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Endosomal escape: A bottleneck for LNP-mediated therapeutics

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Lipid nanoparticles (LNPs) have recently emerged as a powerful and versatile clinically approved platform for nucleic acid delivery, specifically for mRNA vaccines. A major bottleneck in the field is the release of mRNA-LNPs from the endosomal pathways into the cytosol of cells where they can execute their encoded functions. The data regarding the mechanism of these endosomal escape processes are limited and contradicting. Despite extensive research, there is no consensus regarding the compartment of escape, the cause of the inefficient escape and are currently lacking a robust method to detect the escape. Here, we review the currently known mechanisms of endosomal escape and the available methods to study this process. We critically discuss the limitations and challenges of these methods and the possibilities to overcome these challenges. We propose that the development of currently lacking robust, quantitative high-throughput techniques to study endosomal escape is timely and essential. A better understanding of this process will enable better RNA-LNP designs with improved efficiency to unlock new therapeutic modalities.

mRNA | LNPs | endo-lysosomes | endosomal escape | RNA vaccines and therapeutics

Precise delivery of gene-manipulating nucleic acid payloads unlocks a wide range of therapeutic applications. The development of elegant and advanced delivery modalities, such as LNPs, has resolved the challenges related to inefficient delivery and immunogenic responses elicited by naked nucleic acid payloads (1, 2). These delivery vehicles can be classified into biological (mostly viral) and nonbiological (lipid- or peptide-based) classes. Viral vectors are clinically approved but carry the limitations of small payload sizes, antivector immunogenicity, and safety issues due to the risk of insertional mutagenesis. Examples of nonviral delivery vehicles include polymers and LNPs. For example, polyacrylamide-polymer nanoparticles have been applied for the entrapment and delivery of nondiffusible compounds as early as the 1970s, and significantly enhanced accumulation of fluorescein molecules in cultured fibroblast cells was achieved compared to free fluorescein-treated cells (3). Presently, LNPs are the most advanced delivery vehicle for the RNA-based therapeutics (4, 5). The recent rapid development of two mRNA-LNP vaccines for SARS-CoV-2, as well as a previously approved siRNA-LNP therapeutic, Patisiran®, has proved the potential of the LNP platform (6–8). The LNP delivery field initially focused on siRNA delivery, with a recent shift toward mRNA delivery. The shift to mRNA payloads unlocks a myriad of therapeutic applications. These range from expression of antigens of choice as prophylactic viral and bacterial vaccines as well as therapeutic cancer vaccine applications, supplementation of missing proteins for enzyme replacement therapies, and expression of gene editing machinery, such as CRISPR, to gene edit aberrant natively expressed proteins (4, 9–16).

Despite the developments in the LNP field, a commonly overlooked aspect regards the very limited release of the nucleic acid payloads in the cytoplasm (17–19). LNPs internalize into cells, via both clathrin-dependent and clathrin-independent endocytosis mechanisms such as macropinocytosis (17, 20). The majority of the particles are endocytosed by macropinocytosis; however, clathrin-mediated endocytosis is a prerequisite. Endocytosed LNPs are transferred to early endosomes, which mature into late endosomes and eventually into lysosomes (21). For efficient delivery, the nucleic acid payloads must be released into the cytosol before the maturation of late endosomes to lysosomes where the majority of the foreign materials are degraded enzymatically. The release of the payload prior to lysosomal maturation is a crucial stage for efficient delivery and is known as endosomal escape. This process is inefficient and is considered a bottleneck in this field (17, 18). Previous studies showed that the majority of the RNA-LNPs that internalize into target cells are either degraded by lysosomes or recycled outside of the target cells, with only a very limited amount of RNA payloads released into the cytoplasm (17, 20). Currently, there are disagreements on the endocytic stage of RNA payload release into the cytosol (17, 20, 22, 23). Further complicating the matter, the knowledge gained on siRNA delivery does not necessarily translate to mRNA payload release since several studies suggest differences between the escape of siRNA and mRNA payloads (17, 18, 22). A payload-dependent release profile suggests that the mechanism is more complex and requires

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characterization methods that can accommodate this discrepancy in payload nature and size to escape efficiency.

Since endosomal escape is a limiting factor for the development of LNP therapeutics, we argue that first, we need to better understand these processes to provide guidelines and advance us to develop more effective RNA-LNP applications. It will help to develop a better understanding on the lipid structures and formulations that can lead to higher escape efficiency since there is no current capability to predict a lipid formulation that will enable efficient endosomal escape. To achieve this, first, there is still a requirement to develop robust and less complex methods to study endosomal escape, which we currently lack. With this theme in mind, in this perspective, we will critically discuss the theories regarding how endosomal escape occurs and the available methods to characterize these processes.

A Brief Introduction to the Endo-Lysosomal System

To understand endosomal escape and payload release, it is important to understand the endosomal system and its dynamics (21, 24). Early endosomes are the first compartment where the internalized payload resides once it has been endocytosed by a cell. Early endosomes are characterized by a pH of ~6.2, which is maintained by a V-ATPase proton pump and by association of various RAB (Ras-associated binding) proteins in their GTP bound state (RAB 4, 5, 10, 14, 21, 22, EEA1) that progress endosomal fusion and tethering (21). The majority of the cargos internalized by early endosomes are recycled back, and only a fraction of them are trafficked to the late endosome and eventually to lysosomes. The maturation process of early endosomes to late endosomes takes place through various steps, which include RAB switch (from RAB5 to RAB7), acidification (from pH~6.2 to pH 6-4.9), formation of ILVs (intralamellar vesicles), PI conversion (from PI3P to PI2P), and gain of lysosomal hydrolases and membrane proteins among others (21). Thus, for a short interval, there is the presence of both RAB5 and RAB7 on endosome-forming transient early–late endosomes, which is considered an important compartment for release of siRNA-LNPs (17). Late endosomes share many properties with lysosomes which makes it difficult to differentiate between them and is a technical limitation for researchers in this field. Cargos that are transferred to late endosomes are considered to be transferred to a dead end where the molecules will be ultimately transferred to lysosomes and degraded.

An exception to this finding are nanocarriers containing inorganic ions such as Ca^{2+} and Zn^{2+} (25–27). These undergo ionic interaction in the lysosome and release a large number of inorganic ions in the lysosome. This leads to a sharp increase in the internal osmotic pressure of lysosomes disturbing the osmotic balance and release of drugs into the cytosol. Applying this concept, cationic lipid membrane-coated calcium phosphate/siRNA nanoparticles were synthesized together with Ca^{2+}, HPO_4^{2-}, and DOPA (1,2-dioleoy l-sn-glycero-3-phosphate) (28, 29). Upon entry into the lysosome, calcium phosphate dissolved and thus released large number of ions, increasing the internal osmotic pressure of the lysosomes which results in water influx inside the lysosome, eventually releasing the siRNA into the cytosol. However, studies also suggest some beneficial trafficking back to the membrane after late endosome's fusion with the lysosome (21, 24).

Overall, endosomes are highly dynamic with rapid homo- and heterotypic fusion events between different endocytic compartments making it extremely challenging to identify the exact endosomal compartment and the kinetics of payload release to the cytoplasm.

General Theories of Endosomal Escape

There are two general theories to explain the endosomal escape process. One claims that the ionizable lipids, when protonated in the acidic pH of endosomes, interact with anionic lipids present on the luminal side of the endosomal membrane. This interaction induces a nonbilayer (hexagonal H_{II}) structure, which leads to damage in the membrane that releases the nucleic acid payloads into the cytosol (30) (Fig. 1). The ability of lipids to form the H_{II} phase in the acidic pH of the endosome can be used as a surrogate for their endosomal membrane rupture potential and therefore escape efficiency (31). For example, in one study, the bilayer to hexagonal transition temperature (T_{BH}) was measured as a marker for efficient escape by ^{31}PNMR and differential scanning colorimetric analysis (32). Various studies clearly revealed a pKa optimum between 6.2 and 6.5 for efficient in vivo silencing in hepatocytes, and the highest potency is achieved at a pKa of 6.44 (33). However; other research shows that the optimal pKa required for the protein expression from mRNA-LNPs via the intravenous route is lower than the optimal pKa for protein expression administered via the intramuscular route (34, 35). At present, the reason for this difference is unknown. It has been shown before that the LNPs can enter through different endocytic mechanisms in different cell types and these entry mechanisms can affect the route of endocytosed particles (17, 36). In addition, different cell types or even the same cell types cultured under different conditions can differ in their endolysosomal properties, and thus, LNPs with varying pKa may have a different escape efficiency based on the cell type (37, 38). Moreover, our present knowledge on endocytosis as well as endolysosomal trafficking is completely based on in vitro cell culture–based experimental models. It will be highly valuable to understand whether our in vitro knowledge of trafficking pathways also replicates in vivo.

Nevertheless, various LNPs with similar pKa and T_{BH} show significantly different efficacy, suggesting that these two parameters are not the only determinants of efficient escape (30). Various factors, such as distance and flexibility of the charged group relative to the lipid bilayer interface, nature of the linker between head group and lipid tail may also be important. Together with the characteristics of the lipids, various cell-specific parameters such as endosomal pH, size, various genetic factors as well as trafficking kinetics can also impact the escape which will be highlighted in the subsequent sections (23, 39).

The other prevalent endosomal escape mechanism of action is known as the "Proton Sponge Effect." LNPs have a buffering capacity that leads to activation of proton pumps resulting in increased membrane potential. To achieve membrane equilibrium, chloride ions are diffused into the endosomal compartment, further increasing the osmotic pressure

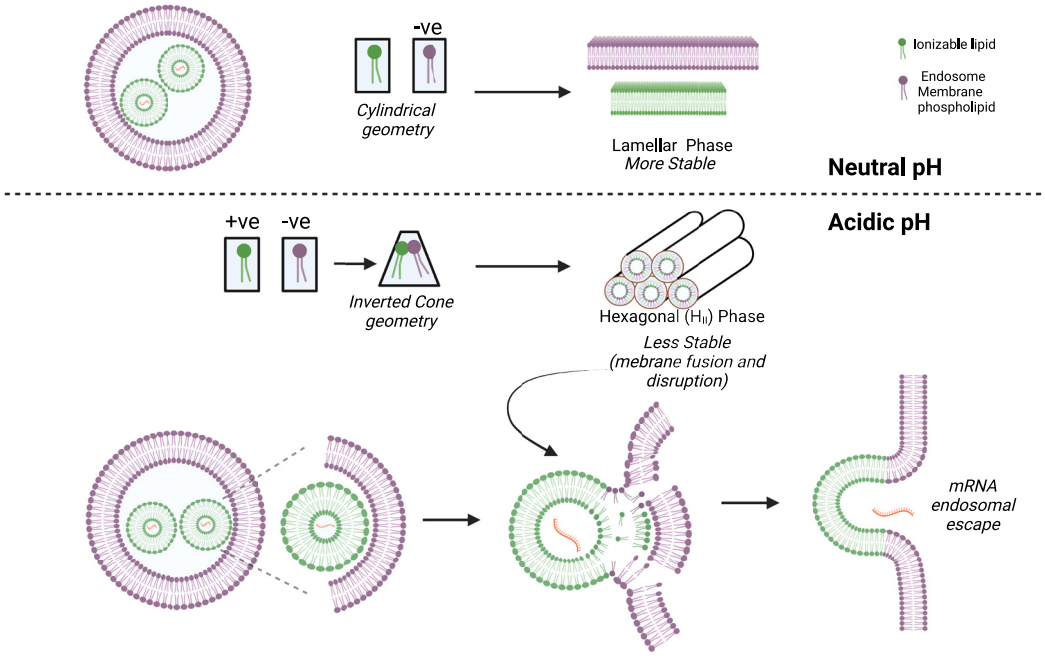

Fig. 1. Schematic representation of the bilayer to hexagonal phase transition of LNPs. Under the acidic pH of endolysosomal compartment, ionizable lipids are positively charged and interact with the anionic lipids present on the inner leaflet of the endosomal membrane. This interaction leads to the transition from bilayer to hexagonal phase transition resulting in endosomal membrane damage and cargo release.

inside the endosomes which leads to swelling and subsequent bursting of the endosomes resulting in escape of the nucleic acid (40) (Fig. 2). The basic parameters of this hypothesis rely on the buffering property of the ionizable lipid, acidification of the endosomes, chloride accumulation, and endosome bursting (41). There are studies to support as well as criticize each of these parameters (42, 43). In several studies, the end point is only transfection ability, whereas a few studies focus only on migration of labeled nucleic acids from the endosome to the cytosol. This limits the development of an overall understanding of this hypothesis. However, based on the studies reported so far, there are other mechanisms also at play for the release of the payload from LNPs beyond the two described (44). Solving the mechanism can bring us closer to optimizing the process for improved therapeutics. Next, we will dive into the current characterization methods.

Methods to Detect Endosomal Escape

Currently, the majority of methods to quantify endosomal escape processes rely on imaging to either directly detect encapsulated payloads or indirectly detect surrogate indications for escape processes. Regarding direct imaging of encapsulated payloads, advances in microscopy enable studying internal RNA-LNP trafficking to higher resolutions. These mostly rely on fluorescently labeled payloads (18, 45–47) or gold siRNA-tagged LNPs imaged by transmission electron microscopy (17). The indirect approach most commonly relies on identifying endosomal escape by imaging endosome damage indicators. In addition to this, membrane mimetics have also been used to evaluate the interaction of nanoparticles with the endosomal membrane.

Direct Imaging of Payloads.

pH-sensitive dye-based microscopic studies. The distinct pH property of endosomal compartments can be exploited for compartment detection by tagging small molecules with pH-sensitive fluorophores. In an interesting study using pH-based probes, the innate property of endosomal trafficking and kinetics of individual cell lines came into focus. Sayers et al. developed a pH probe to identify different endosome compartments (39). A dual-labeled 10-kDa dextran tagged with pH-sensitive and -insensitive fluorophores was utilized. The endocytosed dual-labeled dextran could provide pH response between pH 7.4 and 5.0, and calibrated pH readings were used to provide a qualitative and quantitative graphical pictorial representation of pH analysis. In this study, 30 cell lines were screened and classified on the basis of mRNA delivery efficacy. Further endosome profiling experiments based on pH-sensitive dual-labeled dextran revealed a drastic difference in the endosome properties and function in terms of morphology, localization, uptake, trafficking, endolysosomal pH, and recycling. It was observed that the transfection efficiency of these cells was not correlated with the amount of LNP uptake, but instead, it was dependent on the endosomal properties of the respective cells. A colocalization study of pH-sensitive dual-labeled dextran with fluorescently labeled mRNA-LNPs revealed that poorly transfected cells show defects in endosomal organization and slow trafficking of LNPs from the endosome to the lysosome, whereas in highly transfected cells, the endosomal trafficking to lysosomes was much faster. Such kind of studies underscore the importance of the pH-sensitive dye-based screening strategy before designing nucleic acid delivery applications to get better therapeutic benefit of the payload in the specific target cell.

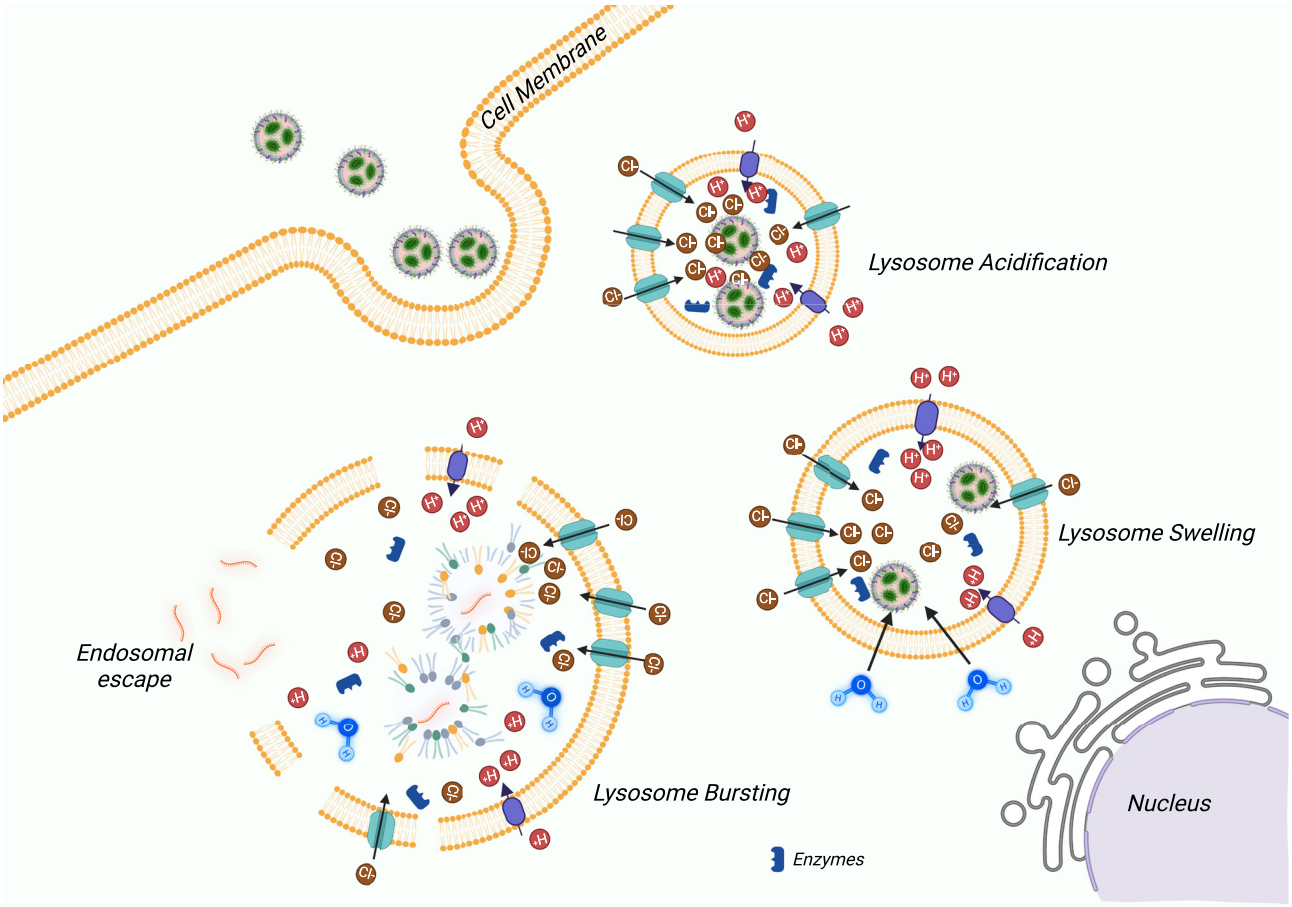

Fig. 2. Schematic representation of the Proton Sponge Effect: Due to the buffering ability of ionizable lipids, there is a huge influx of protons by activation of the proton pump in endolysosomal compartments. To neutralize the membrane potential, an inflow of chloride ion is triggered creating an osmotic imbalance which is followed by water intake. This leads to endolysosomal compartment swelling and eventually burst, which releases the cargo.

Endocytic marker-based microscopic studies. The advent of state-of-the-art confocal and electron microscopes in addition to various fluorescent probes to mark different compartments of the endomembrane complex has helped in better understanding of the complex processes encompassing endosomal escape.

Various image-based studies have shown that the majority of the particles are trapped inside the endolysosomal compartment (48–50). In a ground-breaking study, electron microscopy imaging of gold particle-labeled siRNA clearly showed that only ~2% of the particles are released into the cytoplasm, whereas the majority of the particles were entrapped in the endosomal vesicles (17). In addition, with the application of mathematical models as well as experimental findings, it is clearly shown that endosomal escape takes place at a specific step and is not a continuous process (17, 18, 20).

Furthermore, to determine the compartment of endosomal escape, two milestone reports on LNP-mediated siRNA delivery laid the groundwork for the current understanding of the escape route (17, 20). In one of them, marking different endosomal compartments with respective fluorescent probes suggested the presence of particles in a compartment that has the characteristics of an early as well as late endosome, and such compartments were enriched in response to LNP uptake as compared to control untreated cells (17). Although these compartments gained a lot of attention in further research, they have not been characterized in detail till date. Important questions that arise include the following: What is the pH of this compartment? Is it somewhere between the pH of early and late endosomes? Do these particles share more features with early or late endosomes? Do these vesicles also exist during endocytosis of other agents such as viruses? Such details are necessary to understand the properties of the compartment from where the cargo can be released. These properties can help in the prediction of the LNP interaction with the membrane and can be used to design lipids to achieve higher escape efficiency. To determine the compartment of the cargo release, cells were treated with bafilomycin to block early to late endosome maturation, yet this treatment had no effect on the cytosolic content of gold-labeled LNPs, suggesting that cargo release is not impacted by blocking the maturation of early to late endosomes (17, 51). Based on this observation, endosomal escape was suggested to occur from early endosomes. In addition, RAB5 downregulation led to a significant decrease in LNP uptake. However, effects of bafilomycin and RAB5 knock-down were not linked with functional assays making it hard to determine their actual impact on the release. Findings in various studies over the years contradicted this report with functional readouts (18, 20, 22). It will be hard to claim whether these differences are due to subtle differences in the particle characteristics between studies. Although the use of gold-labeled siRNA-LNPs undoubtedly shed light on our understanding of

the cargo release, this technique also has limited use for screening purpose due to its complexity and high technical demands in terms of methodology and instrumentation.

In another attempt to identify the compartment of cargo release, siRNA-LNPs showed colocalization with recycling endosomes which was associated with a decrease in the colocalization of siRNA with late endolysosomal compartments (20). Overall altered expression of the genes that perturbs transfer of LNP to recycling endosomes or prevents its plasma membrane fusion showed enhanced perinuclear distribution of late endosomes, enhanced LNP retention in these late endosome vesicles which was corroborated with enhanced silencing; thus, late endosomes were predicted as a point of siRNA escape, and it was suggested that higher retention of particles in late endosomes might lead to efficient escape. The presence of LNP-mRNA particles in recycling endosomes was later recapitulated in another study where the extracellular vesicles of LNP-mRNA transfected cells showed the presence of ionizable lipid component as well as transfected mRNA(45).

With further technical advancements of imaging methods, it has become possible to detect a single molecule of fluorescently labeled mRNA by TIRF (total internal reflection fluorescence) microscopy (52), and this advantage has also been used to detect the mRNA release from endosomes. mRNA payloads can also be detected by smFISH (53). This technique was used in a study where the efficiency of LNPs with different ionizable lipids was not correlated with their uptake (23). Analysis of various endosomal compartments revealed that LNPs with higher efficacy showed colocalization with $EEA1^+$, $APPL1^+$, and $RAB11^+$ early/recycling endosomes in contrast to the LNPs with low efficacy which mainly showed accumulation in late endosomes. In addition, the colocalization experiment with pH-sensitive fluorescent dyes showed that a high fraction of many LNPs accumulate in large endosomes with increased pH, blocked acidification, and thus perturbed endosomal maturation resulting in altered progression of the cargo. These endosomal compartments with blocked acidification never showed mRNA escape in further experiments, and LNPs with different efficiency showed a differential effect on these endosomal acidification and maturation blockage. It will be interesting to investigate whether there is a correlation between LNP efficiency with blockage of endosomal acidification/maturation and whether such a tool can provide promising results for lipid screening assays. Besides, a mathematical model predicted the recycling endosomes, RAB11-positive, compartment as the main site of mRNA escape which was confirmed by a single-molecule localization microscope (SMLM). With the help of SMLM, single LNPs were resolved and their subendosomal localization was detected. To avoid fixation which might hamper the membrane and LNP distribution, fluorescent-labeled transferrin and EGF (Epidermal Growth Factor) were used to identify the endosomal compartment. Dispersed Cy5-mRNA signal distinct from condensed and intense endosomal Cy5-mRNA signal was observed outside or close to transferrin-positive structures. Cy5 flashes protruding from transferrin-positive compartments to the cytoplasm were also observed, and based on these findings, recycling endosomes were determined as a point of mRNA escape. Although a significant improvement was achieved in terms of the resolution of LNPs, however, caution should be made while extrapolating these results owing to nonspecificity of dyes. An alternate way to specifically label these compartments without damaging the membrane could be transfection with fluorescent-tagged RAB plasmids to specifically identify the compartment.

Another relevant question regarding the LNPs is whether the particles are disassembled while still in the endosomes or after their release as RNA-LNPs into the cytoplasm. This knowledge can provide information on whether the mRNA observed in the cytoplasm is free for further processing or whether they have to go through another step of LNP disassembly and release after endosomal escape. To answer this question, kinetics of LNP disassembly was measured by FRET (Fluorescence Resonance Energy Transfer)-based probes where identical siRNAs were labeled with different fluorophores to form the FRET pair (20). The FRET signal was obtained only in the intact LNPs where the siRNAs were in proximity, whereas there was a loss of FRET signal within 1 h of internalization, suggesting LNP disassembly. Since at 1 h of internalization, labeled siRNA was mostly entrapped in the vesicular compartments, which indicated that LNPs were disassembled inside the endosomal compartment itself. Disassembly of particles within the endocytic compartment was supported by other studies as well (18).

Eventually, the challenges with direct imaging of payloads include the limit of image resolution and the ability to confidently assume escape. Since the majority of the payload remains in the endocytic compartment and only a small amount is released in the cytosol, it makes it very hard to visualize the low fluorescence intensity of the released labeled siRNA in contrast to the high fluorescence intensity of the particles accumulated in the endosomal compartment. However, some have recently attempted to overcome this with more advanced microscopy techniques (18). Eventually, the direct imaging approach has some limitations such as imaging small cells with low cytoplasmic volumes such as lymphocytes, which are notoriously hard to transfect and challenging to image since these cells are both small at around 5um in diameter and possess a large nucleus that comprises approximately 90% of the cell volume (54).

Indirect Analysis of Endosomal Escape Processes. Due to the current limitations in microscopy, studies have also focused on methods that do not involve high-resolution imaging of phenomena that can help understand escape processes.

Membrane-based studies. Studying the interactions of LNPs with endosomal membranes is challenging due to the small size of the organelle and LNPs, the highly dynamic nature of these vesicles, the complex structure of the membrane, and the presence of many background molecular cellular functions. To this end, there are examples of studies used to model biomembranes as a replica of the endosomal membrane to study the biophysical interaction. Langmuir trough technique with different lipid compositions had been used previously to evaluate the effect of nanoparticles on phase properties of the membrane (55, 56). Model endosomal membranes have also been used to elucidate the mechanism of membrane destabilization by DOPE-containing liposomes (57). In a recent study, in situ optical reflectometry techniques were applied on the Langmuir membrane to replicate early and late endosomal

membranes (58). One of the techniques applied, ellipsometry, provides information on the extent of interaction and lateral inhomogeneities by measuring the changes in polarization of light upon reflection at the interface (59). Another technique, Brewster angle microscopy provides information on the lateral morphology of the monolayer (60). Moreover, the technical understanding combined with surface pressure measurements provide information on LNP binding to the membrane, lipid exchange between the two moieties and from this, the delivery of the lipid-nucleic acid complex is suggested to be derived. Using in situ optical reflectometry technique with Dlin-DMA-MC3 LNPs with various mRNA payloads as well as empty LNPs demonstrated that that the strongest interactions occurs at a pH below 6.5 for early as well as late endosomes. Furthermore, it was observed that the stage of the endosome had no influence on the extent of interaction. Interestingly, they demonstrated that the nature of the mRNA cargo impacted the LNP interaction with the endosomal membrane. Overall, it was shown that for efficient release from endosomes, rapid release of the cargo plays an important role. Although such experimental setups can simplify the process to understand membrane–lipid interactions, it is important to note that these membranes are constructed purely from lipids and lack proteins and other components of endosomes. Furthermore, these models are flat membranes and thus lack the curvature of the circular endosomes. Last, it is expected that the lipid interactions might get impacted by the presence of other molecules in the vicinity of the vesicles as well as the escape is governed by various genes which cannot be ascertained using such models.

Endosomal damage as a reporter of escape processes. The Galectin-based cell line reporter systems are the most common using this approach. Galectin family proteins (such as Gal8 or Gal9) bind glycans found on the inner leaflet of endosomal membranes which become available upon endosomal rupture (61). The cell reporter systems encode for fluorescently tagged Galectins that cluster on endosomes where escape has occurred and can then be imaged (47, 62–65). With this system, endosomal escape of siRNA-LNPs was observed from RAB5-positive compartments which could have been positive or negative for RAB7 (18). As it appears robust and technically simple process, it can be applied for the screening purpose given the validity of the approach and significant correlation.

Genetic manipulation of endosomal maturation facilitating proteins. Another approach involves genetic downregulation of proteins that facilitate maturation of endosomes at known stages (64). Small molecules that inhibit endosomal maturation can be unspecific; to this end, researchers have performed sequence-specific CRISPR knock-out studies to pinpoint the endosomal state of escape. Patel et al. utilized genetically edited Haploid cells (HAP1) to remove the expression of RAB 4, 5 and 7 separately to interrupt the function of recycling, early and late endosomes, respectively (22). It was shown that mRNA expression was significantly reduced in cells lacking RAB7, whereas the absence of RAB4 or 5 had only little effect on lipopolyplex/LNP-mediated mRNA expression. Since RAB7 is a late endosome marker, reduced mRNA expression in response to RAB7 knock-out suggests the importance of late endosomes for efficient mRNA expression. However, in the absence of uptake data, it is difficult to ascertain whether these differences are specifically related to endosomal escape. In spite the release observed from RAB5+ve compartments in various studies, why RAB5 knock-down or introduction of RAB5 dominant negative mutation has no effect on endosomal escape has not been ascertained till date. Furthermore, these perturbations can affect signaling events. For example, mTORC is localized to the late endosome–lysosomal surface on activation and acts as a key point to drive various downstream signaling pathways, including protein translation and ribosome biogenesis. Thus, late endosomes and lysosomes appear to play an important role in efficient translation of synthetic mRNA via mTORC-mediated signaling pathways, regardless of escape processes.

Overall, it is evident that to date, there is no agreement on the nature of the compartment from where the escape takes place (Fig. 3). A few studies suggest that higher retention time might lead to better escape, whereas the majority of the studies suggest that rapid trafficking and release lead to efficient escape. All studies to date unanimously show that a very small amount of cargo is released into the cytosol; however, the underlying mechanisms for this limitation differed in different studies. It is possible that there are multifactorial reasons behind the lack of efficient release. Furthermore, all the major studies unanimously show that cargo release takes place at a specific step and is not a constant process. The majority of the studies suggest that the mRNAs are first disassembled from the LNPs in the endosomal compartment, and subsequently, the cargo is released into the cytosol. Therefore, although some of the steps of endosomal escape show uniform mechanism across the studies, there are also discrepancies on a few steps.

Approaches to Enhance Endosomal Escape. Since endosomal escape is widely accepted as a bottleneck, researchers are searching for schemes to enhance this process by either pharmacological compound or genetic manipulations to interventions.

One example includes manipulation of NPC1 (NPC intracellular cholesterol transporter 1), a protein that plays an important role in exocytosis of lipids from late endosome or lysosomes to extracellular environment. Studies demonstrate that NPC1−/− cells accumulate siRNA inside cells after siRNA-LNP transfection, which was corroborated with increased efficiency of siRNA-directed knockdown in these cells (20). Another example includes genetic manipulation to perturb Golgi/ER (endoplasmic reticulum) secretion (RAB8) or fusion of recycling endosomes to the plasma membrane (RAB27b) as a means to enhance LNP retention in the cell. Eventually, these findings will be interesting if efficient and specific in vivo knock-down will become feasible. Interestingly, differential binding of NPC to various modified or natural cholesterol molecules has been exploited to enhance escape efficiency (66). Replacement of cholesterol with naturally occurring phytosterols in the LNP formulation has shown better mRNA delivery efficiency which is linked with reduced recycling via NPC1 (46). In another study, cholesterol was replaced with hydroxycholesterol in the LNP formulation with the goal to achieve reduced recognition of cholesterol by the NPC1 enzyme (67). The formulation showed reduced recycling endosome formation, which was corroborated with

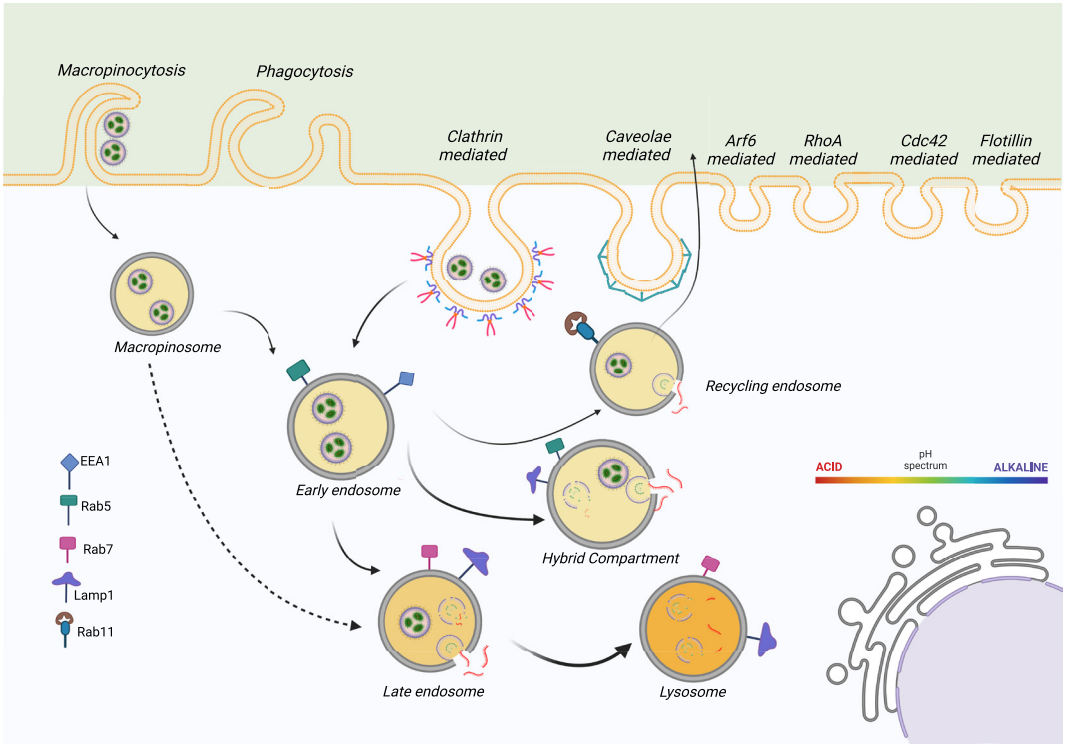

Fig. 3. Schematic representation of endocytosis mechanisms. Figure showing LNP uptake by clathrin-mediated endocytosis and macropinocytosis. Once inside the cell, particles are transferred to early endosomes. From this stage, there are various reports on exact endosomal compartment of escape. Various reports claim that the cargo is released from a hybrid compartment having the properties of early as well as late endosome (17, 18), the late endosomal compartment (20), and RAB11 +ve recycling endosomes (23).

increased late endosome formation, resulting in a twofold increase in mRNA release.

Small molecules that lead to damage of endolysosomal membranous compartments have also been tested to monitor their effect on endosomal escape and siRNA efficiency(47). As mentioned above, Galectin-9 can be utilized as a sensor to this end. Chloroquine, siramesine, and amitriptyline have been harnessed to induce endosomal damage and endosomal escape of cholesterol conjugated siRNA. Membrane damage caused by these small molecules results in efficient release of cholesterol conjugated siRNA, which results in the increase in knockdown efficiency by 47-fold. Such events were also recapitulated in tumor spheroid models, underscoring the value of endosomal escape for better and efficient delivery of nucleic acids. Further investigation indicated membrane damage to the LAMP1-positive compartments in response to small molecule treatments and resulted in the release of the siRNA from the same compartments. Since LNPs accumulate in LAMP1-positive compartments to a large extent from where the end point is mostly degradation in the lysosomes, release of nucleic acids from these late endosomes can be of great benefit to increase the efficiency of this therapy. However, toxicity issues and dose determination will be important factors.

Other examples include a leukotriene inhibitor (MK571) that increased transfection efficiency by 200% (22). Further nano-formulation of LNPs for codelivery of MK571 and mRNA showed higher efficiency in vitro as well as in vivo. Leukotrienes are inflammatory molecules and are known to bind to a GPCR receptor resulting in its internalization in the endolysosomal compartment, but the mechanism by which leukotriene inhibitor enhanced mRNA efficiency is unclear.

These studies are encouraging as they provide an insight into the possible ways of enhancing mRNA-LNP efficiency by encapsulation of various lipid compounds into the LNP system. However, an important issue will be to look into the side effects of such compounds and the dose at which they can elicit their effects. There is a definite goal to enhance the efficiency of escape, as less than 2% is released into the cytoplasm, a large part is recycled out of the cell and the majority of the cargo is entrapped in late endosomes/lysosomes leading to degradation. Retention of the endocytic compartment from where the cargo is released might lead to better release as it may provide longer time to release the payload. In contrast, it has been shown that faster endosomal kinetics leads to better transfection efficiency when the uptake is constant, canceling the possibility of this theory. Nevertheless, it is clear that the endosomal escape efficiency can be enhanced by molecules that can inhibit recycling of the endosomes or the molecules that can induce damage in late endosomes to release the payload which is entrapped in these vesicles and are destined to dead end by degradation in lysosomes.

In addition to LNPs, it is interesting to explore other lipid-based platforms that do not rely on endocytosis mechanisms for cargo delivery. For example, many viruses and bacteria bypass receptor-mediated endocytosis and endosomal trafficking by direct fusion with the plasma membrane and deliver the nucleic acid directly into the cytoplasm of the host cell (68, 69). In a similar fashion, some lipid molecules enable liposomes

to fuse with the cell membrane directly. This leads to efficient and faster delivery of the payload. Fusogenic liposome systems contain a neutral and a positively charged lipid molecule in combination with lipids modified by aromatic groups (70). The neutral lipid component acquires cone shape and supports various fusion intermediate states. The polar head group of positively charged lipid interacts with the glycocalyx and enhances the fusion probability of the liposome with the cell membrane. The delocalized π electrons of the aromatic group are polarized by the strong positive charge of the liposome head groups inducing local dipoles which presumably yield local instabilities and disorders of molecular arrangements in the bilayer. Such fusogenic liposomes had been used to transfer lipids, proteins, polyphenol, and synthetic beads (70–73). The delivery of negatively charged nucleic acids through such fusogenic liposomes is limited as they neutralize the positive charge of the liposomes and thus hamper their cell membrane fusion ability. To overcome this barrier, nucleic acids are incubated with other positively charged lipids before their incorporation into fusogenic liposomes (74). Optimum composition obtained by this method was unaffected by endocytosis blockers confirming their fusion with the cell membrane. Further studies comparing the endosomal-dependent transfection method with the fusogenic pathway did not show any advantage in terms of better transport or expression; however fusogenic liposomes showed reduced immune response (75). Whether such kind of delivery agent can provide better output than the presently available LNPs is yet to be explored. In addition, research on fusogenic liposomes for nucleic acid delivery is at its early stages and in vivo toxicity as well as efficacy data is yet to be determined. Another approach is employment of cell-penetrating peptides (CPP) for direct delivery into the cytoplasm (76–78). While some of these CPPs endocytose with translocation, others are known to penetrate electrostatically through anion phospholipids. Although CPPs are highly efficient in their penetration ability, they have also been related with cytotoxicity, limiting use of such agents for nucleic acid delivery.

These interventions are examples of how better understanding of endosomal escape processes can guide us toward improvement of endosomal escape processes and enhance the therapeutic potential of this powerful platform.

Conclusion

It is clear that despite extensive research to elucidate the mechanism of endosomal escape, our understanding is limited, and research is full of contrasting findings. The highly dynamic nature of endosomal compartments and shared markers between them adds another layer of complexity making it even more difficult to gain common conclusion regarding trafficking and escape of LNPs. Nevertheless, endosomal escape is one of the most important aspects for efficient nucleic acid delivery, and it is also pertinent to pay attention to the requirement of sophisticated tools and probes to understand the basic molecular biology of this process. It is important to develop a consensus on the compartment from where the cargo is released into the cytosol to design smart interventions to enhance this process. Optimally, it will assist us to predict the link between ionizable lipid structures that result in efficient endosomal escape. To achieve this, we need robust methods to study escape efficiency to screen large number of lipids. The techniques such as glycan foci formation can be useful for their ease of methodology as well as read out; however, they are subject to validation and have higher resolution limits. An optimal system could be a "turn-on" system, that does not rely on colocalizations but can emit an easily detected signal following an escape process and that can be quantified by high throughput processes for screening. Current examples include tracking of non-RNA payloads either as a proxy by themselves or coencapsulated with RNA payloads (79–81).

On the other hand, sophisticated assays and advanced high resolution microscope techniques to differentiate between the mRNA that is present in the cytosol in association with ribosomes after escape and the one that is present inside the endolysosomal compartment are also in demand. It will be interesting to understand whether and how different mRNA structures or payload sizes will affect trafficking properties.

We believe that once these methods are established, development of the formulations and intervention strategies that can enhance escape efficiency will be a game changer for expanding the therapeutic application of the mRNA-LNP platform.

Data, Materials, and Software Availability. There are no data underlying this work.

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