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Strategies and mechanisms for endosomal escape of therapeutic nucleic acids

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Despite impressive recent establishment of therapeutic nucleic acids as drugs and vaccines, their broader medical use is impaired by modest performance in intracellular delivery. Inefficient endosomal escape presents a major limitation responsible for inadequate cytosolic cargo release. Depending on the carrier, this endosomal barrier can strongly limit or even abolish nucleic acid delivery. Different recent endosomal escape strategies and their hypothesized mechanisms are reviewed.

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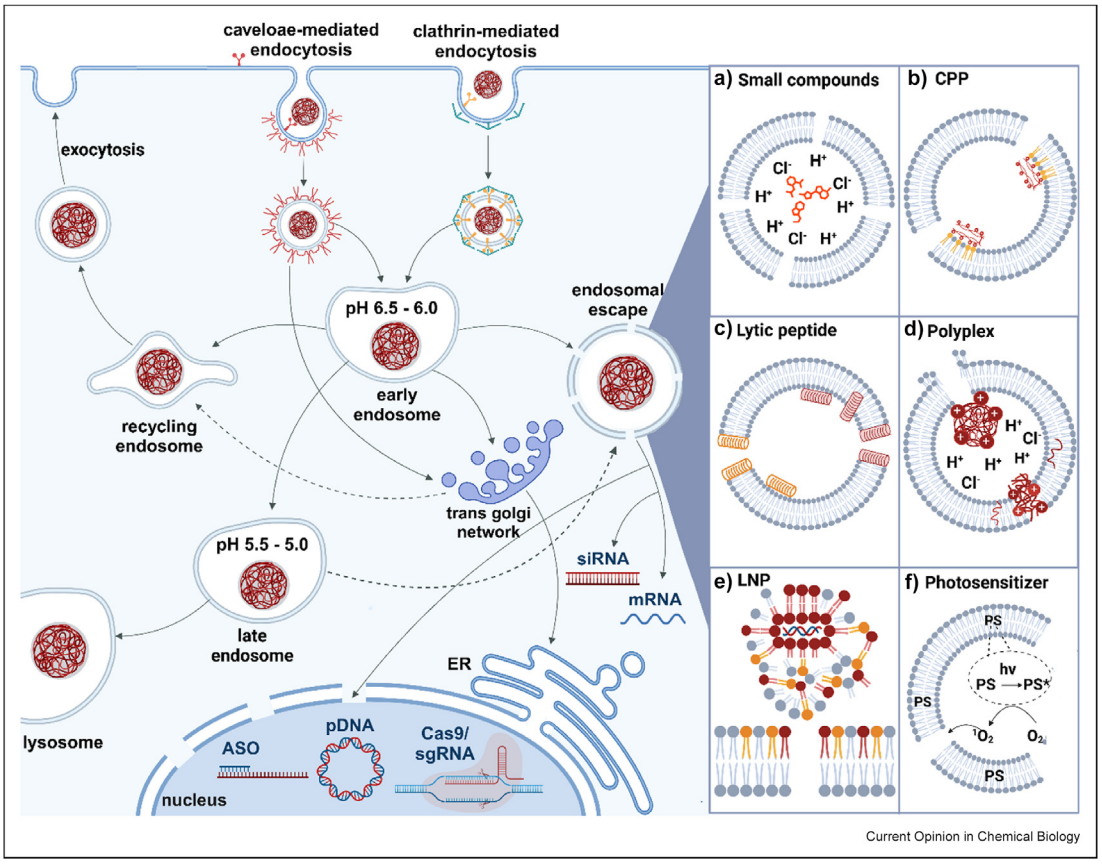
Introduction

Within the last decade, nucleic acids gained importance as drugs for combatting diseases. In order to unleash their therapeutic effect, these macromolecules must traverse multiple extracellular and intracellular barriers including cellular membranes. Endocytosis of nucleic acids into a network of maturating vesicles from early endosomes to late endosomes and lysosomes (Figure 1) displays the major internalization route. Sorting processes may further deliver the engulfed cargo into organelles such as the trans Golgi network and ER. Sorting into vesicles that recycle back to the cell surface or into lysosomes that degrade cargo by lysosomal enzymes, however, present non-productive pathways [1,2]. Consequently, the nucleic acid cargo needs to translocate across the endosomal membrane before nonproductive exocytosis or lysosomal sequestration. Measures for enhancing this process known as endosomal escape [3–9] often capitalize on the physiological acidification process (from pH ~ 7 to pH ~ 5) triggered by vATPase in a variety of intracellular vesicles. Alternatively, pH-independent strategies such as photochemical internalization or fusogenic liposomes have been applied [10–13]. The type of nucleic acid carrier applied for nanoparticle formulation dictates the extent of the delivery bottleneck. Nucleic acid polyplexes with first-generation cationic polymers like polylysine are restricted from release and require added endosomolytic agents [14]. The hurdle is less pronounced for nucleic acid complexes with cationic lipids (lipoplexes) and lipid nanoparticles (LNPs). Nevertheless, endosomal escape efficacies were found to be low in the 3 % range [15]. Herein, we elucidate different endosomal escape strategies involving small chemical compounds, cell penetrating or other lytic peptides, generation of reactive oxygen species (ROS), or next-generation cationizable carriers.

Low molecular weight compounds promoting endosomal escape

Addition of the antimalaria agent chloroquine to the transfection medium was found to strongly enhance gene transfer by DNA/polylysine polyplexes [16,17]. Chloroquine is a weak base that becomes entrapped by protonation in acidifying endosomes, thus reducing acidification and activity of lysosomal enzymes (Figure 1a). Further hypothetical mechanisms for the favorable effect on transfection are endosomal vesicle swelling and membrane destabilization, and a direct effect on nucleic acids by intercalation, displacement of the cationic carrier and improved properties of the released nucleic acid [17,18]. Recently, El Andaloussi and colleagues [19] performed a chemical library screening for endosomolytic compounds. They combined a functional screen (delivery of a splice-switching oligonucleotide) with a microscopy screen, including a galectin-9 endosomal disruption assay. The top agent CMP05-7 (containing three aromatic groups indole, pyrimidine and pyrazole as well as a basic piperidine) triggers endosomal escape of cargo by blocking trafficking to late LAMP1 positive endosomes and endosomal membrane rupture, in parallel with swelling of these endolysosomes. Co-incubation of cells with the

Figure 1



Left, internalization and intracellular delivery routes of nucleic acid carriers (ER, endoplasmatic recticulum). Right, different endosomal escape strategies and their proposed mechanisms. **a)** Small compounds prevent endosomal acidification and induce membrane destabilization by osmotic swelling or other mechanisms. **b)** Electrostatic interactions between arginine rich cell penetrating peptides (CPPs) and specific anionic lipids trigger membrane fusion and subsequent membrane leakage. **c)** Amphipathic lytic peptides mediate membrane destabilization by insertion and rearrangement of lipid bilayers or pore formation through peptide self-assembly. **d)** Cationized free or polyplex-bound polymers decrease the integrity of the endosomal membrane and induce membrane holes. **e)** Ionizable lipids (red) become cationic within acidifying endosomes. Mixing with anionic endosomal lipids (orange) promotes phase transition and formation of transmembrane pores. **f)** Upon light exposure, photosensitizers generate reactive oxygen species (ROS), mainly singlet oxygen ($^1O_2$). ROS oxidize lipid membranes and promote their disruption.

vATPase inhibitor bafilomycin, indicated a dependence of activity with endosomal protonation [19]. Another approach repurposes cationic amphiphilic drugs (CADs) as endosomolytic compounds [20–22]. The efficacy and mechanisms may vary depending on the selected specific CAD, cell type and delivery system. The antihistamine CAD ebastine improved the efficacy of several siRNA formulations [20]. Incorporation of CADs into LNPs enhanced mRNA delivery [21].

Cell penetrating and lytic peptides

Several classes of peptides possess abilities to enhance transfer across endosomal membranes. TAT peptide, derived from the HIV-1 tat protein, and related arginine rich cell penetrating peptides (CPPs) are internalized by micropinocytosis [23], and cytosolic delivery is observed, demonstrating potential for drug and nucleic acid delivery [24,25]. Dimerization of the TAT peptide (e.g. dfTAT) as well as hydrophobic chemical modification greatly enhances efficacy [26,27]. Endosomal escape occurs within late endosomes through electrostatic interactions of TAT with anionic bis(monoacylglycero)phosphate (BMP) (Figure 1b), including an initial fusion process succeeded by membrane leakage [28]. Preclinical trials have demonstrated effective oligonucleotide delivery by CPPs without adverse effects like acute inflammation at therapeutic doses. However, weekly intravenous administration in non-human primates indicate renal toxicity which has to be considered in future applications [29].

A series of amphipathic peptides of 15–30 amino acids with alternating hydrophilic and hydrophobic residues have been exploited for cytosolic delivery [8,30–33]. They are derived from natural viral fusion protein

sequences, defense toxins and antibacterial peptides, or designed as artificial analogs. In their active form, they commonly display amphipathic α-helical secondary structure that either destabilize membranes by insertion and thus increasing curvature strain of lipid bilayers (Figure 1c), or by peptide self-assembly into small transmembrane pores [8,32]. Similar trends were observed in molecular dynamic simulations between native hemagglutinin or fusogenic derivatives and a model lipid bilayer. In contrast, nonfusogenic peptides arranged parallel to the membrane. Notably, comparing hemagglutinin fusion peptide with other sequences revealed structural similarities, indicating that its fold depends on the local environment [34]. Capitalizing on the pH-specific endosomal escape of several viruses, synthetic endosomolytic peptides derived from N-termini of influenza virus hemagglutinin HA2 or rhinovirus VP1 were incorporated into pDNA or siRNA polyplexes, greatly enhancing functional delivery both *in vitro* [35–37] and *in vivo* in a tumor model [38].

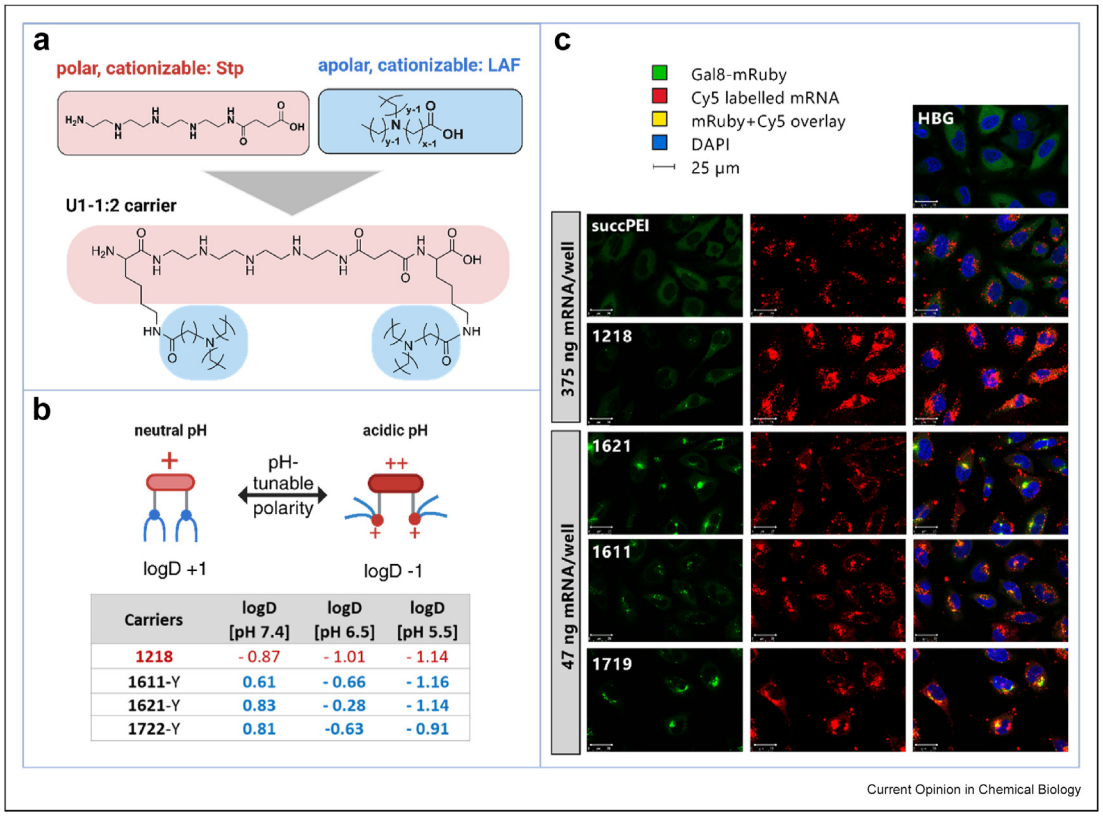
The artificial acidic endosomolytic peptide GALA with repeats of Glu-Ala-Leu-Ala [30] was successfully applied for enhancing pDNA [39] and mRNA [40] polyplexes. Kichler and colleagues systematically explored another artificial amphipathic peptide family based on Leu, Ala and His termed LAH4, which strongly promotes transfection [31]. Membrane pore formation and transfection activities show a clear correlation with the hydrophilic angle (<150°) of the amphiphilic helix [41].

Melittin, a cationic peptide of bee venom, exhibits strong membrane-disruptive behavior. However, its lytic activity is pH-independent, leading to substantial cytotoxicity. This can be prevented by several measures, including conjugation of melittin via the N-terminus [42], or by replacing cationic lysines and arginines or neutral glutamine by glutamic acid. Corresponding analogs enhanced gene transfer by pDNA/PEI polyplexes [43]. Replacement of the glutamic acids of an acidic mutant by histidines resulted in melittin HELP-4H that displays high pDNA, siRNA and mRNA delivery [44]. Alternatively, acid cleavable protective groups and linkers can mask melittin at neutral pH. Within an acidic milieu the endosomolytic potential of unmasked melittin is restored [45,46]. A virus-inspired block-copolymer for endosomal escape (VIPER) hides melittin within a hydrophobic pH-sensitive block, but exposes it upon acidification. Intratumoral pDNA delivery [47] and intratracheal siRNA delivery [48,49] demonstrate the potency of this melittin-containing carrier.

Endosomal escape of polyplexes

Polymers used for polyplex formation may strongly differ in their endosomal release capacity [3,9,50,51]. Polylysine (PLL), a first-generation polycation exhibits weak transfection efficacy. Addition of endosomolytic agents is required to enhance cargo release. Reversibly masking the positive charges of PLL can limit associated cytotoxicity [14]. In contrast, polycations such as polyethylenimine (PEI) or PAMAM dendrimers are potent transfection agents that do not need added endosomolytic agents. These polymers are defined as "proton sponges", because their amines are only partly protonated at neutral pH, but become increasingly cationized when polyplexes accumulate in acidifying endosomes. Simultaneously, chloride ions passively enter the endosome to maintain a charge equilibrium, and protonation drives water influx, inducing endosome and also polyplex swelling. Previously hypothesized osmotic effects are debatable as the cause for endosomal disruption. Instead, the strongly cationized free or polyplex-bound polymer is assumed to destabilize the endosomal membrane (Figure 1d). Based on such considerations, PEI has been modified with hydrophobic units [52–54] to increase polyplex stability at neutral pH outside the cell but enable dissociation from polyplexes at endosomal pH, and to provide sufficient lipophilicity for active interaction with the endosomal target membrane. Blockade of endosomal acidification by bafilomycin, or polymer shielding by permanent PEGylation inhibits the transfection. A significant cytotoxicity of PEI [55] can be reduced by designing novel polymer analogs that present multiple shorter aminoethylene units [56]. In a chemical evolution strategy for optimizing intracellular nucleic acid delivery, precise sequence-defined oligoaminoamides can be generated by standard solid-phase supported peptide synthesis. Successful delivery motifs, such as the cationizable aminoethylene motif derived from PEI, were identified and incorporated as artificial amino acids. In combination with other units such as lipidic residues for hydrophobic stabilization, artificial peptides termed "xenopeptides" with various topologies were obtained and subjected to a selection process for carriers of various nucleic acid cargos [57–59]. For example, hydrophobic balancing between cationizable Stp analogs identified potent carriers for intracellular delivery of Cas9/sgRNA ribonucleoprotein (RNP) [57]. Double pH responsive xenopeptides (Figure 2) were designed by combining ionizable Stp units with at least two ionizable lipo amino fatty acid (LAF) units [58]. LAF presents an extended lipophilic domain with a tertiary amine positioned near the center of the apolar fatty acid unit (Figure 2a). At neutral pH, the cationic Stp-LAF carriers form polyplexes with non-protonated LAF units providing hydrophobic stabilization. Upon endosomal acidification, protonation of LAF units drastically change the polarity of carriers (Figure 2b). mRNA polyplexes containing such LAF units trigger fast and potent endosome disruption as evidenced in mRuby3-galectin-8 endosomal recruitment assays (Figure 2c). Highly potent carriers for polyplex formulation of pDNA, mRNA, siRNA and Cas9 mRNA/sgRNA were identified [58,60]. Efficacy was

Figure 2



LAF-Stp lipo-xenopeptides (XPs) for potent endosomal escape. **a)** The combination of polar (Stp) and apolar, cationizable (LAF) domains results in double pH responsive lipo-XPs. **b)** Upon acidification carriers undergo a sharp transition in polarity as demonstrated in logD (octanol/water) distribution studies. **c)** Endosomal disruption by mRNA polyplexes detected in Hela-Gal8-mRuby3 cells. The Gal8-mRuby fluorescence pattern represents accumulation of galectin-8 at intra-endosomal galactan residues and thus rupture of these vesicles. LAF-free OleA-Stp xenopeptide 1218 and succinoylated PEI (succPEI) present transfection positive controls. **c)** is reproduced from [58] with permission from Wiley.

demonstrated by functional activity of mRNA at ultra-low picogram dosage (only ~2 nanoparticles/cell) *in vitro* and potent *in vivo* expression of mRNA [58] and also Cas9 mRNA/sgRNA mediated gene editing [60] in several organs upon intravenous delivery.

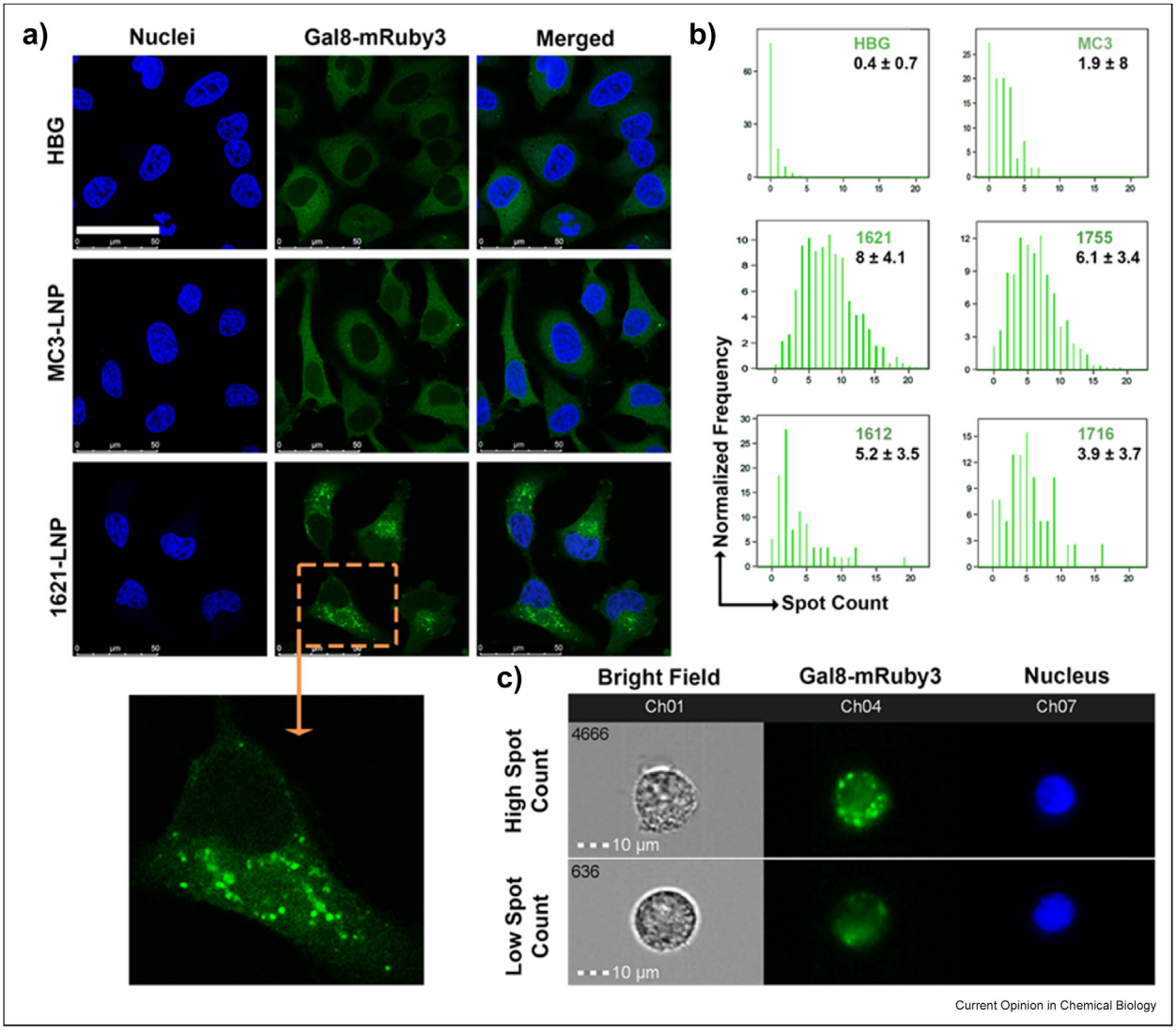
Endosomal escape of lipid nanoparticles (LNPs)

LNPs, originating from cationic lipoplexes, typically contain four carrier components: an ionizable lipid (IL), a phospholipid helper lipid, cholesterol and a polyethylene glycol (PEG)-lipid. The IL is primarily responsible for nucleic acid complexation and endosomal escape. Albeit neutral and insoluble at physiological pH, it becomes cationic and water-soluble upon acidic pH within endosomes. Electrostatic interactions between cationic lipids and endosomal anionic lipids result in the formation of cone-shaped ion pairs. These ion pairs trigger a phase transition of the endosomal membrane, shifting from an intact bilayer to an inverted micelle (hexagonal, H_{II}) structure which triggers destabilization (Figure 1e) [61–63]. Hence, strategies promote the formation of an H_{II} phase. Most efforts involve the development of optimized ILs. In general, an IL is divided into three parts: the headgroup, linker and tail region, thus enabling countless options for variation [64]. For example, introduction of unsaturated bondings and branching points into the tails represent one approach. Combination of both moieties within one IL may initially shift its geometry towards a cone, thus accelerating endosomal escape [65]. Furthermore, the inclusion of degradable groups contributes biocompatibility and promotes LNP disassembly once internalized [64]. Chen et al. designed novel ILs, containing GSH-responsive disulfide bonds within their linker region. FRET and microscopic colocalization assays were conducted to determine their membrane fusion and rupture capabilities. Overall, superior endosomal escape abilities of cone-shaped, disulfide ILs compared to parent and control lipids was found [66]. A different strategy modulates the LNP's helper phospholipid composition. In particular, 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), the phospholipid utilized in clinically approved LNP formulations, is

assumed to form a cylindrical shape, which promotes particle stability, thus resulting in lysosomal cargo sequestering. In contrast, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) enables the formation of a H_{II} phase facilitating endosomal escape [67]. Recent findings support the hypothesis that pH-driven transitions in structurally active lipid nanotopology (cubic Fd3m inverse micellar, or inverse hexagonal phases) in LNPs promote endosomal escape [68,69].

Despite the broad therapeutic application of LNPs, endosomal escape still remains a challenge with efficacies to be in low ~3 % range as reported by Wittrup and colleagues using fluorescent-labeled endosomal markers and galectin recruitment assays [15]. Lipo-XP carriers (compare Figure 2) with a higher lipophilic LAF to Stp ratio were successfully applied as ionizable compounds in LNP formulations of either siRNA, mRNA or Cas9 mRNA/sgRNA *in vitro* and *in vivo* [59,60,70]. High efficiencies at ultra-low doses were observed, consistent with the expected high endosomal escape potency. In fact, endosomal disruption was analyzed at the quantitative level using siRNA LNPs in a Gal8-mRuby recruitment assay (Figure 3). Endosomal

Figure 3



Imaged-based analysis of endosomal disruption capabilities of siRNA XP-LNPs versus MC3-LNPs using HeLa-Gal8-mRuby3 cells [70], reproduced with permission from authors. Cells were analyzed by either CLSM or ImageStream imaging flow cytometry. HBG-treated cells were considered as a negative control. **a)** The fluorescent Gal8-mRuby3 puncta (green) tracked by confocal microscopy. The scale bar is 50 µm. **b)** The Gal8-mRuby3 puncta count per cell quantified by the ImageStream analyzer. **c)** Representative images of transfected cells by 1621-LNP with high and low spot counts. The cells were analyzed in three different channels of bright field (Ch01), Gal8-mRuby3 (Ch04; green), and Hoechst (Ch07; blue).

spot formation was compared for four lipo-XP (1621, 1755, 1612, 1716) LNPs and well-established MC3-LNPs (as applied in the siRNA drug patisiran). 1621-LNPs performed most effective in endosomal disruption, with about 4-fold higher spot frequency compared with MC3-LNPs.

Photochemical internalization and chemical electron transfer

The principles of photochemical internalization (PCI) originate in photodynamic therapy, an approved treatment of various diseases. In general, PCI requires a photosensitizer (PS) containing an extended chromophore, and a light source (Figure 1f). Upon light exposure, the PS accumulated in endosomal vesicles generates reactive oxygen species (ROS), mainly singlet oxygen which oxidizes lipid membranes and promotes their disruption [10,11,71,72]. Recently, this mechanism was applied to facilitate LNP mediated siRNA delivery. In particular, the Onpattro® formulation was modified by embedding a phospholipid-porphyrin conjugate, increasing endosomal escape upon irradiation [73]. Alternatively, ROS can be generated by non-radiative chemical electron transfer (CET), which demands a donor and acceptor probe. For example, lipopolyplexes co-encapsulating siRNA, bis(2,4,6-trichlorophenyl) oxalate (TCPO) as donor and gold nanoparticles (GN) as acceptor induce ROS formation that facilitates endosomal membrane disruption (demonstrated by galectin-8/mRuby) and siRNA cargo release [74].

Techniques to study endosomal escape

Unfolding the mechanisms of endosomal escape, has been of great interest since decades. Yet, previously the complexity of the endolysosomal system along with a lack in standardized endosomal escape detection techniques impaired the field. Current state-of-the art strategies [Table 1] mainly involve visualizing intracellular trafficking of the cargo and delivery system or membrane perturbations associated with endosomal escape [75]. Wittrup et al. [15] developed time-lapse fluorescence imaging using a spinning disc at high dynamic range to monitor fluorescently labeled nanoparticles (siRNA LNPs) simultaneously with various fluorescence-labeled endosomal markers and galectin-fusion proteins. In general, upon membrane rupture, galectins selectively bind to glycans on the inner endosomal leaflet, yielding punctuate fluorescence patterns. Indeed, recruitment of galectin-8 was found associated with siRNA release into the cytosol. Furthermore, high-throughput imaging assays enable simultaneous quantification of nanoparticle uptake, endosomal leakage and efficacy, allowing comparative analysis of different particle formulations at once [76,77].

An alternative technique for detecting endosomal escape employs multicolor single-molecule localization microscopy (SMLM) to visualize single mRNA-LNPs within sub-endosomal compartments. They found that different LNPs vary considerably in endosomal distributions. Prolonged accumulation of LNPs with impaired endosomal acidification resulted in cytotoxicity and was unproductive for mRNA delivery. High resolution imaging indicated that mRNA escape may preferably occur from recycling tubules, while accumulation of LNPs in defective early endosomes inhibits endosomal acidification, leading to increased toxicity [78]. The "split luciferase endosomal escape quantification" (SLEEQ) assay presents a technique for directly quantifying endosomal escape efficiency. It employs a split luciferase system, with one fragment expressed in reporter cells and the other conjugated to the delivery system. Upon endosomal escape, both moieties fuse to a functional enzyme, generating luminescence. This was evidenced by evaluating the escape efficiency of different pH-responsive nanoparticles [79].

Recent novel endosomal escape strategies and detection technologies have raised new critical questions. To which extent can endosomal membrane leakage be achieved without compromising the biocompatibility of delivery systems? And which type of overcoming the endosomal barrier would be most productive and least cytotoxic to cells? Natural viruses have applied a series of different internalization strategies [80];

Table 1

Techniques for detecting endosomal escape.

Technique	Target	Detection	Ref
Time-lapse high resolution imaging	Cellular uptake Endosomal escape Efficacy	Cy3, AF647 Galectin-8 recruitment eGFP	[15]
CLSM Imaging flow cytometry	Endosomal membrane disruption	count of Galectin8-mRuby puncta	[70]
SMLM	localization of LNPs endosomal escape	immunofluorescence smFISH staining	[78]
SLEEQ-assay	Endosomal escape efficiency	Bioluminescence	[79]

internalization by lipid membrane fusion in case of influenza virus and other enveloped viruses; small endosomal pore formation in case of non-enveloped rhinovirus, or large endosome disruption in case of adenovirus. Hypothetically, a pure lipid membrane fusion mechanism would not expose the interior of endosomes and thus not trigger a Gal-8 recruitment. Recent findings investigating LNPs [81] correlate galectin recruitment with the formation of large, irreversible membrane lesions, ultimately triggering inflammatory responses. In contrast, smaller holes can be repaired by the "endosomal sorting complex required for transport" (ESCRT) machinery, thus preventing inflammation after endosomal escape. Considering LNPs, incorporation of biodegradable ionizable lipids appeared to favor the formation of reparable lesions, thus holding potential for enhancing safety without diminishing efficacy.

Conclusion

Efficient endosomal escape represents a critical step for synthetic nucleic acid delivery systems and a major challenge for the clinical translation. Multiple means have been applied to surmount this hurdle, ranging from small chemical compounds, drugs, peptides, lipids and polymers to physical methods. The complexity of the endosomal system and compartments of cells and differences between various tissues present a significant but interesting challenge. To this date, the various different underlying mechanisms of endosomal escape remain to be better understood with cutting-edge high resolution technologies. Open questions to be answered include: why are hepatocytes more permissive for *in vivo* cytosolic delivery than other tissues? Should one aim for maximum endosomal escape or an optimum release at lower level? Which endosomal escape mechanism is most productive and biocompatible? Vesicle lipid membrane fusion versus endosomal pore formation versus larger endosomal destruction? Would a fast and early escape be preferable or a continuous slow release? Future research is expected to provide the answers that help for drug/target-tailored escape strategies.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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