery through GalNAc conjugation.[38]

Additional Examples

Numerous studies have demonstrated the successful use of various other materials as delivery vehicles for RNA. Examples include porous silica nanoparticles,[39] gold nanoparticles,[40] graphene nanotubes,[41] and metal-biomolecule network nanoparticles.[42] While such approaches show potential as RNA delivery vehicles, they have not yet been incorporated into therapeutics approved by medicinal regulatory agencies.

Endosomal Escape of Non-viral Delivery Vehicles

As indicated in the previous section, the number of available delivery vehicles for nucleic acids is vast, but the number of approaches that have led to treatments approved for use in humans remains limited. This underscores the complexity involved in developing delivery vehicles for gene therapy applications, as numerous factors must be considered to ensure clinical viability.[1] These factors include scalability of production processes, storage stability, low batch-to-batch variation, biocompatibility of materials, minimal immunogenicity of delivery vehicles, compatibility with cell-targeting strategies, and, importantly, high delivery efficiency. This thesis focuses mainly on the limited delivery efficiency of currently used vehicles, which is considered to be largely related to the endosomal escape process.[43] Enhanced delivery efficiency would allow for reduced administration doses, thereby mitigating toxicity and immunogenicity concerns, an aspect that is particularly crucial for the design of chronic therapies.

To achieve therapeutic efficacy with RNA-based therapies, the RNA must reach the site of protein translation, the cytosol. Similar to viral infections, most non-viral delivery vehicles enter cells through endocytosis, initially trapping the RNA within an endosomal compartment.[44][45] As with viruses, a crucial step in enhancing RNA delivery efficiency is the successful escape of the RNA from the endosomal compartment, allowing protein translation or RNA interference effects to occur.

To date, most human-made RNA delivery vehicles employ mechanisms for endosomal escape that are distinct from those used by viruses. While viral strategies for endosomal escape typically rely on conformational changes in viral proteins that interact with the endosomal membrane, human-made delivery vehicles generally employ alternative mechanisms to achieve the same goal, as outlined in the following sections.

Electrostatic Attraction Mediated by Cationic Lipids

The cytoplasmic-facing leaflet of the endosomal membrane is enriched with anionic lipids, such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and

bis(monooleoylglycero)phosphate (BMP).[46] The presence of cationic lipids in cationic liposomes or lipid nanoparticles is thought to induce destabilization of the delivery vehicles by promoting electrostatic interactions between the cationic lipids and the anionic lipids on the endosomal membrane. Specifically, for cationic liposomes, a lipid flip-flop mechanism has been proposed, where the cationic lipids in the liposome cause displacement of anionic lipids from the cytoplasmic-facing leaflet of the endosomal membrane to the endosomal-facing leaflet (flip-flop).[47] This translocation of anionic lipids strengthens the electrostatic attraction between the liposome and the endosomal membrane, driving the fusion process that results in the release of RNA into the cytosol. A similar fusion mechanism is thought to contribute to the endosomal escape process of ionizable lipid nanoparticles, where the electrostatic attraction is pH-dependent due to the ionizable nature of the cationic ionizable lipids (Fig.2.3a). Observations from papers A and B of this thesis work suggest that such a fusion mechanism plays a significant role in the endosomal escape of lipid nanoparticles.

Fusion Mediated by Lipids Adopting Inverted Hexagonal Phases

Cationic liposomes used for RNA delivery typically contain both cationic and neutral lipids, where the latter are often referred to as helper lipids. Studies have shown that the type of helper lipid used can significantly influence the transfection efficiency. For example, it is reported that use of the helper lipid DOPE results in enhanced transfection efficiency compared to other neutral lipids like 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC).[48][49]. This difference in transfection efficiency is thought to originate from the molecular shape of those helper lipids, which affects the adoption of specific lipid phases.

DOPE, with its cone-like molecular shape, favors the formation of inverted hexagonal lipid phases, which are believed to i) facilitate the fusion of lipid structures and ii) promote efficient dissociation of nucleic acids from lipid complexes (Fig.2.3b). This, in turn, enhances the ability of the liposomes to fuse with endosomal membranes, aiding the release of the nucleic acid cargo into the cytosol.[50] In contrast, cylinder-shaped lipids such as DPPE or DOPC prefer the formation of lamellar lipid phases, which are less effective in promoting these processes.

Furthermore, cholesterol has been identified as a component that can affect the formation of inverted hexagonal lipid phases. Specifically, reports indicate that cholesterol can stabilize such inverted hexagonal phases and thus play a role in enhancing the efficiency of lipid-based nucleic acid delivery systems, such as liposomes or LNPs.[51]

Specifically for LNPs, insight into preferential lipid phases and transitions are

considered an important factor for the ability to undergo fusion with endosomal membranes.[52] Therefore, also the design of novel ionizable cationic lipids for LNPs is guided by considerations about lipid shape.[53]

Osmotic Swelling of Endosome Induced by pH-buffering Moieties

Numerous studies have investigated nucleic acid delivery using cationic polymers. Poly-L-lysine (PLL) was among the first cationic polymers employed for this purpose; however, its transfection efficiency was relatively low. Subsequent studies demonstrated that other cationic polymers containing protonable residues, such as amine groups, including poly(amidoamine) (PAMAM) dendrimers and poly(ethyleneimine) (PEI), exhibit significantly higher transfection efficiencies.[54] This improved performance is commonly attributed to the presence of these protonable groups, which contribute to the so-called "proton sponge" effect.

According to this model, polymers with buffering capacity can sequester protons that are pumped into the endosome during acidification. The rising proton concentration leads to an influx of counter ions, such as chloride. This results in an increased osmotic pressure within the endosome, leading to water influx, swelling, and eventual destabilization or rupture of the endosomal membrane. Consequently, the nucleic acid cargo is released into the cytosol, where it can exert its therapeutic effect (Fig.2.3c).[55]

In addition to polymer-based strategies, nucleic acid delivery has been shown to benefit from the use of small-molecule additives *in vitro*. Chloroquine, a well-characterized weak base, can readily cross cellular membranes and accumulate in acidic organelles, such as endosomes. Its ability to neutralize endosomal pH is thought to promote osmotic swelling and membrane disruption, thereby enhancing the release of nucleic acids into the cytosol. More recently, small-molecule compounds with superior transfection-enhancing capabilities compared to chloroquine have been identified.[56] Similar to chloroquine, these agents are believed to induce endosomal swelling by buffering endosomal pH, though distinct mechanisms, such as alteration of cellular trafficking can not be excluded.

Membrane-destabilization Induced by Cell-penetrating Peptides

Many viruses rely on protein-based strategies to facilitate endosomal escape, enabling the release of their genetic material into the cytoplasm. Inspired by these mechanisms, similar approaches have been incorporated into non-viral nucleic acid delivery systems. One such approach involves the use of cell-penetrating peptides (CPPs), which were initially derived from viral proteins, for example, the TAT peptide from the HIV-1 virus, and later expanded to include a variety of synthetic peptides, such as the pH-sensitive GALA peptide.[57]

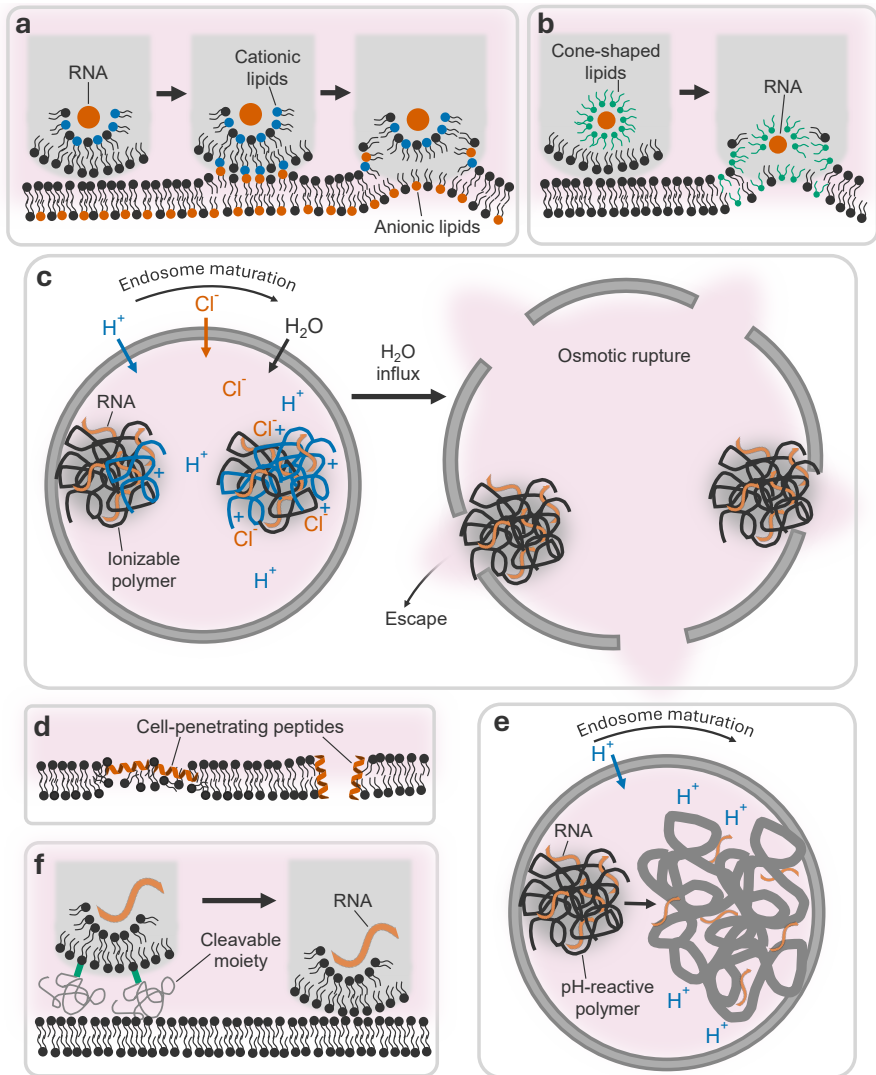

Figure 2.3 Schematic representation of the mechanisms employed by non-viral delivery vehicles to facilitate endosomal escape. The endosomal lumen and cytosol are indicated by purple and white background, respectively. **(a)** Electrostatic interactions between anionic lipids located in the cytoplasmic-facing leaflet of the endosomal membrane and cationic lipids in the delivery vehicle are thought to induce lipid flip-flop events, facilitating fusion between the delivery vehicle and the endosomal membrane.
(*Caption continues on next page*)

(*Continuated caption*)
(b) Lipids that adopt inverted hexagonal phases, typically with conical molecular shapes, are considered to promote fusion between delivery vehicle and the endosomal membrane. **(c)** The presence of protonable groups, for example in polymer-based delivery vehicles, can influence endosomal acidification by sequestering protons. This, in turn, drives chloride ion influx into the endosome, resulting in osmotic stress that can cause expansion and rupture of the endosomal membrane. **(d)** Cell-penetrating peptides interacting with the endosomal lipid membrane can disrupt its integrity, potentially leading to the formation of pores. **(e)** pH-responsive polymers can undergo swelling or disassembly upon acidification of the endosomal lumen, increasing the likelihood of endosomal membrane disruption and cargo release. **(f)** Acidification of the endosomal lumen can trigger pH-induced cleavage of molecules. For instance, cleavage of particle-stabilizing PEG molecules may facilitate closer interaction between delivery vehicle and endosomal membrane to promote fusion.

The membrane-disruptive activity of CPPs is thought to be mediated by two mechanisms: i) Cationic CPPs, like TAT, promote electrostatic interactions between the delivery vehicle and the anionic lipids of the endosomal membrane, facilitating close contact and potentially triggering destabilization, and ii) Amphipathic CPPs, such as GALA, which possess distinct hydrophobic and hydrophilic regions that enable them to insert their hydrophobic residues into the endosomal membrane. This insertion can disrupt membrane integrity, promoting the formation of pores or inducing membrane fusion events, ultimately allowing the nucleic acid cargo to escape into the cytoplasm (Fig.2.3d).[58]

Membrane-destabilization Induced by Swelling of Delivery Vehicle

For some polymer-based delivery vehicles, endosomal escape is thought to be facilitated through mechanisms such as swelling and/or disassembly of the vehicle in response to the acidic environment within the endosome. Disassembly of the delivery vehicle is believed to assist in the release of the nucleic acid cargo, whereas swelling is thought to exert mechanical pressure on the endosomal membrane, potentially leading to membrane disruption and the subsequent release of the nucleic acid into the cytosol (Fig.2.3e).[59][60]

Although successful nucleic acid delivery has been achieved using pH-responsive polymeric nanoparticles, the evidence supporting specific mechanisms of endosomal escape is limited. In addition to the mechanical pressure hypothesis, the endosomal escape of these delivery vehicles may also be enhanced by osmotic swelling due to buffering effects of the polymers, similar to the proton-sponge effect discussed above.[61]

Alternative Mechanisms

Other delivery vehicles have been designed with chemical moieties that undergo pH-dependent changes, such as cleavage reactions. One such strategy involves the use of PEG-lipids that detach from the delivery vehicle under acidic conditions. This detachment is aimed at destabilizing the vehicle once it reaches the acidic environment of the endosomal lumen, thereby potentially improving the release of the cargo (Fig.2.3f).[62]

In addition to pH-sensitive approaches, photochemical strategies to enhance endosomal escape have been explored. These strategies employ light-activated compounds that trigger endosomal membrane destabilization upon exposure to specific wavelengths of light. Photosensitizers used in these approaches generate reactive oxygen species upon illumination, which can disrupt the lipid bilayer of endosomes. This disruption facilitates the release of the encapsulated cargo into the cytoplasm, offering a controlled mechanism to enhance delivery efficiency.[63]

2.3 Protein Corona Formation on Delivery Vehicles

When nucleic acid delivery vehicles are used for *in vivo* administration, it is necessary to consider their interaction with components of body fluids. Upon administration, these synthetically engineered nanostructures, designed to meet stringent criteria such as purity, structure, size, polydispersity, and nucleic acid content, encounter fluids containing a high concentration of electrolytes and various biological molecules such as lipids and proteins. The composition of these fluids varies depending on the administration route: lung surfactant for inhalation, interstitial fluid upon local injections, or blood plasma when injected intravenously. Regardless of the specific biological fluid, it is widely accepted that proteins adsorb onto delivery vehicles, although their physicochemical properties can influence factors such as the affinity and composition of the adsorbed proteins. The adsorption of proteins is a dynamic process that leads to the formation of a protein layer, often referred to as the "protein corona", due to its appearance in electron microscopy images.[64]

The presence and composition of the protein corona can substantially alter the physicochemical and biological properties of lipid-based delivery vehicles, influencing their circulation time, biodistribution, cellular uptake, immune recognition, and overall therapeutic efficacy. Specific proteins within the corona play critical roles in determining the biological fate of the particles. For example, adsorption of opsonins such as immunoglobulins and complement proteins can trigger immune responses and promote clearance by phagocytic cells, reducing circulation half-life. In contrast, enrichment with apolipoproteins like ApoE can facilitate receptor-mediated uptake through interactions with low-density lipoprotein receptors (LDL-R), enhancing de-

livery efficiency and targeting. Understanding the implications of the presence of a protein corona is thus a necessary consideration in the design of advanced delivery vehicles.[8]

Stealth Polymers

Upon administration, antibodies such as immunoglobulins M and G (IgM and IgG), along with complement system proteins, are known to bind to delivery vehicles. This process, referred to as opsonization, facilitates recognition by immune cells such as macrophages, leading to accelerated clearance of delivery vehicles from circulation.[65] A reduced circulation time lowers the probability of delivery vehicles reaching their target sites, diminishing their potential therapeutic effects. A common strategy to mitigate opsonization and premature clearance is the incorporation of hydrophilic "stealth" polymers, such as PEG, which shield delivery vehicles from opsonins and thereby prolong circulation.[66]

Beyond prolonging systemic circulation, stealth polymers can modulate interfacial interactions with cells, potentially affecting cellular uptake and endosomal escape due to steric hindrance.[67] To overcome this limitation, advanced delivery vehicles such as LNPs are often engineered with sheddable stealth coatings that degrade or desorb upon interaction with biological fluids. A common approach is the use of PEG that is conjugated to lipid molecules, where the lipid's acyl chains enable a thermodynamically stable association of these PEG-lipids with lipid-based delivery vehicles. Upon interaction with serum proteins, particularly serum albumin, the association of PEG-lipids to the delivery vehicles is weakened, eventually leading to their desorption from the delivery vehicle. This desorption is believed to be driven by the binding of serum albumin to the acyl chains that tether the PEG-lipids to the delivery vehicle.[68]

Furthermore, the rate of desorption for such PEG-lipids has been shown to critically depend on acyl chain length, with PEG-lipids possessing 14-carbon chains desorbing significantly faster than those with 18-carbon chains.[69] This is leveraged in common LNP formulations, which typically use 14-carbon chain PEG-lipids that demonstrate superior therapeutic efficacy compared to their longer-chain counterparts.[29]

Forces and Dynamics of Protein Corona Formation

Protein adsorption onto nanoscopic delivery vehicles is governed by a complex interplay of thermodynamic and molecular interactions. Among these, electrostatic interactions are recognized as a key determinant of protein adsorption onto nanoparticles. The surface charge of nanoparticles significantly influences the composition

and structure of the resulting protein corona. For example, Lundqvist et al. demonstrated that nanoparticle surface charge alters the identity and quantity of adsorbed proteins.[70] Specifically, in the case of LNPs, incorporating positively or negatively charged lipids leads to distinct differences in the profiles of adsorbed proteins. The incorporation of negatively charged lipids has been shown to promote the adsorption of proteins with isoelectric points (pI) above physiological pH 7.4. At this pH, such proteins carry a net positive charge, resulting in enhanced electrostatic attraction to negatively charged nanoparticle surfaces.[71]

In addition to electrostatic forces, hydrophobic interactions play a significant role in protein adsorption onto nanoparticles. This is supported by observations demonstrating that nanoparticle surfaces with increased hydrophobicity tend to enhance protein binding.[72] Furthermore, van der Waals forces and hydrogen bonding are also considered to contribute to both nonspecific and specific protein adsorption, with their magnitude depending on various physicochemical properties of the nanoparticle.[73]

Protein corona formation is also recognized as a dynamic process. Specifically, the adsorption of proteins onto nanoparticle surfaces is often described by the Vroman effect, which proposes that smaller, more abundant proteins adsorb first but are then subsequently displaced by larger, slower diffusing proteins with higher surface affinity. These dynamic exchange processes are thought to lead to the formation of two distinct layers within the protein corona: a "soft" corona, consisting of loosely bound proteins that exchange rapidly with the surrounding environment, and a "hard" corona, composed of tightly bound proteins that exhibit slower exchange dynamics. The hard corona generally develops over prolonged incubation times as high-affinity proteins gradually replace those with weaker interactions.[74]

Protein Corona Composition for Lipid-based Delivery Vehicles

Accurately characterizing the proteins adsorbed onto nanoparticles vehicles remains a significant challenge. Furthermore, experimental outcomes are highly influenced by factors such as type and concentration of the biological fluid used for incubation, temperature, and processing steps required for the measurement technique of choice.[75] Notably, the last point is specifically relevant for weakly bound proteins, the soft corona, which are expected to be especially susceptible to disruption by procedures such as washing, filtration, or separation steps. In situ measurement techniques can provide a more accurate representation of the protein corona under near-physiological conditions. However, the availability of such methods is currently very limited and further requires refined methodology, such as the combination of physicochemical fishing techniques with mass spectrometry.[76]

The protein corona of lipid-based delivery vehicles is considered to contain hundreds of distinct proteins.[77] Among the most abundant are apolipoproteins, which are major constituents of endogenous lipoprotein particles such as high-density lipoproteins (HDL). In addition to apolipoproteins, serum albumin is another protein frequently identified in the corona. Beyond these, typically detected in smaller amounts, are proteins such as coagulation factors, immunoglobulins, complement proteins, and acute phase proteins, which are mostly related to immune system and inflammation reactions.[78] An overview of proteins frequently identified in the coronas of lipid-based delivery vehicles, along with their reported relative abundances, is provided in table 2.1.

Table 2.1 A selection of commonly identified proteins and their reported relative abundances in the protein corona of lipid-based nanoparticles. Data are compiled from multiple sources, with the origin indicated by color. Black: Liu et al.[78], using lipid nanoparticles, orange: Pattipeiluhu et al.[79] using anionic liposomes, blue: Dilliard et al.[71] using lipid nanoparticles, and green: Pozzi et al.[77] using cationic liposomes.

Protein	Relative abundance (%)
Lipoproteins	
Apolipoprotein E	46, 3.8, 14, 2.6
Apolipoprotein C-II	8, 0.6
Apolipoprotein A-II	5, 0.7
Coagulation	
Clusterin	0.2, 0.7, 0.5
Complement	
Complement C1qB	0.9, 6.5, 0.7
Complement C3	0.3, 12.5, 1.1
Immunoglobulins	
Immunoglobulin heavy constant Mu	2.0, 4.2, 7
Acute phase	
Alpha-1-inhibitor 3	0.5,
Alpha-2-HS-glycoprotein	0.5, 0.9
Peroxiredoxin-4	0.5, 0.1
Others	
Vitronectin	0.4, 0.4
Ferritin	0.3
Serotransferrin	0.2, 2.1, 1.0
Serum albumin	4, 17, 5.5, 5.2

CHAPTER 3

Methods

3.1 Nanoporous-silica Thin Film Preparation

Thin films with structured pores in the nanometer size range can be prepared using an amphiphilic copolymer and silica precursors. Amphiphilic copolymers, such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymers (e.g., Pluronic P123), serve as templating agents for porous networks by forming liquid crystalline phases, including lamellar, hexagonal, or cubic bicontinuous structures.[80] When a silica precursor such as tetraethyl orthosilicate (TEOS) is introduced, the silica species condense and adapt to the structure imposed by the copolymer template, forming a crosslinked siloxane network. Thin films can be fabricated by depositing the copolymer-silica solution onto planar substrates using spin coating, followed by extended crosslinking. A prerequisite for silica crosslinking via siloxane bonds is the hydrolysis of the silicate precursors. The extent of hydrolysis in the silica solution is a critical parameter affecting the quality and uniformity of the nanopores and is influenced by factors such as aging time and conditions. After condensation of the silica into a crosslinked network, the thin film is typically calcined at several hundred degrees Celsius to remove the copolymer template and further promote crosslinking.[81]

3.2 Lipid Nanoparticles

Lipid nanoparticles (LNPs) are typically prepared through self-assembly processes that occur upon mixing an aqueous RNA solution with an ethanol-based lipid solution. A critical parameter in the formulation is the pH, as it directly influences the charge state of one commonly used lipid component for LNPs, so called cationic ionizable lipids. Specifically, the RNA solution is prepared at an acidic pH of approximately 2–4, which is well below the pK_{A} of commonly used ionizable lipids, ensuring that their headgroups are positively charged. Mixing of the RNA and lipid solutions can be performed by bulk methods, but microfluidic systems are more commonly used to achieve rapid and controlled mixing at high flow rates. These microfluidic approaches facilitate the production of LNPs with low size polydispersity and diameters typically below 100 nm.[82]

Following LNP formation at acidic pH, a dialysis step is employed to increase the pH to near-physiological conditions (pH 7.4). Studies have demonstrated that pH elevation significantly alters both the internal structure and overall size of LNPs. For example, internal structures with characteristic dimensions of approximately 5–6 nm, which are minimally present immediately after acidic preparation, become more prominent during the pH increase.[83] Additionally, an increase in LNP size during dialysis has been observed,[84] potentially due to LNP fusion events, which have been experimentally confirmed.[85] These findings indicate that pH modulation following LNP assembly plays a crucial role in determining particle size and internal morphology.

Common quality attributes used to characterize LNPs include particle size, polydispersity, and RNA encapsulation efficiency. Nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS) are established techniques for determining particle size. While both methods rely on measuring diffusion properties, they differ in their measurement principles, which can result in variations in reported size distributions. In particular, the assessment of size polydispersity differs between the two techniques. For NTA, polydispersity can be quantified using the coefficient of variation (CV), defined as

$$CV = \frac{\sigma}{\mu}, \tag{3.1}$$

where σ is the standard deviation and μ is the mean particle size. In contrast, DLS typically reports polydispersity via the polydispersity index (PDI), calculated directly by DLS analysis software. Based on CV or PDI values, polydispersity can be categorized as low (CV or PDI ≤ 0.1), medium (0.1–0.4), or high (CV or PDI $\geq$ 0.4).[86], [87]

RNA encapsulation efficiency (EE) quantifies the proportion of RNA enclosed within LNPs versus free RNA remaining in solution after synthesis. A widely used

method to assess EE employs the RiboGreen dye, which fluoresces upon binding to RNA. By comparing the RiboGreen fluorescence intensity of a pristine LNP sample (RG_{pristine}) to that of a lysed sample (RG_{lysed}), where all RNA is exposed in solution, the EE can be calculated as

$$\text{EE} = 1 - \frac{RG_{\text{pristine}}}{RG_{\text{lysed}}}. \tag{3.2}$$

For standard LNP formulations, RNA encapsulation efficiencies greater than 85% (EE > 0.85) are commonly achieved.[88]

3.3 Nanoparticle Tracking Analysis

The size of nanoparticles can be determined by analyzing their motion in a colloidal solution, commonly using a method called nanoparticle tracking analysis (NTA). For this, particle positions are tracked based on their scattering signals, which are detected through optical microscopy. NTA is based on molecular diffusion theory, which describes the random motion of particles due to thermal fluctuations, commonly referred to as diffusion. Diffusion leads to a net flux of particles from regions of high concentration to regions of low concentration, a phenomenon that can be mathematically described by Fick's second law:[89]

$$\frac{\partial c(x,t)}{\partial t} = D\frac{\partial^2 c(x,t)}{\partial x^2}, \tag{3.3}$$

where $c(x,t)$ is the particle concentration as a function of position x and time t, and D is the diffusion coefficient. For simplicity, we consider diffusion in one spatial dimension. The solution to equation (3.3) has the form of a Gaussian distribution,

$$c(x,t) = \frac{1}{\sqrt{4\pi Dt}}, \exp\left(-\frac{x^2}{4Dt}\right), \tag{3.4}$$

which describes the probability of finding a particle at position x at time t, having started at $x = 0$ at $t = 0$. The second moment of this probability distribution yields the mean square displacement (MSD),

$$\langle x^2 \rangle = 2Dt. \tag{3.5}$$

To relate the diffusion coefficient D to particle size, the Einstein relation is employed:

$$D = k_{\text{B}} T \nu, \tag{3.6}$$

where k_{B} is the Boltzmann constant, T is the absolute temperature, and ν is the

particle mobility. The fluctuation-dissipation theorem connects mobility to friction f, where $\nu = 1/f$. For a spherical particle of hydrodynamic radius r_H in a solution of dynamic viscosity η, Stokes' law gives the friction as $f = 6\pi\eta r_\mathrm{H}$. Combining these relationships yields the Stokes-Einstein equation:

$$D = \frac{k_\mathrm{B}T}{6\pi\eta r_\mathrm{H}}. \tag{3.7}$$

In practice, diffusion in d spatial dimensions is considered, and the MSD becomes

$$\langle(\mathbf{r}(t) - \mathbf{r}(0))^2\rangle = 2dDt, \tag{3.8}$$

where $\mathbf{r}(t)$ is the particle position at time t. From particle tracking data, the MSD is estimated and used to calculate D, which is then converted to the hydrodynamic radius r_H via equation (3.7). It is important to note that the hydrodynamic radius may differ from the particle's physical radius, as it reflects how the particle diffuses in solution rather than its geometric dimensions.

Several considerations apply to tracking-based particle size determination. The estimation of the MSD can be affected by errors in locating particle centers and by the limited length of the recorded trajectories.[90] Additionally, commercial instruments such as NanoSight often implement data corrections before reporting particle size distributions. These may include enforcing a minimum track length and smoothing the resulting distributions.[91]

3.4 Fluorescence Microscopy

Fluorescence

Fluorescent molecules, or fluorophores, are commonly used in microscopy to enhance image contrast by specifically labeling the sample. Fluorophores typically absorb photons in the visible spectrum, which excites them from the electronic ground state (S_0) to higher electronic states (e.g., S_1 or S_2). Excitation generally occurs into a higher vibrational level of these excited states, followed by rapid relaxation (internal conversion, $\sim 10^{-12}$ s) to the vibrational ground state of S_1. From there, the fluorophore relaxes back to the electronic ground state, emitting a photon in the process (fluorescence emission, $\sim 10^{-8}$ s). This sequence of absorption, internal conversion, and emission is illustrated in the Jablonski diagram shown in figure 3.1a.

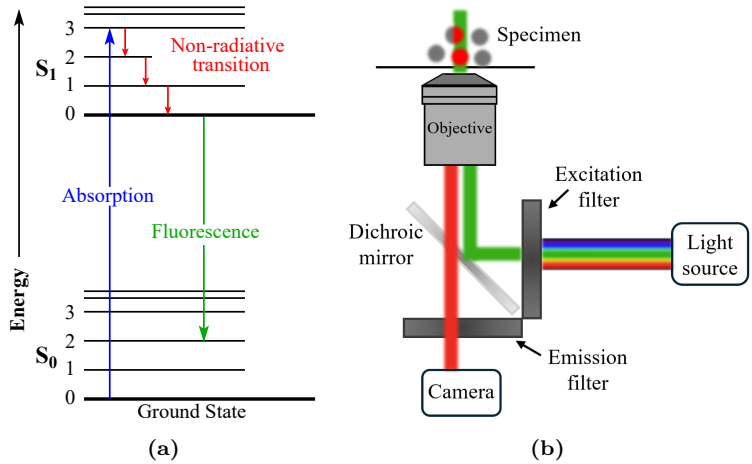

Figure 3.1 Principles of fluorescence microscopy. (a) Jablonski diagram illustrating the fluorescence absorption-emission cycle. Image adapted from [92]. (b) Schematic light path and components of a fluorescence microscope.

Because energy is dissipated during internal conversion, the emitted photon has lower energy, and thus a longer wavelength, than the absorbed photon. This difference in wavelength is known as the Stokes shift. A key limitation of fluorophores is photobleaching, a process by which prolonged illumination leads to chemical changes that irreversibly eliminate fluorescence. Photobleaching can significantly affect fluorescence imaging by reducing signal intensity over time.([93], chapter 1)

Total Internal Reflection Fluorescence Microscopy

Introducing fluorescently labeled species into the specimen allows for highly specific imaging using fluorescence microscopy. Under appropriate conditions, it is possible to achieve strong contrast between regions that are enriched in fluorophores and those that are not, which can enable the detection, and in some cases the visualization, of individual fluorophores. Additionally, different illumination techniques can be applied to enhance imaging specificity. In conventional epifluorescence illumination, fluorophores are excited throughout the entire optical path, including areas outside the focal plane. This leads to fluorescence emission from out-of-focus regions, which contributes to background noise and reduces image contrast (Fig. 3.1b).

Total internal reflection fluorescence (TIRF) microscopy addresses this challenge by using an evanescent field that is generated when light undergoes total internal

reflection at the interface between the specimen and the coverslip. This selective excitation minimizes background fluorescence from areas further away from the interface. As a result, TIRF microscopy produces high-contrast images with minimal signal from out-of-focus regions.

Total Internal Reflection and the Evanescent Field

When light propagates from medium two into medium 1 it refracts through the interface, which is described by Snell's law $n_2 \sin\theta_2 = n_1 \sin\theta_1$ with n_2, n_1 being the refractive index (RI) of medium 2 and 1, and θ_2, θ_1 being the angle of the light propagation measured from the normal of the interface. Assuming that the incident light is an "infinitely" extended plane wave, and medium 2 has a bigger RI than medium 1, one can observe total internal reflection at the interface if the angle θ_2 is greater than the "critical angle" θ_c, given by:

$$\theta_c = \arcsin \frac{n_1}{n_2}. \tag{3.9}$$

The assumption of a plane wave is a good approximation for unfocused light, where the beam width is many times the wavelength of the light. Total internal reflection occurs, e.g., if a light beam propagates in glass and hits an interface to water at an incident angle $\theta > \theta_c$ (measured from the normal to the interface). With a RI of glass $n_1 = 1.52$ and water $n_2 = 1.33$, light with an incident angle larger than $\theta_c = 61.0^\circ$ is the subject of total internal reflection. All of the light reflects back into the solid. However, some of the incident energy penetrates through the interface and propagates parallel to the surface in the plane of incidence. The non-radiative field in the liquid, called the "evanescence field", has the same frequency as the incident light and is capable of exciting fluorophores. Significantly, for TIRF microscopy, the evanescence field intensity I (for an infinitely wide beam) exponentially decays with perpendicular distance z from the interface:

$$I(z) = I(0) \exp(-z/d), \tag{3.10}$$

where the characteristic penetration depth d of the evanescence field is given by

$$d = \frac{\lambda_0}{4\pi} (n_2^2 \sin\theta - n_1^2)^{-1/2}, \tag{3.11}$$

with λ_0, the wavelength of the light in vacuum. The characteristic penetration depth is in the order of λ_0, d decreases for increasing θ, and $d \to \infty$ for $\theta \to \theta_c$.[94]

3.5 Supported Lipid Bilayers

An attractive approach to investigate the chemical and physical properties of lipid membranes is the use of supported lipid bilayers (SLBs). SLBs are lipid bilayers formed on solid substrates, providing a high degree of experimental control while retaining key characteristics of biological membranes. As such, they serve as versatile model systems for studying membrane-associated processes.

Several established methods are available for preparing SLBs, including Langmuir-Blodgett deposition,[95] solvent-assisted bilayer formation,[96] and the vesicle adsorption and fusion method.[97] In this thesis, SLBs were prepared using the vesicle adsorption and fusion method. In this process, a hydrophilic substrate is incubated with a suspension of small unilamellar vesicles. The formation of an SLB proceeds through a sequence of steps: (1) vesicle approach to the substrate, (2) adhesion of vesicles to the surface, (3) rupture and fusion of vesicles to form localized bilayer patches, and (4) lateral spreading of these patches until a continuous bilayer is established.

The suitability of a substrate for SLB formation depends on fundamental surface properties, including surface energy, chemistry, charge, and roughness. In practice, SLBs can be formed on various materials such as borosilicate glass, SiO_2, mica, aluminum, gold, Teflon AF, and monolayer graphene. Generally, SLB formation is favored on hydrophilic surfaces.[98]

A key feature of SLBs as model membranes is the lateral mobility of lipids, which is preserved due to the lack of permanent adhesion between the lipids and the support. Neutron reflectometry studies reveal the presence of a thin water layer, typically a few nanometers thick, between the SLB and the substrate.[99] This water layer enables lipid diffusion, although the diffusion coefficients for SLBs (approximately 1 to $4\,\mu m^2\,s^{-1}$) are generally lower than those of free-standing bilayers (approximately $8\,\mu m^2\,s^{-1}$).[100] This reduction reflects residual adhesive interactions and steric constraints imposed by the substrate. Such constraints can limit the incorporation of transmembrane proteins and hinder processes like membrane translocation.

To mitigate these limitations and improve the biomimetic properties of SLBs, porous substrates have been explored. For instance, porous silica supports have been shown to enhance lateral lipid mobility and allow the observation of membrane translocation events involving small molecules.[101]

CHAPTER 4

Summary of Results

4.1 Paper A

The work presented in this paper aims to elucidate the endosomal escape process of lipid nanoparticles using a simplistic model system representing endosomal conditions. Specifically, lipid nanoparticles were molecularly tethered to an anionic lipid bilayer, which is formed on a solid nanoporous support embedded in a microfluidic channel that allows a stepwise decrease of pH through solution exchange.

Time-resolved imaging of the fluorescently labeled LNPs revealed that a decrease in pH induces fusion of LNPs with the anionic-supported lipid bilayer (Fig.4.1a). The fusion efficiency displayed a pH dependency, with the highest efficiency observed for a pH decrease from 6.6 to 6.0. This aligns with the protonation of ionizable lipids in the LNPs, with 50% protonized ionizable lipids at pH 6.4, which was obtained with the 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS) charge titration method (Fig.4.1b).

An inspection of the fluorescence signal of individual LNPs upon fusion indicates that, upon fusion, >90% of the LNP lipid molecules escape from the fusion site through lateral diffusion in the supported lipid bilayer. Inspection of the mRNA fluorescence signal indicates that mRNA is displaced from the site of fusion, however, to a lesser extent of approximately 70% compared to the lipid molecules. The data

suggests that the main path for mRNA escape from the fusion site is diffusion into the aqueous bulk phase and not through translocation across the lipid bilayer.

The fate of mRNA released from the LNP fusion sites is of interest as this is a potential pathway that leads to cytosolic and, thus, effective delivery of mRNA. Another data set presented in this paper indicates that individual mRNA molecules rebind to the supported lipid bilayer after being released into the bulk solution. Observation of the diffusivity of rebound mRNA molecules at different pH values between 5.6 and 7.4 suggests a pH-dependent interaction strength of mRNA and supported lipid bilayer. The most probable explanation is that mRNA rebinding is driven by electrostatic attraction to protonated ionizable lipids that escape into the supported lipid bilayer upon LNP fusion. The pH dependency can then be explained by a varying density of protonated ionizable lipids in the supported lipid bilayer, where notably the apparent pK_A of the ionizable lipids is significantly increased if not associated with nucleic acids, from $pK_A = 6.4$ to 9.4.[102] Translating this into the endosomal environment, the escape of mRNA into the endosomal volume could be followed by rebinding and diffusion along the endosomal membrane, where translocation across the membrane is potentially possible through, e.g., a local membrane disruption. Considering the importance of mRNA translocation for the overall performance of LNPs, this type of electrostatically-mediated rebinding may be an undesired effect and, therefore, could be the subject of future investigations.

Lastly, the paper discusses the observation that LNP fusion efficiency depends on the lipid composition of the LNPs. Specifically, LNPs containing 10 mol% of the phospholipid DSPC display a lower efficiency for fusion than LNPs containing 4.65 mol% DSPC (Fig.4.1c). DSPC is located primarily at the LNP surface, and as such, varying amounts of this lipid are expected to affect the molecular organization at the LNP surface. The observed difference in fusion efficiency indicates the important role of prevalent lipid phases for efficient fusion, especially at the LNP surface. Notably, the same LNP compositions showed a significant difference in cellular transfection efficiency, which was attributed to poorer endosomal escape capacity of the LNPs with higher DSPC content of 10 mol%. The qualitative agreement between cell transfection and fusion efficiency highlights the value of observations made with the model system and, thus, potentially a tool to assist in the design of improved LNP compositions.

In conclusion, this paper presents a method that enables the investigation of fusion between LNPs and lipid bilayers. This method was used to gain insights into mechanisms potentially important for the escape of the LNP's mRNA cargo from the endosomal compartment, which is considered a major bottleneck for currently used LNP-based therapeutics and vaccines. Insights into the mechanisms of endosomal escape processes and the availability of relevant model systems, as presented in this paper, could thus contribute to the development of improved LNP compositions.

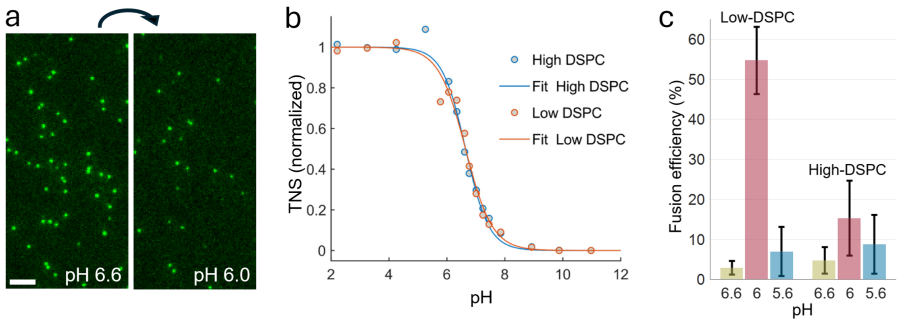

Figure 4.1 Selection of results from paper A. **(a)** Fluorescence micrographs showing individual LNPs labeled with the lipid-conjugated dye Rhodamine-DOPE, tethered to an anionic lipid bilayer. The near-complete disappearance of fluorescence signal from individual LNPs indicates fusion with the lipid bilayer, triggered by a decrease in bulk phase pH from 6.6 to 6.0. **(b)** Degree of ionization of LNPs, quantified using 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS) fluorescence intensity. The inflection point at pH $\approx$ 6.4, observed for both high (10 mol%) and low (4.65 mol%) DSPC content LNPs, coincides with the pH range at which the highest fusion efficiency is observed, as shown in **(c)** Fusion efficiency of LNPs in response to a pH decrease from 7.4 to 6.6 (yellow), 6.6 to 6.0 (red), and 6.0 to 5.6 (blue). While both LNP types show maximal fusion near their respective ionization inflection points, low-DSPC LNPs display significantly lower fusion efficiency at pH decrease from 6.6 to 6.0 than their high-DSPC counterparts.

4.2 Paper B

When an LNP-based therapeutic is administered to patients, the LNPs readily interact with bodily fluids such as blood serum upon intravenous injections. It is known that the interaction with bodily fluids leads to the adsorption of biological molecules, and in particular proteins, onto the LNP surface. The adsorption is considered to result in a layer of proteins on the LNP surface, sometimes called a protein corona, that can alter the interfacial interactions of the LNPs with their environment.

The work presented in this paper aims to improve the understanding of the impact of protein corona formation on LNPs. A part of this work specifically focuses on the effect of a protein corona on interfacial interactions between LNPs and an anionic lipid bilayer, which is relevant for the endosomal escape process and is currently considered a major bottleneck for the efficiency of LNP-based therapeutics, as described in 2.

Observations presented in the paper indicate that the formation of a protein corona can affect the capability of LNPs to retain their mRNA cargo. While the average hydrodynamic size of the LNPs remained mostly unaffected by protein corona formation (Fig.4.2a), fluorescence microscopy imaging of individual LNPs revealed a decrease of mRNA fluorescence signal upon protein corona formation, which indicates partial mRNA release (Fig.4.2b). This observation may be explained through a disruption of the lipid organization on the LNP surface by adsorbed proteins that make the LNP surface more susceptible to translocation of hydrophilic mRNA from a lipiduous LNP core into an aqueous environment outside the LNP.

Current LNP formulations often contain lipids that are conjugated with a PEG molecule. These PEG-lipids are designed to be released through the interaction with serum albumin, a major protein component of serum. The ability to release PEG-lipids, sometimes called PEG-shedding, has been shown to enhance the performance of LNP-based therapeutics. In this work, PEG-shedding was quantified for individual LNPs using time-resolved fluorescence microscopy imaging. Specifically, incubation in 10% fetal bovine serum for 180 minutes led, on average, to the release of $36 \pm 6\%$ of the PEG-lipid. Practically all PEG-lipid release took place 60 minutes after the start of incubation and could be described by an exponential decay law with a half-life time of 11 ± 1 minutes (Fig.4.2c).

When molecularly tethered to an anionic lipid bilayer that is supported by a nanoporous substrate, LNPs displayed reduced lateral mobility if preincubated in 10% fetal bovine serum for 3 hours. This suggests altered interfacial interactions due to the modifications of LNPs and/or the presence of proteins on the LNP surface after serum preincubation. Simulating the environment for LNPs in an endosome by acidifying the bulk solution (as described in the summary of paper A) leads to fusion of LNPs with the anionic lipid bilayer. A comparison of the pH-dependent

fusion efficiency of serum preincubated and pristine LNPs showed that serum preincubated LNPs preferentially fused at more moderate acidification (Fig.4.2d). This observation suggests that the presence of a protein corona does not hamper the fusion of LNPs with an endosomal membrane and is thus likely not a limiting factor for the endosomal escape efficiency of current LNP formulations. The results rather indicate that the interactions with serum proteins could enhance the LNP fusion efficiency at moderate acidification of the endosomal lumen, which is considered an advantage for the successful endosomal escape of the LNP mRNA cargo.

In conclusion, the observations presented in this paper contribute to a better understanding of the impact of protein adsorption on LNPs. Specifically, the adsorption can decrease the capability of LNPs to retain their mRNA cargo, which can be considered an undesired effect. Further, the release of PEG-lipids from LNPs changes the surface composition of LNPs within minutes after the start of serum incubation. Finally, results indicate that alterations of LNPs through interaction with serum proteins are not a limiting factor for the capacity of LNPs to perform endosomal escape but potentially even enhance its capacity.

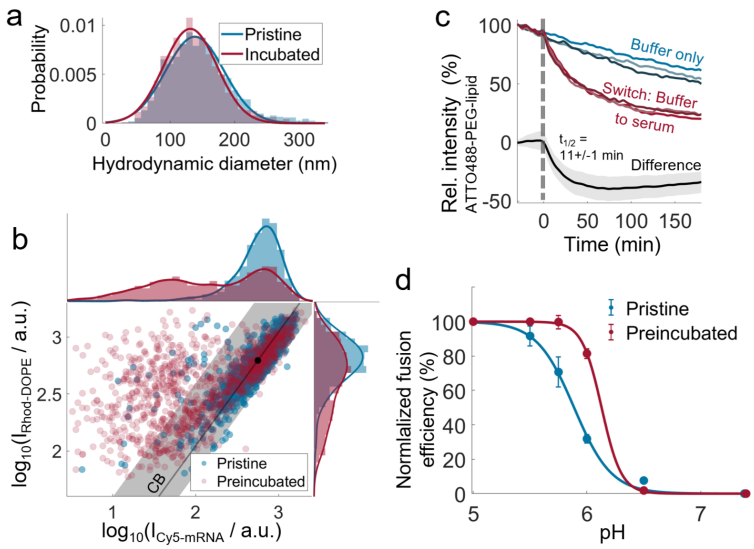

Figure 4.2 Selection of results from paper B. **(a)** Hydrodynamic diameter distribution determined with nanoparticle tracking analysis of LNPs incubated for 3h at room temperature in buffer (pristine) or 10% (v/v) fetal bovine serum (preincubated). Mean and standard deviation of 138 ± 45 nm and 131 ± 41 nm for pristine and preincubated LNPs, respectively, determined using a Gaussian fit. **(b)** Log-log plot of the single-particle fluorescence signal (labeled lipid- and mRNA-moieties: Rhodamine-DOPE and Cy5-mRNA) of tethered LNPs shows a significant difference between pristine and preincubated LNPs, with 32% of preincubated LNPs located outside the 95% confidence band (CB) of pristine LNPs (shaded area). **(c)** Time-resolved fluorescence signal of ATTO488-labeled PEG-lipid for representative individual LNPs subjected to either buffer only (blue palette) or a buffer-to-serum switch at t = 0 (red palette; dashed line indicating switch). The average signal difference between LNPs subjected to buffer only and to the buffer-to-serum switch shows serum-induced PEG-shedding (black line; shaded area ±1 s.d.). **(d)** Fusion efficiency as a function of pH, assessed based on the cumulative percentage of fused LNPs, normalized with total fusion efficiency. Solid lines represent sigmoidal fits to the data and uncertainty based on three replicates.

CHAPTER 5

Outlook

The results presented in papers A and B contribute to a deeper understanding of the processes involved in the cellular processing of LNPs used for gene therapies. In particular, the findings address the behavior of LNPs within endosomal compartments, where their cargo is frequently entrapped, ultimately limiting the overall efficacy of this gene delivery approach. This chapter provides a brief outline of potential future research directions aimed at further elucidating the physicochemical mechanisms underlying LNP-mediated gene delivery.

5.1 Can LNP Surface Modifications Enhance Fusion?

One major takeaway from the observations made in papers A and B is that characteristics of the LNP surface alter the interactions with anionic lipid membranes, which is believed to be a key process for efficient endosomal escape. Characteristics of the LNP surface can be, e.g., lipid phases mediated by specific lipid types, passivation through inert molecules such as PEG, or the presence of adsorbed proteins. These considerations could hold the potential for the design of LNPs with improved gene delivery performance by further investigation of the impact of LNP surface characteristics on the capability to fuse with anionic membranes. However,

it should be noted that the design of LNPs and gene delivery vehicles, in general, must respect a vast number of different processes related to, e.g., the biocompatibility of used materials, clearance from circulation, adverse immune responses, storage stability, scalability of manufacturing processes, and many more. Nevertheless, investigating the effects of modifications that alter the LNP surface characteristics could be one possible way to address the current limitations of LNP delivery efficiency. Modifications affecting the LNP surface characteristics could be achieved through:

- Altered lipid compositions
- Altered molecules used for passivation
- Adsorption of proteins on LNP surface

5.2 Does LNP Fusion Enable mRNA Translocation?

In vitro assays indicate that disruption of the endosomal membrane can serve as a proxy for the escape of LNP cargo from endosomes.[103] However, to our knowledge, it remains unclear whether such membrane disruption is confined to the immediate site of interaction between LNPs and the membrane, or whether it results in a more global loss of endosomal membrane integrity. In the former case, local membrane disruption, potentially triggered by close LNP-membrane interactions or fusion events, could create transient openings that permit the translocation of LNP cargo. In contrast, a more widespread disruption could arise from mechanisms such as osmotic stress due to the presence of protonatable molecules (e.g., cationic ionizable lipids) within the endosome, interference with endosomal maturation, or modulation of intracellular trafficking. Additionally, lipid transfer upon LNP fusion could significantly alter the composition of the endosomal membrane, changing membrane properties.

Given the size and negative charge of RNA molecules, particularly mRNA, their translocation across the hydrophobic core of a lipid membrane, especially one enriched in anionic lipids like the endosomal membrane, can be considered a low-probability event. Nevertheless, the demonstrated efficacy of LNP-based delivery systems confirms that such translocation does occur, albeit with low efficiency. Gaining mechanistic insight into how these rare but essential translocation events are facilitated could guide the rational design of more efficient delivery vehicles. Therefore, investigating how LNPs mediate cargo translocation across anionic membranes is a key step toward advancing RNA therapeutics.

The model system mimicking the endosomal environment used in Papers A and B

was designed to isolate one potential contributor to endosomal escape: pH-triggered fusion of LNPs with anionic lipid membranes. This fusion process was shown to promote the release of significant amounts of mRNA cargo at the fusion site. However, direct evidence that fusion itself facilitates mRNA translocation across the lipid membrane is still lacking. Since crossing the endosomal membrane is a prerequisite for successful cytosolic delivery, an important question remains: Does LNP fusion directly result in mRNA translocation?

One consideration in evaluating mRNA translocation is the potential influence of the solid support used in our current model system. While the nanoporous substrate includes nanoscale pores that allow for the study of small molecule translocation,[101] the steric and spatial constraints may hinder the passage of larger cargos such as mRNA. Additionally, the simplified lipid composition of the model membrane, although representative of key features of the endosomal membrane, remains rudimentary compared to the complexity of native endosomal membranes. Preliminary results from our group suggest that forming phase-separated lipid domains may play a role in the endosomal escape, potentially facilitating translocation at domain boundaries where membrane order is disrupted. These findings highlight the need for further studies that incorporate more complex and physiologically relevant membrane compositions to better understand the factors that govern mRNA translocation during LNP-mediated delivery.

References

[1] T. R. Damase, R. Sukhovershin, C. Boada, F. Taraballi, R. I. Pettigrew, and J. P. Cooke, "The limitless future of rna therapeutics," *Frontiers in Bioengineering and Biotechnology*, vol. 9, p. 628 137, 2021.

[2] A. Yen, Y. Cheng, M. Sylvestre, H. H. Gustafson, S. Puri, and S. H. Pun, "Serum nuclease susceptibility of mrna cargo in condensed polyplexes," *Molecular pharmaceutics*, vol. 15, no. 6, pp. 2268–2276, 2018.

[3] S. F. Dowdy, R. L. Setten, X.-S. Cui, and S. G. Jadhav, "Delivery of rna therapeutics: The great endosomal escape!" *Nucleic acid therapeutics*, vol. 32, no. 5, pp. 361–368, 2022.

[4] J. Staring, M. Raaben, and T. R. Brummelkamp, "Viral escape from endosomes and host detection at a glance," *Journal of Cell Science*, vol. 131, no. 15, jcs216259, 2018.

[5] S. A. Smith, L. I. Selby, A. P. Johnston, and G. K. Such, "The endosomal escape of nanoparticles: Toward more efficient cellular delivery," *Bioconjugate chemistry*, vol. 30, no. 2, pp. 263–272, 2018.

[6] J. A. Kulkarni, P. R. Cullis, and R. Van Der Meel, "Lipid nanoparticles enabling gene therapies: From concepts to clinical utility," *Nucleic acid therapeutics*, vol. 28, no. 3, pp. 146–157, 2018.

[7] K. Paunovska, D. Loughrey, and J. E. Dahlman, "Drug delivery systems for rna therapeutics," *Nature Reviews Genetics*, vol. 23, no. 5, pp. 265–280, 2022.

[8] V. Francia, R. M. Schiffelers, P. R. Cullis, and D. Witzigmann, "The biomolecular corona of lipid nanoparticles for gene therapy," *Bioconjugate chemistry*, vol. 31, no. 9, pp. 2046–2059, 2020.

[9] M. G. Rossmann, "Structure of Viruses: A Short History," *Q. Rev. Biophys.*, vol. 46, no. 2, pp. 133–180, 2013.

[10] J. E. Jones, V. Le Sage, and S. S. Lakdawala, "Viral and Host Heterogeneity and Their Effects on the Viral Life Cycle," *Journal of Virology*, vol. 99, no. 3, e01234–25, 2025.

[11] M. S. Maginnis, "Virus–Receptor Interactions: The Key to Cellular Invasion," *J. Mol. Biol.*, vol. 430, no. 17, pp. 2590–2611, 2018.

[12] J. Huotari and A. Helenius, "Endosome Maturation," *EMBO J.*, vol. 30, no. 17, pp. 3481–3500, 2011.

[13] S. C. Harrison, "Viral membrane fusion," *Nature Structural Molecular Biology*, vol. 15, no. 7, pp. 690–698, 2008.

[14] C. M. Wiethoff, H. Wodrich, L. Gerace, and G. R. Nemerow, "Adenovirus protein vi mediates membrane disruption following capsid disassembly," *Journal of Virology*, vol. 79, no. 4, pp. 1992–2000, 2005.

[15] A. Panjwani, M. Strauss, S. Gold, *et al.*, "Capsid protein vp4 of human rhinovirus induces membrane permeability by the formation of a size-selective multimeric pore," *PLoS Pathogens*, vol. 10, no. 8, e1004294, 2014.

[16] S. Suikkanen, M. Antila, A. Jaatinen, M. Vihinen-Ranta, and M. Vuento, "Release of canine parvovirus from endocytic vesicles," *Virology*, vol. 316, no. 2, pp. 267–280, 2003.

[17] C. E. Hulseberg, L. Fénéant, K. M. Szymańska, and J. M. White, "Lamp1 increases the efficiency of lassa virus infection by promoting fusion in less acidic endosomal compartments," *mBio*, vol. 9, no. 1, e01818–17, 2018.

[18] Z. Antošová, M. Macková, V. Král, and T. Macek, "Therapeutic application of peptides and proteins: Parenteral forever?" *Trends in Biotechnology*, vol. 27, no. 11, pp. 628–635, 2009.

[19] M. F. Naso, B. Tomkowicz, W. L. Perry, and W. R. Strohl, "Adeno-associated virus (aav) as a vector for gene therapy," *BioDrugs*, vol. 31, no. 4, pp. 317–334, 2017.

[20] S. Daya and K. I. Berns, "Gene therapy using adeno-associated virus vectors," *Clinical Microbiology Reviews*, vol. 21, no. 4, pp. 583–593, 2008.

[21] J. Y. Shin, "Recent advances in rna therapeutics and rna delivery systems based on nanoparticles," *Journal of Controlled Release*, vol. 272, pp. 96–113, 2018.

[22] J. D. Kulkarni, D. Witzigmann, S. Chen, P. R. Cullis, and R. Van Der Meel, "The current landscape of nucleic acid therapeutics," *Nature Nanotechnology*, vol. 16, no. 6, pp. 630–643, 2021.

[23] B.-C. Ma, S.-B. Zhang, H.-M. Jiang, B.-D. Zhao, and H.-T. Lv, "Lipoplex morphologies and their influences on transfection efficiency in gene delivery," *Journal of Controlled Release*, vol. 123, no. 3, pp. 184–194, 2007.

[24] Y. Hattori, Y. Machida, M. Honda, *et al.*, "Small interfering rna delivery into the liver by cationic cholesterol derivative-based liposomes," *Journal of Liposome Research*, vol. 27, no. 4, pp. 264–273, 2016.

[25] Y. Liu, X. Zhang, S. Li, J. Li, X. Wang, and Z. Zhang, "Barriers and strategies of cationic liposomes for cancer gene therapy," *Molecular Therapy: Methods Clinical Development*, vol. 18, pp. 1–14, 2020.

[26] L. Sercombe, T. Veerati, F. Moheimani, S. Y. Wu, and A. Sood, "Advances and challenges of liposome assisted drug delivery," *Frontiers in Pharmacology*, vol. 6, p. 286, 2015.

[27] M. Yanez Arteta, T. Kjellman, S. Bartesaghi, *et al.*, "Successful reprogramming of cellular protein production through mrna delivered by functionalized lipid nanoparticles," *Proceedings of the National Academy of Sciences*, vol. 115, no. 15, E3351–E3360, 2018.

[28] M. Jayaraman, S. M. Ansell, B. L. Mui, *et al.*, "Maximizing the potency of sirna lipid nanoparticles for hepatic gene silencing in vivo," *Angewandte Chemie International Edition*, vol. 51, no. 52, pp. 13 053–13 057, 2012.

[29] B. L. Mui, Y. K. Tam, M. Jayaraman, *et al.*, "Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of sirna lipid nanoparticles," *Molecular Therapy - Nucleic Acids*, vol. 2, e139, 2013.

[30] K. Garber, "Alnylam launches era of rnai drugs," *Nature Biotechnology*, vol. 36, no. 9, pp. 777–778, 2018.

[31] F. P. Polack, S. J. Thomas, N. Kitchin, *et al.*, "Safety and efficacy of the bnt162b2 mrna covid-19 vaccine," *New England Journal of Medicine*, vol. 383, no. 27, pp. 2603–2615, 2020.

[32] L. R. Baden, H. M. El Sahly, B. Essink, *et al.*, "Efficacy and safety of the mrna-1273 sars-cov-2 vaccine," *New England Journal of Medicine*, vol. 384, no. 5, pp. 403–416, 2021.

[33] M. Zhao, M. Li, Z. Zhang, T. Gong, and X. Sun, "Induction of hiv-1 gag specific immune responses by cationic micelles mediated delivery of gag mrna," *Drug Delivery*, vol. 23, no. 7, pp. 2596–2607, 2016.

[34] M. Molina, M. Asadian-Birjand, J. Balach, J. Bergueiro, E. Miceli, and M. Calderón, "Stimuli-responsive nanogel composites and their application in nanomedicine," *Chemical Society Reviews*, vol. 44, no. 17, pp. 6161–6186, 2015.

[35] A. Kumari, S. Yadav, and S. Yadav, "Biodegradable polymeric nanoparticles based drug delivery systems," *Colloids and Surfaces B: Biointerfaces*, vol. 75, no. 1, pp. 1–18, 2010.

[36] L. Zhang, J. M. Chan, F. X. Gu, and J.-W. Rhee, "Self-assembled lipidpolymer hybrid nanoparticles: A robust drug delivery platform," *ACS Nano*, vol. 2, no. 8, pp. 1696–1702, 2008.

[37] P. R. Desai, S. Marepally, A. R. Patel, C. Voshavar, A. Chaudhuri, and M. Singh, "Topical delivery of anti-tnfα sirna and capsaicin via novel lipid-polymer hybrid nanoparticles efficiently inhibits skin inflammation in vivo," *Journal of Controlled Release*, vol. 170, no. 1, pp. 51–63, 2013.

[38] L. J. Scott, "Givosiran: First approval," *Drugs*, vol. 80, no. 9, pp. 955–961, 2020.

[39] J.-H. Lee, S. Kang, M. Ahn, H. Jang, and D.-H. Min, "Development of dual-pore coexisting branched silica nanoparticles for efficient gene-chemo cancer therapy," *Small*, vol. 14, no. 7, p. 1 702 564, 2018.

[40] D. Zheng, D. A. Giljohann, D. L. Chen, M. D. Massich, X.-Q. Wang, H. Iordanov, *et al.*, "Topical delivery of sirna-based spherical nucleic acid nanoparticle conjugates for gene regulation," *Molecular Therapy*, vol. 20, no. 3, pp. 488–495, 2012.

[41] N. W. S. Kam, Z. Liu, and H. Dai, "Functionalization of carbon nanotubes via cleavable disulfide bonds for efficient intracellular delivery of sirna and potent gene silencing," *Journal of the American Chemical Society*, vol. 127, no. 36, pp. 12 492–12 493, 2005.

[42] W. Xu, Z. Lin, and F. Caruso, "Assembly and biological functions of metal-biomolecule network nanoparticles formed by metal-phosphonate coordination," *Science Advances*, vol. 10, no. 1, eads9542, 2024.

[43] S. F. Dowdy, "Endosomal escape of rna therapeutics: How do we solve this rate-limiting problem?" *RNA*, vol. 29, no. 4, pp. 474–480, 2023.

[44] A. Wittrup, A. Ai, X. Liu, *et al.*, "Visualizing lipid-formulated sirna release from endosomes and target gene knockdown," *Nature Biotechnology*, vol. 33, no. 8, pp. 870–876, 2015.

[45] F. Cardarelli, L. Digiacomo, C. Marchini, *et al.*, "The intracellular trafficking mechanism of lipofectamine-based transfection reagents and its implication for gene delivery," *Scientific Reports*, vol. 6, p. 25 879, 2016.

[46] C. Bissig and J. Gruenberg, "Lipid sorting and multivesicular endosome biogenesis," *Cold Spring Harbor Perspectives in Biology*, vol. 5, no. 1, a016758, 2013.

[47] Y. Xu and F. C. Szoka, "Mechanism of dna release from cationic liposome/dna complexes used in cell transfection," *Biochemistry*, vol. 35, no. 10, pp. 5616–5623, 1996.

[48] H. Farhood, N. Serbina, and L. Huang, "The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1190, no. 2, pp. 133–139, 1994.

[49] I. S. Zuhorn, U. Bakowsky, E. Polushkin, *et al.*, "Nonbilayer phase of lipoplex-membrane mixture determines endosomal escape of genetic cargo and transfection efficiency," *Molecular Therapy*, vol. 11, no. 5, pp. 801–810, 2005.

[50] D. C. Drummond, M. Zignani, and J.-C. Leroux, "Current status of ph-sensitive liposomes in drug delivery," *Progress in Lipid Research*, vol. 39, no. 6, pp. 409–460, 2000.

[51] M. Ramezanpour and D. P. Tieleman, "Computational insights into the role of cholesterol in inverted hexagonal phase stabilization and endosomal drug release," *Langmuir*, vol. 38, no. 24, pp. 7462–7471, 2022.

[52] J. Philipp, J. O. Raedler, *et al.*, "Ph-dependent structural transitions in cationic ionizable lipid mesophases are critical for lipid nanoparticle function," *Proceedings of the National Academy of Sciences*, vol. 120, no. 50, e2310491120, 2023.

[53] Z. Chen, Y. Tian, J. Yang, *et al.*, "Modular design of biodegradable ionizable lipids for improved mrna delivery and precise cancer metastasis delineation in vivo," *Journal of the American Chemical Society*, vol. 145, no. 44, pp. 24 302–24 314, 2023.

[54] N. D. Sonawane, F. C. Szoka, and A. S. Verkman, "Chloride accumulation and swelling in endosomes enhances dna transfer by polyamine-dna polyplexes," *Journal of Biological Chemistry*, vol. 278, no. 45, pp. 44 826–44 831, 2003.

[55] A. K. Varkouhi, M. Scholte, G. Storm, and H. J. Haisma, "Endosomal escape pathways for delivery of biologicals," *Journal of Controlled Release*, vol. 151, no. 3, pp. 220–228, 2011.

[56] J. P. Bost, M. Ojansivu, M. J. Munson, *et al.*, "Novel endosomolytic compounds enable highly potent delivery of antisense oligonucleotides," *Communications Biology*, vol. 5, no. 1, p. 185, 2022.

[57] S. M. Farkhani and A. Valizadeh, "Cell penetrating peptides: Efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules," *Peptides*, vol. 57, pp. 78–94, 2014.

[58] D. Mudhakir and H. Harashima, "Learning from the viral journey: How to enter cells and how to overcome intracellular barriers to reach the nucleus," *The AAPS Journal*, vol. 11, no. 1, pp. 65–77, 2009.

[59] J.-O. You and D. T. Auguste, "Nanocarrier cross-linking density and ph sensitivity regulate intracellular gene transfer," *Nano Letters*, vol. 9, no. 12, pp. 4467–4473, 2009.

[60] P. Andreozzi, E. Diamanti, K. Rapp Py-Daniel, *et al.*, "Exploring the ph sensitivity of poly(allylamine) phosphate supramolecular nanocarriers for intracellular sirna delivery," *ACS Applied Materials & Interfaces*, vol. 9, no. 44, pp. 38 242–38 254, 2017.

[61] M. Grau, Y. Lin, M. Höhn, U. Lächelt, and E. Wagner, "Strategies and mechanisms for endosomal escape of therapeutic nucleic acids," *Advanced Drug Delivery Reviews*, vol. 176, p. 113 888, 2024.

[62] C.-L. Chan, R. Majzoub, A. M. Klibanov, and R. Langer, "Endosomal escape and transfection efficiency of pegylated cationic liposome–dna complexes prepared with an acid-labile peg-lipid," *Biomaterials*, vol. 33, no. 13, pp. 3583–3590, 2012.

[63] M. A. Saad and T. Hasan, "Spotlight on photoactivatable liposomes beyond drug delivery: An enabler of multitargeting of molecular pathways," *Bioconjugate Chemistry*, vol. 33, no. 11, pp. 2041–2064, 2022.

[64] G. Caracciolo, "Liposome–protein corona in a physiological environment: Challenges and opportunities for targeted delivery of nanomedicines," *Nanomedicine: Nanotechnology, Biology and Medicine*, vol. 11, no. 3, pp. 543–557, 2015.

[65] D. Boraschi, P. Italiani, R. Palomba, *et al.*, "Nanoparticles and innate immunity: New perspectives on host defence," in *Seminars in immunology*, Elsevier, vol. 34, 2017, pp. 33–51.

[66] T. U. Wani, S. N. Raza, and N. A. Khan, "Nanoparticle opsonization: Forces involved and protection by long chain polymers," *Polymer Bulletin*, vol. 77, pp. 3865–3889, 2020.

[67] B. Pelaz, P. del Pino, P. Maffre, *et al.*, "Surface functionalization of nanoparticles with polyethylene glycol: Effects on protein adsorption and cellular uptake," *ACS nano*, vol. 9, no. 7, pp. 6996–7008, 2015.

[68] H. Liu, K. D. Moynihan, Y. Zheng, *et al.*, "Structure-based programming of lymph-node targeting in molecular vaccines," *Nature*, vol. 507, no. 7493, pp. 519–522, 2014.

[69] S. C. Wilson, J. L. Baryza, A. J. Reynolds, *et al.*, "Real time measurement of peg shedding from lipid nanoparticles in serum via nmr spectroscopy," *Molecular Pharmaceutics*, vol. 12, no. 2, pp. 386–392, 2015.

[70] M. Lundqvist, J. Stigler, G. Elia, I. Lynch, T. Cedervall, and K. A. Dawson, "Nanoparticle size and surface properties determine the protein corona with possible implications for biological impacts," *Proceedings of the National Academy of Sciences*, vol. 105, no. 38, pp. 14 265–14 270, 2008.

[71] S. A. Dilliard, Q. Cheng, and D. J. Siegwart, "On the mechanism of tissue-specific mrna delivery by selective organ targeting nanoparticles," *Proceedings of the National Academy of Sciences*, vol. 118, no. 52, e2109256118, 2021.

[72] Q. Yu, L. Zhao, C. Guo, B. Yan, and G. Su, "Regulating protein corona formation and dynamic protein exchange by controlling nanoparticle hydrophobicity," *Frontiers in bioengineering and biotechnology*, vol. 8, p. 210, 2020.

[73] A. E. Nel, L. Mädler, D. Velegol, *et al.*, "Understanding biophysicochemical interactions at the nano–bio interface," *Nature materials*, vol. 8, no. 7, pp. 543–557, 2009.

[74] E. Casals, T. Pfaller, A. Duschl, G. J. Oostingh, and V. Puntes, "Time evolution of the nanoparticle protein corona," *ACS nano*, vol. 4, no. 7, pp. 3623–3632, 2010.

[75] M. Mahmoudi, "The need for improved methodology in protein corona analysis," *Nature Communications*, vol. 13, no. 1, p. 49, 2022.

[76] D. Baimanov, J. Wang, J. Zhang, *et al.*, "In situ analysis of nanoparticle soft corona and dynamic evolution," *Nature Communications*, vol. 13, no. 1, p. 5389, 2022.

[77] D. Pozzi, G. Caracciolo, A. L. Capriotti, *et al.*, "Surface chemistry and serum type both determine the nanoparticle–protein corona," *Journal of proteomics*, vol. 119, pp. 209–217, 2015.

[78] K. Liu, R. Nilsson, E. Lázaro-Ibáñez, *et al.*, "Multiomics analysis of naturally efficacious lipid nanoparticle coronas reveals high-density lipoprotein is necessary for their function," *Nature Communications*, vol. 14, no. 1, p. 4007, 2023.

[79] R. Pattipeiluhu, S. Crielaard, I. Klein-Schiphorst, B. I. Florea, A. Kros, and F. Campbell, "Unbiased identification of the liposome protein corona using photoaffinity-based chemoproteomics," *ACS central science*, vol. 6, no. 4, pp. 535–545, 2020.

[80] N. K. Raman, M. T. Anderson, and C. J. Brinker, "Template-based approaches to the preparation of amorphous, nanoporous silicas," *Chemistry of Materials*, vol. 8, no. 8, pp. 1682–1701, 1996.

[81] P. C. Alberius, K. L. Frindell, R. C. Hayward, E. J. Kramer, G. D. Stucky, and B. F. Chmelka, "General predictive syntheses of cubic, hexagonal, and lamellar silica and titania mesostructured thin films," *Chemistry of Materials*, vol. 14, no. 8, pp. 3284–3294, 2002.

[82] I. V. Zhigaltsev, N. Belliveau, I. Hafez, *et al.*, "Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing," *Langmuir*, vol. 28, no. 7, pp. 3633–3640, 2012.

[83] J. Gilbert, F. Sebastiani, M. Y. Arteta, *et al.*, "Evolution of the structure of lipid nanoparticles for nucleic acid delivery: From in situ studies of formulation to colloidal stability," *Journal of colloid and interface science*, vol. 660, pp. 66–76, 2024.

[84] T. Terada, J. A. Kulkarni, A. Huynh, *et al.*, "Characterization of lipid nanoparticles containing ionizable cationic lipids using design-of-experiments approach," *Langmuir*, vol. 37, no. 3, pp. 1120–1128, 2021.

[85] A. Kamanzi, Y. Zhang, Y. Gu, *et al.*, "Quantitative visualization of lipid nanoparticle fusion as a function of formulation and process parameters," *ACS nano*, vol. 18, no. 28, pp. 18 191–18 201, 2024.

[86] S. Bhattacharjee, "Dls and zeta potential–what they are and what they are not?" *Journal of controlled release*, vol. 235, pp. 337–351, 2016.

[87] M. Baalousha and J. R. Lead, "Nanoparticle dispersity in toxicology," *Nature nanotechnology*, vol. 8, no. 5, pp. 308–309, 2013.

[88] G. B. Schober, S. Story, and D. P. Arya, "A careful look at lipid nanoparticle characterization: Analysis of benchmark formulations for encapsulation of rna cargo size gradient," *Scientific Reports*, vol. 14, no. 1, p. 2403, 2024.

[89] N. Lorén, J. Hagman, J. K. Jonasson, *et al.*, "Fluorescence recovery after photobleaching in material and life sciences: Putting theory into practice," *Quarterly reviews of biophysics*, vol. 48, no. 3, pp. 323–387, 2015.

[90] C. L. Vestergaard, P. C. Blainey, and H. Flyvbjerg, "Optimal estimation of diffusion coefficients from single-particle trajectories," *Physical Review E*, vol. 89, no. 2, p. 022 726, 2014.

[91] J. A. Gallego-Urrea, J. Tuoriniemi, and M. Hassellöv, "Applications of particle-tracking analysis to the determination of size distributions and concentrations of nanoparticles in environmental, biological and food samples," *TrAC Trends in Analytical Chemistry*, vol. 30, no. 3, pp. 473–483, 2011.

[92] W. Commons, *Jablonski diagram of fluorescence only*, Accessed: March 11, 2025, 2025.

[93] J. R. Lakowicz and J. R. Lakowicz, "Introduction to fluorescence," *Principles of fluorescence spectroscopy*, pp. 1–23, 1999.

[94] D. Axelrod, "Total internal reflection fluorescence microscopy in cell biology," *Traffic*, vol. 2, no. 11, pp. 764–774, 2001.

[95] L. K. Tamm and H. M. McConnell, "Supported phospholipid bilayers," *Biophysical journal*, vol. 47, no. 1, pp. 105–113, 1985.

[96] S. R. Tabaei, J.-H. Choi, G. Haw Zan, V. P. Zhdanov, and N.-J. Cho, "Solvent-assisted lipid bilayer formation on silicon dioxide and gold," *Langmuir*, vol. 30, no. 34, pp. 10 363–10 373, 2014.

[97] P. S. Cremer and S. G. Boxer, "Formation and spreading of lipid bilayers on planar glass supports," *The Journal of Physical Chemistry B*, vol. 103, no. 13, pp. 2554–2559, 1999.

[98] S. Jõemetsa, K. Spustova, K. Kustanovich, *et al.*, "Molecular lipid films on microengineering materials," *Langmuir*, vol. 35, no. 32, pp. 10 286–10 298, 2019.

[99] S. J. Johnson, T. M. Bayerl, D. C. McDermott, *et al.*, "Structure of an adsorbed dimyristoylphosphatidylcholine bilayer measured with specular reflection of neutrons," *Biophysical journal*, vol. 59, no. 2, pp. 289–294, 1991.

[100] M. Przybylo, J. Sỳkora, J. Humpolickova, A. Benda, A. Zan, and M. Hof, "Lipid diffusion in giant unilamellar vesicles is more than 2 times faster than in supported phospholipid bilayers under identical conditions," *Langmuir*, vol. 22, no. 22, pp. 9096–9099, 2006.

[101] P. Joyce, S. Jõemetsa, S. Isaksson, *et al.*, "Tirf microscopy-based monitoring of drug permeation across a lipid membrane supported on mesoporous silica," *Angewandte Chemie*, vol. 133, no. 4, pp. 2097–2101, 2021.

[102] M. J. Carrasco, S. Alishetty, M.-G. Alameh, *et al.*, "Ionization and structural properties of mrna lipid nanoparticles influence expression in intramuscular and intravascular administration," *Communications biology*, vol. 4, no. 1, p. 956, 2021.

[103] M. J. Munson, G. O'Driscoll, A. M. Silva, *et al.*, "A high-throughput galectin-9 imaging assay for quantifying nanoparticle uptake, endosomal escape and functional rna delivery," *Communications biology*, vol. 4, no. 1, p. 211, 2021.