Journal of Controlled Release 335 (2021) 465–480

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Review article

Escaping the endosome: assessing cellular trafficking mechanisms of non-viral vehicles

Emily Xu [a], W. Mark Saltzman [b,c,d,e,**], Alexandra S. Piotrowski-Daspit [b,*]

[a] *Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06511, United States of America*
[b] *Department of Biomedical Engineering, Yale University, New Haven, CT 06511, United States of America*
[c] *Department of Chemical and Environmental Engineering, Yale University, New Haven, CT 06511, United States of America*
[d] *Department of Cellular & Molecular Physiology, Yale School of Medicine, New Haven, CT 06510, United States of America*
[e] *Department of Dermatology, Yale School of Medicine, New Haven, CT 06510, United States of America*

A R T I C L E I N F O

Keywords:
Endosomal escape
Non-viral vehicle
Polymeric vehicle
Nanoparticle

A B S T R A C T

Non-viral vehicles hold therapeutic promise in advancing the delivery of a variety of cargos *in vitro* and *in vivo*, including small molecule drugs, biologics, and especially nucleic acids. However, their efficacy at the cellular level is limited by several delivery barriers, with endolysosomal degradation being most significant. The entrapment of vehicles and their cargo in the acidified endosome prevents access to the cytosol, nucleus, and other subcellular compartments. Understanding the factors that contribute to uptake and intracellular trafficking, especially endosomal entrapment and release, is key to overcoming delivery obstacles within cells. In this review, we summarize and compare experimental techniques for assessing the extent of endosomal escape of a variety of non-viral vehicles and describe proposed escape mechanisms for different classes of lipid-, polymer-, and peptide-based delivery agents. Based on this evaluation, we present forward-looking strategies utilizing information gained from mechanistic studies to inform the rational design of efficient delivery vehicles.

1. Introduction

Nanomedicine in the form of non-viral delivery vehicles has tremendous potential to treat a myriad of diseases. Viral vectors have been commonly used for gene delivery because of their high transfection efficiency, but they also pose the risk of provoking immune responses and causing aberrant insertional mutagenesis [1]. Therefore, research on alternative non-viral methods has expanded. Advances in these delivery systems incorporate improvements in biocompatibility, targetability, and the ability to deliver larger payloads [2–4]. For example, due to their modifiable properties, polymeric nanoparticles (NPs) have been used to enhance delivery to the central nervous system [5] and for intravenous delivery of cancer drugs [6,7]. Further, there has been great interest in using polymeric NPs and lipid NPs (LNPs) to deliver therapeutic nucleic acids [8,9], gene editing agents such as CRISPR/Cas9 [10,11], and most recently, mRNA-based vaccines [12]. The first two FDA-approved vaccines against SARS-COV-2, the Moderna mRNA-1273 [13] and the Pfizer BNT162b2 [14], both rely on LNP carriers for safe and efficacious delivery [15,16].

Despite continued progress, the clinical translation of non-viral

Abbreviations: AFM, atomic force microscopy; BMP, bis(monoacylglycerol)phosphate; CHEMS, cholesteryl hemisuccinate; CME, clathrin-mediated endocytosis; CPP, cell penetrating peptide; ddRLuc, deglycosylation-dependent Renilla luciferase; DOPE, dioleoylphosphatidyl-ethanolamine; DOPC, dioleoylphosphatidylcholine; DSPE-PEG_{2000}, distearoylphosphatidylethanolamine-polyethyleneglycol$_{2000}$; EEA1, early endosome antigen 1; FRET, Fluorescence resonance energy transfer; Gal8, galectin 8; GEVPG, Glucoevatromonoside containing peracetylated glucose hydroxyl groups; GIGI, Glucocorticoid-Induced eGFP Translocation; HPMA, N-(2-hydroxypropyl)methacrylamide; LAMP1, lysosome-associated membrane protein 1; LNP, lipid nanoparticle; MβCD, methyl-β-cyclodextrin; MPEG, methoxy poly (ethylene glycol); MS, mass spectrometry; mTOR1, mechanistic target of rapamycin complex 1; NGLY1, N-glycanase-1; NP, nanoparticle; NPC1, Neimann-Pick type C1; PACE, poly(amino-co-ester); PAMAM, poly(amindoamine); PBAE, poly(β-amino ester); PCL, poly(ε-caprolactone); PEI, polyethyleneimine; PEG, polyethylene glycol; PHBHHx, 3-hydroxybutyrate-co-3-hydroxyhexanoate; pHLIP, pH (low) insertion peptide; PLGA, poly(lactic-co-glycolic acid); PTMS, PEG-PTTMA-P(GMA-S-DMA); RPE, retinal pigment epithelium; RS, Raman spectroscopy; SERS, surface-enhanced Raman scattering; STORM, stochastic optical reconstruction microscopy; TAT, transactivator of transcription; TGN, trans-Golgi Network; TEM, transmission electron microscopy; TP10, transportan 10.

* Co-Corresponding author at: Malone Engineering Center Room 401E, 55 Prospect Street, New Haven, CT 06511, United States of America.
** Co-Corresponding author at: Malone Engineering Center Room 413, 55 Prospect Street, New Haven, CT 06511, United States of America.
E-mail addresses: mark.saltzman@yale.edu (W.M. Saltzman), alexandra.piotrowski-daspit@yale.edu (A.S. Piotrowski-Daspit).

https://doi.org/10.1016/j.jconrel.2021.05.038
Received 16 February 2021; Received in revised form 26 May 2021; Accepted 26 May 2021
Available online 30 May 2021
0168-3659/© 2021 Published by Elsevier B.V.

vectors continues to be hindered by several challenges related to delivery. Both extra- and intracellular barriers to delivery after systemic administration have been reviewed extensively [17,18], and the ability to control delivery to target tissues, cell types, and even certain sites within cells remains a desirable characteristic for many formulations [19]. At the intracellular level, cellular uptake and endosomal escape are the major hurdles for nanomedicines [20]. Polymeric NPs, for example, are typically taken up by cells via endocytosis pathways and are therefore subject to the endolysosomal pathway [21] (Fig. 1). The relative acidity of endosomes—compared to interstitial fluid or cytoplasm—can lead to significant degradation of NPs and their cargo, and so they must be able to leave this compartment, i.e. "escape" the endosome, to reach their intracellular target in a viable state. This process remains the prohibitive barrier for efficient gene delivery at the cellular level [20]. There are a number of proposed underlying mechanisms to describe how non-viral vehicles are released into the cytosol. However, understanding the mechanism of intracellular delivery is complicated by the likelihood that it is not only vehicle dependent, but also cell-type dependent. Different cell types have different gene expression profiles and endosomal trafficking patterns (Fig. 1), so they each interact with vehicles in unique ways. Other factors, such as cell density, polarity, and differentiation, can also modulate the trafficking patterns [22,23]. These complications build upon the complexity of the process of endocytosis itself, as there are still questions about the number of possible uptake pathways in cells. Although endocytosis has been the object of study for many decades, new pathways and interactions, particularly for clathrin-independent endocytosis, are still being elucidated [22,24]. Following vehicle uptake, additional crosstalk between the endolysosomal pathway and other processes, such as autophagy and exosome biogenesis [25], further complicates the picture. Therefore, a generic, universal mechanism for nanomedicine trafficking might not exist. To pursue rational design strategies that enhance endosomal escape, it is important to understand each nanocarrier in its intended delivery context, especially with respect to the cell-type target of interest.

In this review, we focus on quantitative and qualitative methods to assess the intracellular fate of non-viral vehicles. We describe the most common techniques for quantifying endosomal escape and use this understanding of methods to evaluate what is currently known about the intracellular trafficking mechanisms of lipid-, polymer-, and peptide-based vehicles. With this as background, we describe ways that current knowledge of endosomal escape mechanisms can be used to design smarter, more efficient delivery systems.

2. Experimental methods for assessing endosomal escape and cellular uptake mechanisms

There is no standard technique to determine the mechanisms of intracellular trafficking or endosomal escape. Rather, commonly used experimental tools have been used to illuminate different aspects of the process. Current methods to assess endosomal escape can be subdivided into the following categories: leakage assays, complementation assays, cytosolic-activation assays, inhibitors and genetic screens, and assessments of co-localization. These experimental methods are described below; advantages and shortcomings are summarized in Table 1. One conclusion is clear: to gain a comprehensive understanding of the intracellular fate of nanomedicines, multiple methods must be used in

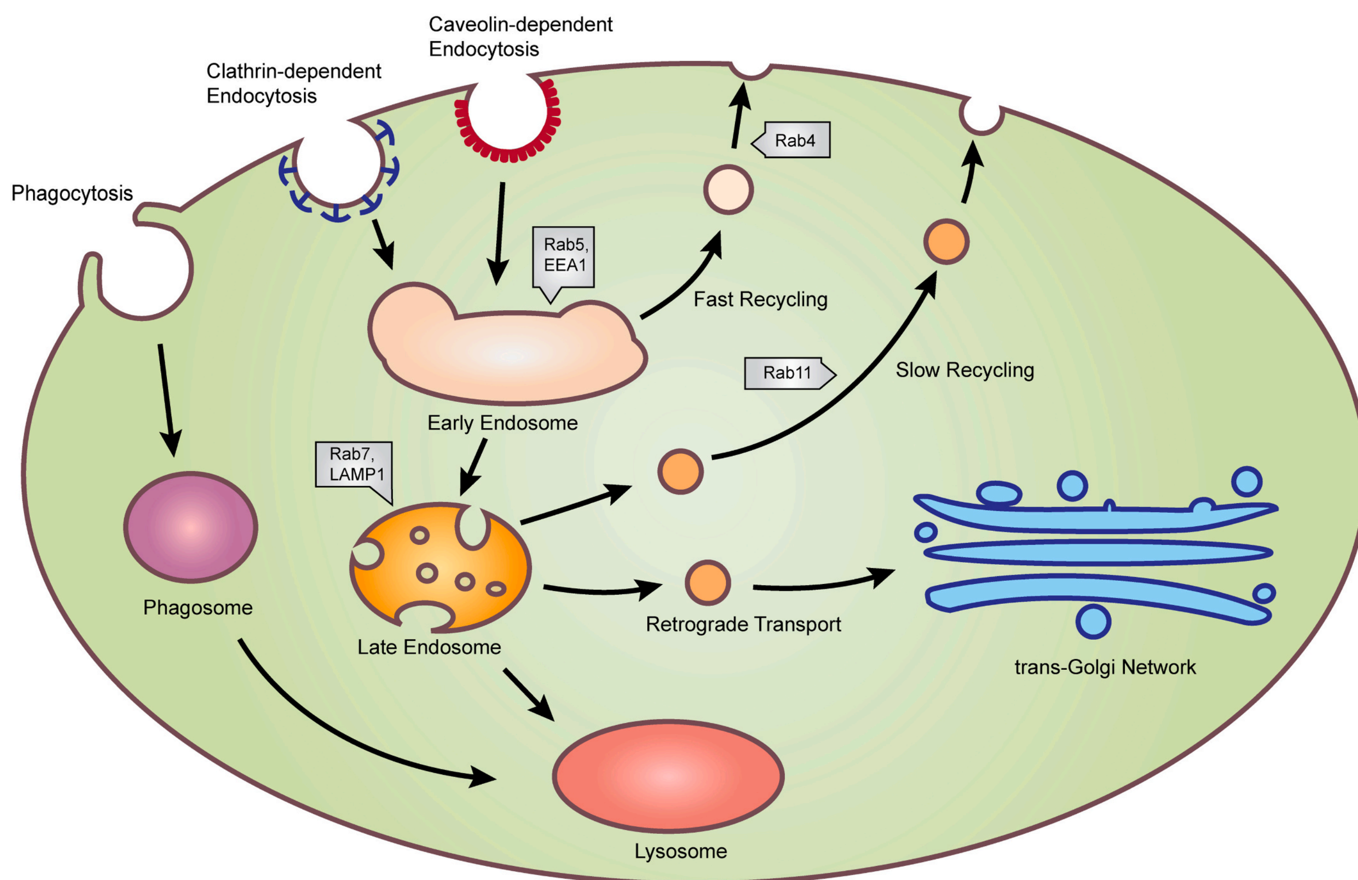

Fig. 1. A schematic overview of the endolysosomal pathway. After uptake, endocytic vesicles fuse with the early endosome, which facilitates sorting of materials that have been taken up. At this stage, ingested materials can be recycled back to the plasma membrane, sent to the trans-Golgi Network (TGN), or degraded through endosomal acidification. Each compartment in the pathway is marked by characteristic proteins, such as early endosome antigen 1 (EEA1), lysosomal-associated membrane protein 1 (LAMP1), and Rab family GTPases.

Table 1
Summary of experimental techniques to assess intracellular trafficking and endosomal escape.

Technique	Description	Advantages	Limitations	Examples from Reported Experimental Data	Ref
Dye Leakage Assay	Measures disruptions in a dye-loaded compartment by quantitating how much dye leaks out	• Can be done *in vitro* or *ex vivo* • Simple procedure that can be used in preliminary experiments to demonstrate endosomal escape potential	• Dye can leak prematurely, leading to false positives • Does not indicate if the cargo has escaped	• A calcein leakage assay was used to compare the endosomal escape of PBAE vs. PLGA NPs in dendritic cells. The results were quantitated by flow cytometry. After 1 h incubation, escape by PBAE NPs was detected in 50% of the cells, compared to 0% of the cells treated with PLGA NPs [28]. • Dye-loaded liposomes were incubated with gold NPs to study the mechanism of membrane disruption. Results suggested that gold NPs primarily interacted with the membrane through electrostatic interactions and that disruption was an all-or-none mechanism [79].	[28,79]
Cytosolic Activation Assay	Uses properties or molecules present in the cytosol to produce a detectable signal to measure endosomal escape	• Can distinguish cytosolic delivery from endosomal disruption • Does not necessarily require transcription or translation machinery	• Requires delivery of a probe, which could affect vehicle properties and intracellular trafficking	• A pH-sensor nanoprobe was co-incubated with PEI NPs over 25 h. Compared to a nanoprobe only control, PEI was able to prevent complete acidification in the endosome, which remained at a pH of 5.5. After 15 h, the pH rapidly increased and eventually reached the starting pH of 7.4, indicating escape into the cytosol [39]. • The delivery efficiency of a library of peptides was assessed based on a biotin ligase assay. Upon cytosolic delivery in cells expressing the bacterial enzyme biotin ligase, the cargo was biotinylated at a specific avi-tag. Levels of biotin were then quantified via western blotting. TAT-based CPPs underperformed bacterial toxin-based transporters and escape varied between four different cell lines [80].	[39,43,44,80]
Split-Protein Complementation Assay	Depends on the binding of two protein fragments to generate a functional probe to measure endosomal escape	• Can distinguish cytosolic delivery from endosomal disruption	• Need to stably transfect cell lines beforehand • Transfected fragments can prematurely bind to one another, leading to false positives	• A split-GFP complementation assay was used to understand the dynamics of TAT peptide-mediated delivery. GFP fluorescence was observed by microscopy as early as 20 min post-treatment and stabilized after 2 h [33].	[33,34,38,81]
Chemical Inhibition	Uses small-molecule inhibitors to disrupt intracellular trafficking pathways	• Easy and quick to conduct	• Non-specific and cell line-dependent	• Chemical inhibitors have been used to investigate the dynamics and mechanisms of PEI-mediated plasmid delivery. Pre-treatment with bafilomycin A1 decreased transfection 30-fold, whereas addition of the inhibitor 4 h after PEI treatment only decreased transfection efficiency by 33%, suggesting that the majority of endosomal escape occurs before 4 h [46]. • The internalization pathway of CPP-functionalized iron oxide NPs was probed through incubation with energy inhibitors, a dynamin inhibitor, MβCD, and chlorpromazine. In all treatment groups, uptake decreased, suggesting that endocytosis is mediated by clathrin and caveolae. However, treatment with another clathrin inhibitor, pitstop 2, did not significantly impact NP uptake, indicating that there are nonspecific effects of chemical inhibitors [82].	[47]
CME Inhibition: Chlorpromazine	Sequesters clathrin and AP2 away from the cell membrane and into endosomes		• Can also affect clathrin-independent pathways • Decreases cell viability		[83,84]
Caveolae-Mediated Inhibition: methyl-β-cyclodextrin (MβCD)	Complexes with and depletes cholesterol in the cell membrane		• Known to impact CME and other endocytosis pathways		[85,86]
Endosome Maturation Inhibition: Bafilomycin A1	Inhibits vacuolar proton ATPases		• Can cause accumulation of protons in the cytoplasm, resulting in acidosis		[46,82,87]
Genetic Screen	Uses genetic manipulation, such as through RNA interference or CRISPR/	• High-throughput • Can identify factors required for trafficking and escape	• Possible off-target effects • Knockdown/knock-out limitations	• A genetic screen using an siRNA library revealed a novel role for Rab33b in polystyrene NP delivery, as knockdown of this marker decreased	[51,52]

(*continued on next page*)

Table 1 (*continued*)

Technique	Description	Advantages	Limitations	Examples from Reported Experimental Data	Ref
	Cas9, to disrupt intracellular trafficking pathways		• Depending on the genes targeted, could alter multiple pathways	NP transport to the late endosome by around 20% [51].	
Co-localization by Fluorescence Microscopy	Microscopy of fluorescently-labeled vehicles and other labeled cellular components to determine co-localization within the cell	• Can identify factors required for trafficking and escape • Can monitor trafficking dynamics in real-time with live imaging	• Low-throughput • Requires fluorescent labels that are susceptible to photo-bleaching, concentration/pH-dependent fluorescence quenching	• Colocalization analysis showed that around 20% of chitosan NPs colocalized with a lysosomal marker after 1 h in HeLa cells. Compartments containing NPs were observed moving towards the perinuclear region [57]. • The endosomal escape efficiency of fluorescently labeled LNPs was estimated at 15% by tracking the carriers through single particle imaging and following their mRNA cargo through single-molecule fluorescence *in situ* hybridization. Escape efficiency was calculated by dividing the number of cytosolic mRNA molecules by the number of internalized LNPs in a single cell [88].	[56–58,88]
FRET	Microscopy technique that uses fluorescent donor and acceptor probes that activate each other when in close proximity	• Can detect interactions within 10 nm resolution	• Requires conjugation and/or delivery of two fluorescent probes	• FRET was used to monitor fusion between liposomes and the endosomal membrane. Protein-modified liposomes were able to mediate fusion, as measured by changes in the fluorescence intensity ratio, while fusion for plain liposomes was not observed [62]. • Nuclear delivery by different sizes of chitosan NPs was investigated using FRET spectroscopy. Hoechst nuclear dye and fluorescein (loaded in the NPs) were used as the donor and acceptor probes, respectively. Whereas the 25 nm NPs did not require a nuclear localization sequence modification for nuclear delivery, the larger 150 nm NPs achieved maximum delivery with functionalization [89].	[62,89,90]
TEM	Microscopy technique that uses an electron beam to image subcellular compartments and labeled vehicles at high magnification	• Offers higher resolution than confocal microscopy and visualization of subcellular structures	• Time-intensive sample preparation • Requires an electron-dense probe	• TEM was used to visualize CPP-mediated protein delivery. Out of the subcellular compartments shown to contain the vehicle, 20% were caveolin-positive. Images also showed that CPP-protein complexes increased the number of caveosomes by two-fold [67].	[67–69]
OTHER					
AFM	Microscopy technique that uses force measurements from a probe to map sample surface topography	• Provides lateral resolution of 1 nm and axial resolution of 0.2 nm • Easier sample preparation than electron microscopy methods	• Cannot be used to image the inner structure of cells	• Researchers compared the uptake of unmodified and poly-L-lysine modified PLGA NPs by AFM imaging of the cell surface. Modified NPs were endocytosed in 20 min, while unmodified NPs remained on the cell surface. The max adhesion force of unmodified NPs on the cell membrane was 280 pN, while modified NPs exerted 1200 pN [91].	[73,74,91]
MS	Spectrometry technique that identifies proteins and other molecules by measuring their mass-to-charge ratio	• Can identify factors required for trafficking and escape • Does not require staining or a label	• Sensitive to impurities and requires stringent subcellular fractionation methods	• Quantitative MS identified Rab7a, Rab9a, and VAMP7 to be enriched in endosomal compartments containing fluorescently labeled iron oxide polystyrene NPs [75].	[69,75]
RS	Spectroscopy technique that analyzes a chemical sample by detecting its vibrational modes	• Does not require staining or a label	• Can have weak signals	• Spectral changes determined by SERS tracked the interactions of gold NPs with the endosome. At 8 h post-treatment, peaks for S-S and C-C bonds emerged, which correspond to the formation of the endolysosome, and at longer incubation times, decreases in C-C stretch bonds indicated a decrease in interaction between the NPs with the endosomal membrane [76].	[76–78]

combination.

2.1. Leakage assays

Leakage assays are simple methods to detect disruptions in endosomal or model membranes by measuring the presence of a fluorescent dye or other detectable molecule outside of the compartment. For example, Manganiello et al. used an *in vitro* hemolysis assay to demonstrate the membrane disruption potential of a diblock copolymer micelle. Free polymer was incubated with erythrocytes in buffers of varying pH to mimic the acidifying endosome. The amount of hemoglobin released was detected using absorbance measurements and used to determine the extent of membrane disruption [26]. Another leakage-based strategy utilized formulated vesicles similar in composition to endosomes, instead of erythrocytes, to more closely model escape [27]. However, these strategies cannot adequately simulate the complexity of the acidifying endosome within the cell and may produce misleading results that do not recapitulate cellular processes. To address this problem, more physiologically-relevant approaches to the leakage-based assay have been developed in cell culture. In one study by Su et al., the impermeable fluorescent dye calcein was co-incubated with lipid-enveloped poly(β-amino ester) (PBAE) NPs. Calcein is trafficked by the endolysosomal pathway once taken up by the cell. It is both self-quenched and quenched by the acidic pH in endosomes, but this phenomenon is reversed once the dye is released into the cytosol (Fig. 2a). Flow cytometry can be used to distinguish cell populations based on fluorescence intensity [28]. However, like most leakage assays, this method is limited by possibility of dye leakage without release of the NP or the cargo.

2.2. Complementation assays

Split-protein complementation assays are another strategy to measure endosomal escape. In these assays, reporter proteins are engineered into two non-functional halves that produce a measurable readout only once they bind to each other. This assay has most commonly been used to investigate protein-protein interactions [29,30], subcellular protein localization [31], and protein assembly [32], but it can also be used to measure cell penetrating peptide (CPP)-mediated delivery to the cytosol [33,34]. Milech and colleagues developed their own version of a split GFP complementation assay to measure the endosomal escape of a library of CPPs fused to cargo proteins. The cells were first transfected with half of a GFP protein, so that the nonfunctional fragment was stably expressed in the cytosol. Then, the other half of the GFP was conjugated to the cargo-CPP fusion. Once the CPPs mediated endosomal escape, the two GFP components complemented each other and generated a fluorescent signal (Fig. 2b). This assay has the advantage of minimal background signal and a direct readout independent of enzymatic processes. The protocol can also be relatively high-throughput, if the fluorescence signal is measured using automated microscopy, flow cytometry, or a

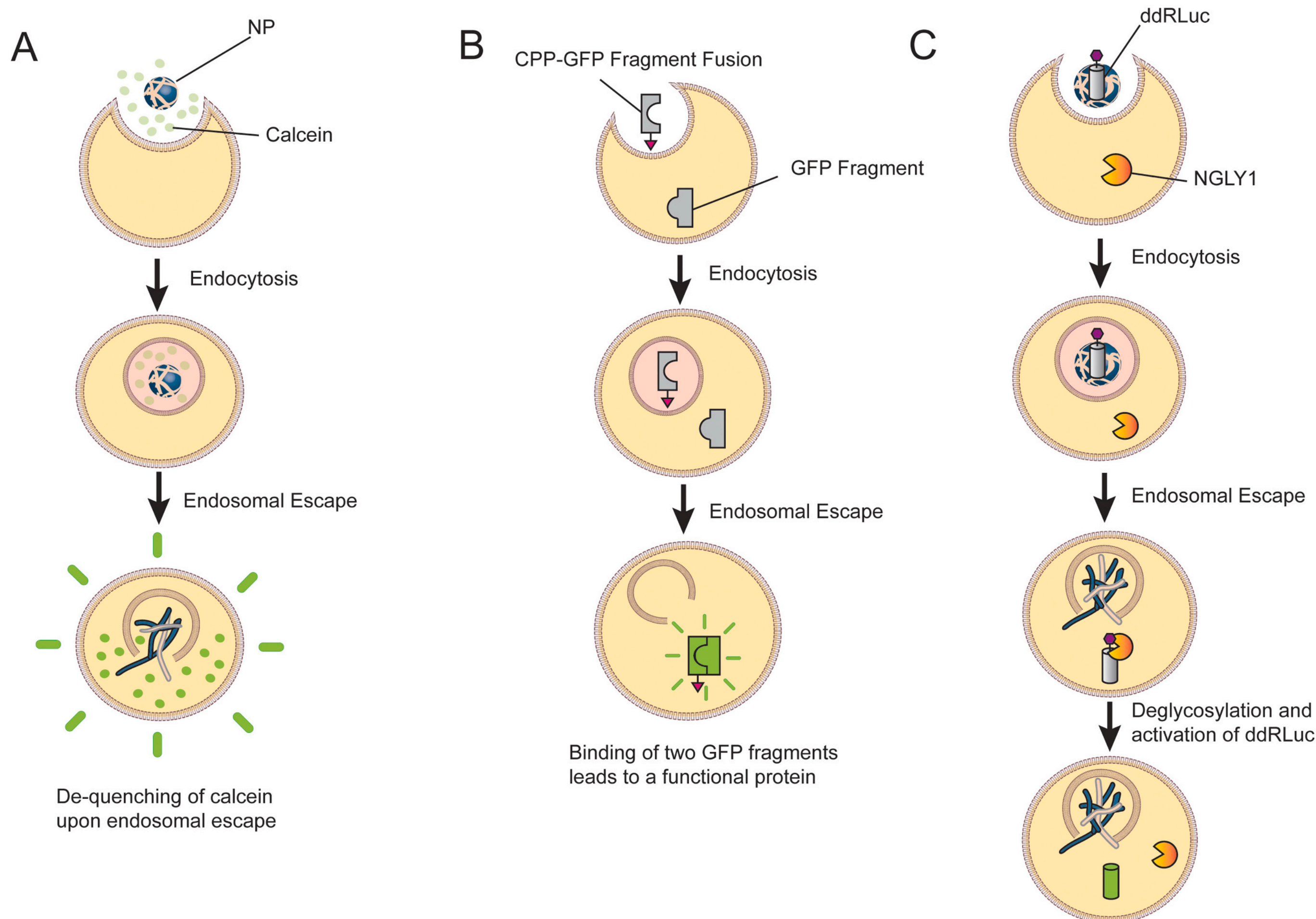

Fig. 2. A schematic of fluorescence- and luminescence-based assays to measure endosomal escape. In these diagrams, the endosomal compartment is pink and the cytoplasm is yellow. (A) NPs and calcein dye are endocytosed by cells and trafficked into endosomes. Calcein is quenched until released into the cytosol upon NP-mediated endosomal escape. (B) A split GFP complementation assay can be adapted to measure the endosomal escape of a CPP by fusing the CPP to a GFP fragment. Endosomal escape allows for the reconstitution of a functional GFP reporter. (C) A deactivated Renilla luciferase probe (ddRLuc) is delivered into the cell, and once it is released into the cytosol, the NGLY1 enzyme restores the functional luciferase and a luminescent signal.

plate reader. Similar protein complementation assays have been expanded to measure the escape of other vehicles, such as cationic lipids [35] and polyplexes [36].

Another variant of the GFP complementation assay directly measures endosomal disruption. Kilchrist et al. developed two split-luciferase assays that rely on the protein interactions of the marker galectin 8 (Gal8), which is recruited to damaged endosomes and lysosomes [37]. In the first assay, Gal8 is fused to an N-terminal luciferase fragment, while another protein, CALCOCO2, is bound to the C-terminal fragment. Upon endosomal disruption, Gal8 localizes inside endosomes and then recruits CALCOCO2, uniting the two luciferase fragments into a functional enzyme with a luminescent readout. The second assay is similar in concept but uses the formation of Gal8 dimers to bring together the luciferase fragments. This was demonstrated for both liposomal- and polymer-based delivery systems, although insufficient sensitivity constrained its use *in vivo* [38]. While this method is rapid, quantitative, and usable without labeling carriers or cargo, it is also limited in that it can only detect endosomal disruption and cannot determine whether the cargo has actually escaped.

2.3. Cytosolic-activation assays

Another class of assays to measure endosomal escape relies on properties or components of the cytosol to produce a signal. For example, certain fluorescent probes take advantage of the difference in pH between the acidic endosome and the neutral cytosol as an indicator of escape. These probes can be co-incubated with the vehicles [39] or directly conjugated to a polymeric [40] or micellar carrier [41]. Other assays rely on particular proteins abundant in the cytosol. One such assay, termed Glucocorticoid-Induced eGFP Induction (GIGI), exploits the tight interaction between a glucocorticoid receptor-transcription factor fusion protein and the Hsp90 heat-shock protein. In the absence of the glucocorticoid ligand, such as dexamethasone or a peptide tagged with dexamethasone, the fusion protein remains tightly bound to Hsp90 in the cytosol. However, once the ligand is successfully delivered into the cytosol by a vehicle, the fusion protein is released and can drive the expression of a reporter gene, which then can be detected using flow cytometry or microscopy [42]. One disadvantage of this approach is the time delay from the release of the fusion protein to detection due to transcription and translation of the reporter gene.

An approach that circumvents this time delay utilizes a deglycosylation-dependent Renilla luciferase (ddRLuc) probe engineered with two key amino acid substitutions that render it enzymatically inactive. The probe can be encapsulated into the vehicle being investigated, and upon endosomal escape, the cytosolic enzyme N-glycanase-1 (NGLY1) activates the luciferase (Fig. 2c). When the vehicle was loaded with mRNA, this release assay was shown to correlate with *in vitro* mRNA transfection efficiency [43]. Another variation of this cytosolic activation concept, which mimics gene delivery closely, is the splicing reporter system developed by Guterstam et al. [44]. A HeLa cell line was stably transfected with a non-functional luciferase with an aberrant splice-site. The complementary oligonucleotide that could mask the splice-site was then delivered, allowing for the production of functional luciferase that could be detected using a luminometer.

2.4. Inhibitors and genetic screens

Pharmacologic inhibitors have been some of the earliest tools developed to elucidate endocytosis and intracellular trafficking mechanisms. By disrupting certain pathways and then observing the resulting effects on vehicle trafficking, researchers can potentially understand which processes and molecules are crucial for intracellular delivery. For example, uptake mechanisms can be determined by applying inhibitors to different endocytosis pathways. Common clathrin-mediated endocytosis (CME) inhibitors include chlorpromazine, hypertonic sucrose, and potassium depletors [45]. Caveolae-mediated endocytosis can be inhibited by cholesterol depletors, such as statins or methyl-β-cyclodextrin [45]. Inhibitors can also be used to investigate specific theories of endosomal escape. Testing the proton sponge hypothesis for polyethyleneimine (PEI)-mediated delivery, Kichler et al. used proton pump inhibitors, bafilomycin A1 and concanamycin A, to determine if endosomal escape decreased [46]. The inhibition strategy is highly amenable to high-throughput studies. For example, Sahay et al. screened a library of small molecule inhibitors in cell culture and used microscopy to discover the effectors required for LNP cellular entry [47]. Unfortunately, most inhibitors can interfere with multiple intracellular processes, limiting the specificity of this approach [48,49]. Some studies have shown that the effects of chemical inhibitors can also be highly dependent on the cell line used [50].

Gene silencing or gene knockouts can offer a more specific platform to study trafficking mechanisms. For example, RNA interference can be used to probe cellular pathways. Panarella et al. developed two siRNA libraries targeting relevant cytoskeletal and endosomal genes and then utilized an automated high-throughput microscopy protocol to determine the effects on NP delivery [51]. Ross-Thriepland et al. conducted a similar screening experiment, except instead of using siRNA, the researchers used the CRISPR/Cas9 gene editing platform to interrogate LNP-mediated mRNA delivery. Through a pooled design, they were able to screen 7795 genes and found 44 hits that either increased or decreased transfection [52]. As with any CRISPR/Cas9-based method, there is potential for off-target effects, so additional validation steps are needed. Some endosomal proteins also play a role in many different trafficking and cellular processes, so similar to pharmacologic inhibitor screens, genetic screens cannot guarantee that only one specific target/pathway is disrupted. Lastly, gene trapping has been successfully implemented to elucidate mechanisms of vesicle trafficking [53] and viral infection [54], but has yet to be explored in the context of non-viral delivery.

2.5. Colocalization assessments

Co-localization studies using immunohistochemistry and fluorescence microscopy enable detailed observations of the intracellular trafficking process of non-viral vehicles. Dyes, such as those used for the leakage assays described above, can be used to distinguish specific intracellular compartments, whereas immunofluorescence staining can label particular proteins, such as EEA1 and LAMP1, that mark vesicles in particular stages of the endolysosomal pathway (Fig. 1). Using these methods and dye-conjugated delivery vehicles, labeled nucleic acid cargo, and/or reporter nucleic acids, non-viral carriers can be systematically tracked throughout intracellular pathways, as demonstrated with poly(lactic-co-glycolic acid) (PLGA) NPs [55]. In one study, NPs loaded with coumarin-6 were screened for co-localization with ~30 Rab GTPase proteins, as well as other factors specific to clathrin-independent and clathrin-dependent uptake pathways [56]. Similar strategies have also been used to probe the pathways of chitosan NPs [57] and LNPs [58]. Unfortunately, colocalization does not directly measure endosomal escape or interactions with trafficking markers, although the use of 3D laser scanning microscopy and live cell imaging offer better opportunities for spatial and temporal observation [59,60].

Fluorescence resonance energy transfer (FRET) is another tool used to visualize molecular interactions between delivery vehicles and cellular components. FRET utilizes two fluorophores: a donor in an excited state that transfers energy via long-range dipole coupling to an acceptor [61]. Because the transfer can only occur if the donor and acceptor are within a 10 nm range, this method can reveal the spatial location of delivery vehicles with high sensitivity. For example, Wang et al. formulated liposomes with the FRET labels Rho-PE and NBD-DOPE. Changes to the fluorescence intensity ratio of these two labels indicated fusion of the vehicle with the endosomal membrane, enabling observation of endosomal escape in real-time [62]. By labeling different molecules with donor and acceptor fluorophores, other questions

regarding trafficking mechanisms can be answered, such as when the cargo and delivery vehicle are separated [63] and to which subcellular compartments the cargo is delivered [64].

Computational image analysis can facilitate quantification of co-localization when used in combination with fluorescence microscopy. One common approach utilizes algorithms to correlate input signals from different fluorescent channels based on image coordinates. Each channel corresponds to a different labeled NP, marker, or compartment. This protocol can determine the percentage of NPs that co-localize with certain factors and also give an intracellular spatial distribution of the vehicle and/or cargo [65]. Another algorithm, termed pair correlation analysis, inputs a series of rapidly scanned images along a designated path from the extracellular space to the nucleus and measures the change in fluorescence intensity at each pixel location across time. These fluctuations are correlated with the concentration of the labeled delivery vehicle, reporting the number of NPs in the extracellular space, cytoplasm, and nucleus at each time point. This analysis can be extended to measuring the movement of NPs in and out of subcellular compartments, making it useful for providing the subcellular location and kinetic profile of cargo release in the cell [66].

Transmission electron microscopy (TEM) has been employed as a complementary technique to optical microscopy. Due to its higher resolution, TEM can be used to detect vehicle interactions with cell membranes, organelles, and even some macromolecules, providing valuable information on intracellular trafficking that is not detectable using fluorescence microscopy. For example, Gilleron et al. used TEM to image LNP-mediated siRNA delivery and then developed a program to automatically count the number of gold-tagged NPs in each image; they found that only 1-2% of siRNA escapes from the endosome, a miniscule amount that would have been undetectable by fluorescence [58]. In addition to tracking *in vitro* delivery by CPPs [67], gold NPs [68,69], and other vehicles [70], TEM has been implemented to track intracellular localization in tissues after administration of NPs *in vivo* [71]. The additional resolution comes at a cost: TEM requires extensive sample preparation and an electron dense label, which could affect vehicle properties and influence cellular uptake and intracellular trafficking.

Atomic force microscopy (AFM) is another high-resolution technique that maps out the topography of a sample by running a cantilever probe across the sample surface [72]. While this method is mainly used for characterization of vehicle morphology, imaging of the differences in polyplex structures in cytosolic and endosomal conditions can be used to make inferences about endosomal escape [73]. In addition, AFM has also been used to directly visualize the lipid bilayer after exposure to dendrimers [74].

Other quantitative methods do not involve microscopy. For example, Hofmann et al. investigated the uptake and trafficking of iron oxide polystyrene NPs by quantitative mass spectrometry (MS). They used the magnetic properties of the NPs to isolate NP-containing intracellular compartments and analyzed the fractions by MS to identify associated proteins [75]. While MS is particularly useful for marker identification, Raman spectroscopy (RS) can be used for monitoring the endosomal activity. Surface-enhanced Raman scattering (SERS) was used to look at spectral changes to track the maturation of endosomes after treatment with gold NPs [76]. RS can also be used in combination with optical microscopy [77] and electron microscopy [78] to obtain sub-nanometer and chemically-specific spatial resolution.

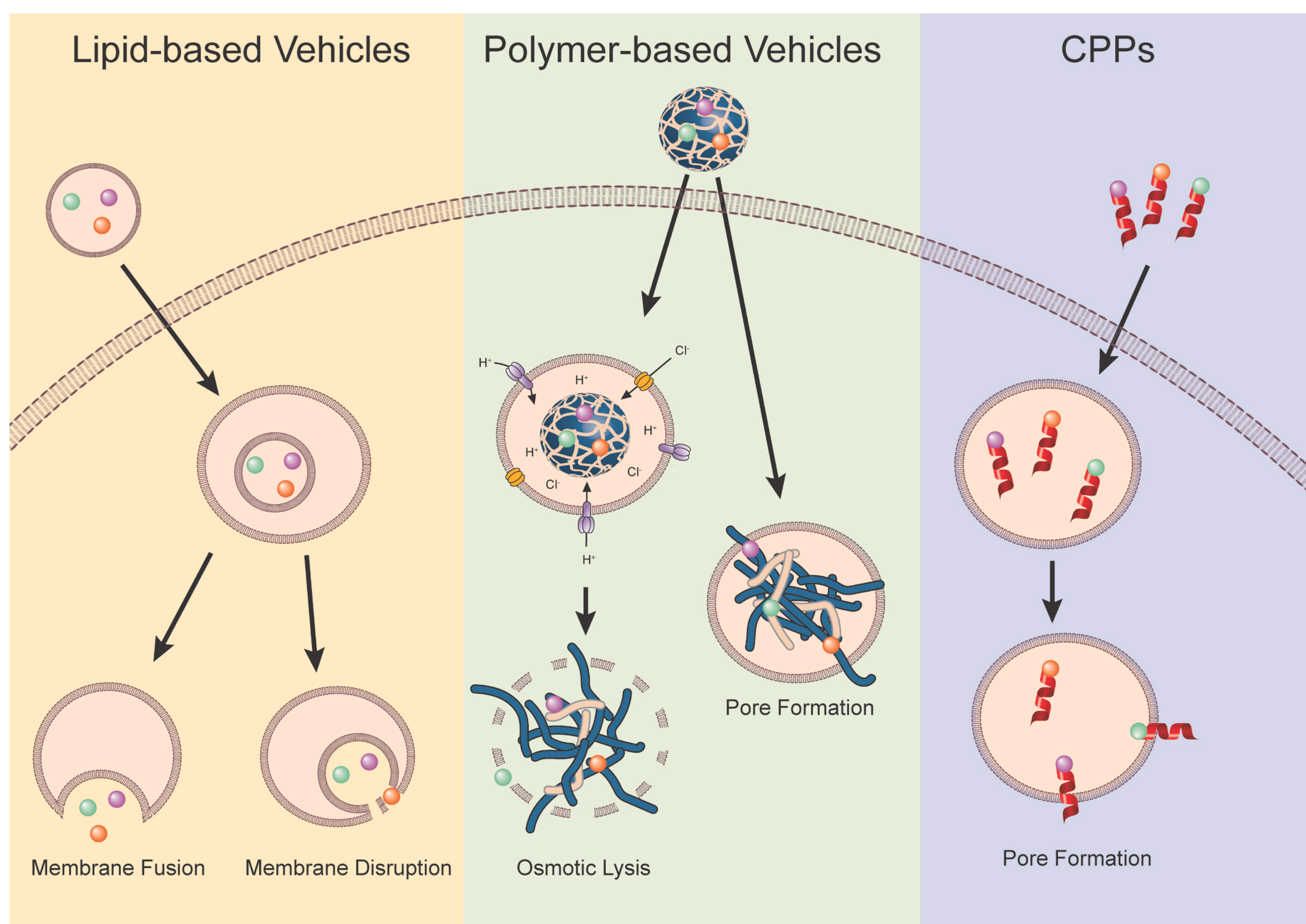

Fig. 3. Summary of proposed endosomal escape mechanisms based on non-viral vehicle type.

3. Endosomal escape mechanisms of non-viral delivery vehicles

Several proposed endosomal escape mechanisms have emerged in the literature for non-viral vehicles. However, despite decades of intense research, the topic remains controversial, probably due to the influence that different vehicle compositions and surface chemistries can have on the mode of escape (Fig. 3). We summarize the current understanding of endosomal escape and uptake mechanisms below, organizing the information according to delivery vehicle type (lipid-, polymer-, and peptide-based carriers). Each section is further divided by the specific sub-types of each class of delivery vehicle.

3.1. Lipid-based vehicles

3.1.1. Liposomes

Liposomes are artificial phospholipid vesicles that have long been used for drug delivery [92], due to their physical properties and low toxicity [93,94]. After uptake by endocytosis, it has been suggested that liposomes mainly rely on membrane fusion to escape the endosome [210]. The surface charge of liposomes can modulate uptake and trafficking mechanisms [97,98]. Anionic liposomes, for example, appear to be processed differently [99] and rely on endosomal acidification to escape [100], which would most likely preclude the fusion model. However, anionic liposomes are still not well-characterized and trafficking mechanisms are unclear.

3.1.2. Lipoplexes

Lipoplexes are heterogeneous complexes formed by mixing preformed cationic liposomes with nucleic acids. Using FRET microscopy, Zelphati et al. studied the trafficking of cationic lipoplexes and hypothesized a three-step mechanism: (1) the cationic lipids of the lipoplex cause the predominantly cytosolic-facing anionic lipids in the endosomal membrane to flip-flop, (2) the anionic lipids complex with the cationic lipids to form an inverted hexagonal structure, and (3) the cargo is then displaced and can be released [96]. This proposed mechanism is bolstered by later evidence suggesting that the formation of an inverted hexagonal structure, with single DNA strands encapsulated in lipid tubules [101], leads to more effective release through fusion [102–104]. The incorporation of the helper lipid DOPE is also known to increase the transfection efficiency of lipoplexes by promoting the formation of the hexagonal structure [105–107]. Therefore, this observation along with confocal microscopy data [108] supports the fusion hypothesis for lipoplexes. However, subsequent data has called for some to suggest revisions to the fusion model. Early EM studies showed that intact lipoplexes were present in the cytosol, supposedly due to endosomal membrane disruption instead of fusion [109]. Live cell fluorescence imaging of lipoplex-mediated delivery revealed a gradual release of the cargo into the cytosol in several stages with no accompanying release of the carrier lipids. This led the authors to propose the idea that multiple transient pores are formed in the endosomal membrane [110]. While the fusion hypothesis also assumes the formation of pores, it is thought to lead to a sudden release rather than the "leakage-like" release that was observed, although further studies are needed to clarify the distinction. Another study by Wittrup et al. found similar results [111]: a highly sensitive microscopy technique was used to observe a rapid cytosolic diffusion of siRNA from the endosome, suggesting that free siRNAs, instead of intact lipoplexes, escaped. Each cell was observed to have one to five release events and the releasing vesicles still displayed fluorescence, indicating that the endosome was not completely lysed. Treatment with proton pump inhibitors decreased the amount of siRNA released, indicating the dependence of escape on endosomal acidification. This study, and others [112], also demonstrated that, upon endosomal escape, autophagy was triggered by galectin recruitment. Degradation by the autophagy pathway forms yet another formidable challenge for lipoplex-mediated delivery [112]. Apart from trafficking through the endosomal pathway, other studies have shed light on alternative pathways for uptake and release of cargo. Lu et al. demonstrated that a minority of siRNA lipoplexes bypass the endosomal pathway and directly fuse with the plasma membrane to reach the cytosol [113]; <5% of siRNA enters the cell by this mechanism and the endocytosis pathway is still the predominant mechanism [95].

These mechanisms have been primarily studied in cell culture. What is the relevance of these theories *in vivo*? Extracellular factors also have the potential to modulate vehicle characteristics and trafficking. For example, serum is known to interfere with lipoplex delivery [114–116]. Zuhorn et al. suggested that serum changes the structure of lipoplexes to a more open conformation that exposes the DNA cargo to digestion [114]. Interestingly, Tandia et al. demonstrated that some of the lipoproteins in serum were able to inhibit lipoplex transfection by enhancing lipid mixing [117], which would seem counterintuitive under the fusion model.

3.1.3. Lipid NPs

LNPs are homogenous NPs that encapsulate the nucleic acid cargo in their core. When compared to lipoplexes of identical lipid composition, LNPs loaded with siRNAs have been shown to have higher cellular uptake: this difference was hypothesized to be due to the difference in structure, as LNPs take on a more spherical shape [118]. Several other studies indicate that surface morphology modulates interactions with the endosomal membrane and may enhance fusion [119,120]. Some LNPs are also formulated with ionizable cationic lipids so that they are neutral at physiological pH and become protonated in the endosomes. Once charged, the LNP can either escape through the proton sponge effect or directly disrupt the negatively-charged endosomal membrane [121]. One study that compared the trafficking patterns of lipoplexes and ionizable LNPs found that the LNPs were always released before the compartments became positive for LAMP1, which is a slightly earlier release point than for lipoplexes [111]. However, a different microscopy study found that ionizable LNPs localized to compartments marked with LAMP1, EEA1, and Rabankyrin-5, a micropinocytosis regulator. This result suggests that LNPs are sequestered in a hybrid vesicle that demonstrates delayed maturation into a lysosome. Additionally, time-lapse confocal fluorescence microscopy showed no "burst" in release that would be expected of a fusion or lysis event. Instead, the release was limited to a few siRNAs escaping from multiple early endocytic compartments [58]. Another study used a CRISPR/Cas9-based approach to knock out Rab5, Rab4, or Rab7, which control formation of the early, recycling, and late endosomes, respectively. While decreases in Rab5 and Rab4 had minimal effect, cells with deficient Rab7 were shown to have significantly impaired LNP-mediated mRNA delivery. However, the discrepancy was not due to differences in endosomal escape, but rather downstream coordination of translation mediated by the mechanistic target of rapamycin complex 1 (mTOR1) [122]. These results reinforce the conclusion that genetic expression or transfection is not an adequate proxy for measuring endosomal escape of nucleic acid cargo and that there remain other barriers to efficient delivery.

Another barrier for LNPs are the recycling pathways, which are used to return receptors from the endosome to the plasma membrane [123]. A study on LNP-mediated siRNA delivery showed that as much as 70% of the delivered siRNA undergoes exocytosis [47]. Neimann-Pick type C1 (NPC1), a lysosomal membrane protein that regulates cholesterol trafficking, was determined to be the regulator of these LNP recycling pathways. Knocking out NPC1 expression prevented the exocytosis of LNPs and increased their retention inside late endosomes/lysosomes, allowing more time for siRNA to diffuse into the cytosol. Another group has further expanded upon these results, following LNPs as they were processed from the endosome into extracellular vesicles and secreted [124]. These extracellular vesicles also had the potential to transfect surrounding cells.

Some of the difficulty in establishing a consistent mechanism could also be due to differences between cell lines, as Sayers et al. have shown. Comparing three different human and mouse cell lines (HCT116, H358,

and CT26), researchers found differences in internalization rates, transfection rates, lysosomal pH, recycling rates, and lysosomal localization rates for the delivery of mRNA by LNPs. Immunofluorescence microscopy also showed that differences in the spatial organization of late endosomal compartments in the cell lines were correlated with transfection rates [125]. The level of differentiation of cells presents another variable in these mechanistic studies. Cells that are highly differentiated are more difficult to transfect by lipid-, polymer-, and peptide-based vehicles [126–128]. Some of the difference can be attributed to decreases in uptake as determined by microscopy studies, but there are morphological factors as well. For example, in a study on siRNA delivery to retinal pigment epithelium cells, researchers found that there was low transfection in 4-week matured lines due to sequestration of the cargo inside melanosomes. Melanosomes were absent in the 2-week matured lines, so there was a larger amount of cytosolic cargo observed in the less-differentiated cells [129]. Comparisons with primary cells also revealed differences in endocytic profiles [130], which is relevant in the context of target cell types *in vivo*. All of these results indicate that *in vitro* data should be interpreted with skepticism and may not be relevant when considering *in vivo* applications. Vehicles should be tailored for their specific target tissue, as endosomal escape not only appears to be vehicle-specific, but also cell-type specific.

3.2. Polymer-based vehicles

3.2.1. Polyplexes

Polyplexes are polymeric vehicles formed by the electrostatic interactions between cationic polymers and anionic nucleic acids. The “proton sponge” hypothesis is often cited to explain the escape of cationic polymer NPs, such as PEI [131,132]. According to this theory, ionizable moieties such as amine groups, which are present in the polymer backbone, buffer protons during endosomal acidification, so that ATPase pumps can continue transporting protons into the compartment. To balance the charge, chloride ions are also transported into the endosome, increasing the osmotic pressure until the membrane finally ruptures [133]. A recent study used stochastic optical reconstruction microscopy (STORM) to track silica NPs functionalized with PEI. STORM confers up to 20 nm lateral resolution and allows for single NP tracking. Together with observations of the rigidity of the PEI chains, the study indicated that the endosomes underwent osmotic swelling caused by the proton sponge effect [134]. Others have reported that smaller endosomal size increases the chance of endosomal escape and that membrane leakiness inhibits it [135], which is consistent with an escape mechanism based on osmotic pressure. However, there is another body of literature disputing the proton sponge effect. Studies have demonstrated that the buffering ability of PEI is not able to increase the pH of intracellular compartments, suggesting that these polymers are unlikely to cause the influx of ions needed for the proton sponge effect [136]. Additionally, the “PEG dilemma” has presented similar challenges. Though the addition of polyethylene glycol (PEG) has long been used to prolong blood circulation time in both polymer- and lipid-based vehicles [137,138], PEG has the unfortunate tradeoff of limiting endosomal escape [139–141]. Since PEGylation does not alter the polymer's buffering capability, the proton-sponge hypothesis is likely not the only mechanism of escape. An alternative explanation for escape is that PEI and other cationic polymers create pores in endosomal membranes that allow cargo to escape. PEI has been shown to permeabilize the membranes of bacteria [142] and these results were further supported using coarse-grained simulations. These computational models confirmed that cationic polymer chains could penetrate across the membrane and create a hydrophilic pore allowing water molecules and ions to pass [143]. Additionally, other studies have used AFM to show that poly (amindoamine) (PAMAM) dendrimers caused the formation of 15-40 nm diameter holes in bilayers. Once the amine end-groups were replaced with acetamide groups, hole formation was abrogated [144]. Given that PEI also has repeating amine groups, it is possible that a similar mechanism of escape occurs. Rehman et al. further shed light on trafficking mechanisms [110]. Through live cell fluorescence imaging, they found that PEI polyplexes exhibited a “burst” of release from the endosome, although the endosomes were only locally ruptured and were not completely lysed. Furthermore, they were able to determine that the nucleic acid cargo likely separates from the polymeric carrier while inside the endosomal compartment, as some of the polymer remnants could still be detected within the endosome after the release event, supporting the pore formation hypothesis.

One interesting comparison with the LNP-mediated trafficking pathway involves the role of NPC1. The same group that initially studied its effect on the exocytosis of LNPs also investigated its role with the cationic polymer PBAE. Whereas previously a knockout of NPC1 led to an increase in transfection efficiency of LNPs, it had the opposite effect for PBAE polyplexes, leading to a 10-fold decrease in uptake [145]. PEI was not affected by the decrease in NPC1 [145], indicating that trafficking and endosomal escape mechanisms for NPs are likely specific to polymer chemistry and the properties of the vehicle as a whole.

3.2.2. Polymeric NPs

In contrast to polyplexes, polymeric NPs are solid NPs that have uniform cargo loading. Panyam et al. compared the mechanism of escape between PLGA NPs and polystyrene NPs [146]. Unlike the PLGA NPs, very few polystyrene NPs were observed inside the cytosol, indicating poor escape. The authors hypothesized that the difference may be due to surface charge. Polystyrene NPs have a negative surface charge at all pH values which preclude them from interacting with the similarly negative endosomal membrane. PLGA is negatively charged at a neutral pH, but it becomes cationic in the acidic endosome. TEM images of PLGA NPs show interaction between the NPs and the membrane of the endosome prior to escape. A follow-up study by the same group employed AFM to show that adhesion forces between the NP and membrane could determine the efficiency of delivery [91]. One interesting study on polystyrene NPs compared the trafficking patterns of 24 nm NPs versus larger 43 nm NPs. Whereas the larger NPs mainly accumulated and degraded in the lysosomes, the smaller ones were able to bypass the endolysosomal pathway entirely and accumulate in the perinuclear region [147]. Controlling the size of NPs may be a useful strategy to achieve higher delivery efficiency but could also affect cargo loading. With regards to the relevance of the endosomal recycling pathway, polymeric NPs such as 3-hydroxybutyrate-co-3-hydroxyhexanoate (PHBHHx) were shown to colocalize with recycling and GLUT4 exocytosis vesicles in addition to experiencing degradation through endosomes [148]. Another study on PLGA NP trafficking also showed that as much as 85% of internalized NPs undergo exocytosis, with most of the NPs exiting within 30 min of uptake [149]. As with LNP-mediated trafficking, endosomal recycling may present a greater challenge than degradation. However, another investigation by Sandin and colleagues found that less than 18% of carboxylated polystyrene NPs colocalized with Rab11-positive membranes, markers of the recycling pathway [60]. These differences in intracellular trafficking are most likely due to different surface properties. For example, conjugation of transferrin onto PLGA NPs is known to increase intracellular retention [150].

3.2.3. Dendrimers

Dendrimers are highly branched polymers that have been used to deliver both small-molecule drugs and nucleic acids. Most dendrimer vehicles are formed with PAMAM [151]. Some studies have suggested that PAMAM can escape into the cytoplasm through the proton sponge effect [152–154]. One study showed that PAMAM itself was unable to induce endosomal escape unless it was modified by histidine residues. Additionally, proton pump inhibition decreased the performance of the histidine modified-PAMAM, suggesting that the improved performance was due to the increased buffering capacity and the proton sponge effect [155]. Furthermore, acetylation of PAMAM dendrimers, which reduces the amount of ionizable primary amines and therefore weakens the

proton sponge effect, was shown to decrease the efficiency of siRNA delivery due to endosomal entrapment [156]. Other models of dendrimer-mediated escape have also been proposed. Studies on interactions between PAMAM and anionic vesicles support a membrane bending model, whereby the rigid, positively-charged dendrimer bends the anionic membrane and disrupts it [157]. Others have also reported on PAMAM's ability to create transient pores in the bilayer [158,159]. The mechanism may also be a combination of several forces. Using coarse-grained molecular dynamic simulations, Tian et al. showed that dendrimers could escape the endosome by increasing the internal osmotic pressure and locally penetrating the membrane [160]. Ainalem et al. compared the effect of dendrimer size on membrane penetrating abilities and showed that the largest dendrimer destroyed the membrane whereas the smaller dendrimers were able to pass through the membrane while leaving it intact [161]. Most of these studies rely on model membranes and the mechanisms by which dendrimers specifically cause endosomal escape in cells remain unclear.

3.3. Peptide-mediated delivery

CPPs are short peptides of around 6-30 amino acid residues. There are two general classes of CPPs that are known to permeabilize membranes: cationic and amphiphilic peptides. The TAT peptide (RKKRRQRRR), which was derived from the HIV transactivator of transcription, is the prototypical cationic CPP. TAT can deliver cargo to the cytosol, albeit with low efficiency [162–164]. This finding suggests that arginine-rich CPPs are unable to mediate endosomal escape [165,166]. Nevertheless, their low performance in cells makes it difficult to adequately detect and assess endosomal escape. Many studies have circumvented this issue by examining the interaction between CPPs and formulated liposomes. For example, Hitz et al. used this approach to elucidate the interaction between oligoarginines with anionic vesicles; they proposed a two step-mechanism with an initial weak electrostatic interaction, followed by a bilayer rigidification that leads to leakiness and disruption of the membrane [167]. Another study by Yang and colleagues supported this theory of membrane permeabilization. The authors highlighted that membrane interactions only occurred in bilayers rich in bis(monoacylglycerol)phosphate (BMP), which is an anionic lipid present in late endosomes [168]. Subsequent work studying the endosomal escape of modified versions of TAT peptides have corroborated this BMP-specific leaky fusion model [169,170].

Amphiphilic peptides can also disrupt lipid bilayers, but they do so through hydrophobic interactions. The “barrel-stave” and “toroidal-pore” models suggest that these peptides create an ordered pore that allows escape [171], while the “carpet” model outlines an accumulation of peptides until a critical concentration is reached, which then leads to a detergent-like disruption [172,173]. Fasoli et al. used whole-cell patch-clamp recording to investigate how modifications to peptides can alter which of the three mechanisms is employed. They found that CM_{18} (KWKLFKKIGAVLKVLTTG), which is an amphipathic α-helical sequence developed from melittin, was able to induce membrane destabilization via the “toroidal-pore” theory, but once it was fused with TAT, it escaped through the “carpet” mechanism [174]. Other studies into the mechanism of pH-responsive CPPs have highlighted the importance of low pH in inducing membrane insertion and leakage [175]. One such CPP is the HA2 peptide (GLFGAIAGFIENGWEGMIDGWYG), which is derived from hemagglutinin [176]. The protonation of glutamic acid and aspartic acid residues in an acidic environment allows for HA2 to insert into the membrane and create transient pores [177,178]. Another family of CPPs that work through a similar mechanism are pH (low) insertion peptides (pHLIPs), which were originally based on the C-helix of the bacteriorhodopsin protein [179]. As their name suggests, in acidic environments, pHLIPs are able to change their conformation into a helix and insert into the membrane. This change is triggered through the protonation of aspartic acid residues [180], and can be used to selectively localize pHLIP-conjugated agents in tumor tissues [181].

While CPPs can be used by themselves to mediate delivery, they are also commonly conjugated or formulated with other vehicle types, which could potentially alter the escape mechanism. El-Sayed and coworkers compared the endosomal escape mechanism of octaarginine (R8)- and octalysine (K8)-modified liposomes [182]. Using FRET imaging, they found that both hybrid vehicles were able to escape via membrane fusion. However, inhibition of endosomal acidification decreased R8-liposome escape, while enhancing K8-liposome escape. Further, live spectral imaging and leakage experiments supported the conclusion that escape by K8-liposomes mainly occurs when the endocytic vesicle is still at a neutral pH, which is soon after uptake, while R8-liposomes can mediate fusion at both an acidic and neutral pH. The authors hypothesized that in the acidic endosome, some of the amino acid groups on hybrid K8-liposomes may become deprotonated due to electrostatic repulsion and would not be able to interact with the negatively charged endosomal membrane. R8-liposome, on the other hand, is able to maintain its positive charge and use its guanidinium group to hydrogen bond with the membrane [182]. Another study by Gomes dos Reis et al. examined PLGA NPs coated with CPPs [183]. Using confocal microscopy and image analysis programs, they observed that 61.5% of all internalized NPs were able to escape the endosome. Their observation of a sudden release of the NPs and the increased surface charge due to the CPP addition led the authors to hypothesize that their vehicle uses the proton sponge effect to lyse the endosome [183]. For vehicles that combine many different materials, the endosomal escape mechanism seems to be largely determined by the surface charge, which should be an important consideration for future vehicle design.

4. Improving vehicle design for enhanced endosomal escape

Endosomal entrapment presents a key bottleneck in delivery, and so designing vehicles with enhanced endosomal escape properties is of increasing interest, particularly in the context of forward-engineering strategies for the rational design of nanomedicines. Some have taken inspiration from strategies employed by viruses, while others have utilized expanding synthetic approaches. In the following section, we briefly outline some recently developed strategies for improving delivery vehicles according to proposed mechanisms of endosomal escape (Table 2).

4.1. Membrane permeabilization

Many NPs have low cytosolic delivery efficiency because they are trapped in the lysosomes, and so a simple way of promoting escape is by co-treatment with lysosomotropic agents, such as chloroquine, to permeabilize the membrane. These molecules are able to freely diffuse into lysosomes, but once they are protonated by the acidified environment, they then cause membrane disruption [184,185]. Du Rietz et al. delivered chloroquine to improve the transfection efficiency of a cholesterol-conjugated siRNA by 47-fold. Using galectin-9 as a sensor for membrane damage, they were able to confirm that chloroquine targets late endosomes or lysosomes to facilitate escape [186].

Another way to improve the escape of vehicles is through the conjugation of CPPs. One example is the work of Tanaka et al. [187]. The group conjugated a TAT analog to a methoxy PEG (MPEG)-poly(ε-caprolactone) (PCL) diblock copolymer. While the polymer MPEG-PCL itself was not able to complex with pDNA, the MPEG-PCL-TAT NP was able to form a stable complex and achieve transfection *in vitro* and *in vivo* with minimal toxicity. Another CPP-polymer conjugate composed of transportan 10 (TP10) and PEI was shown to have superior plasmid delivery compared to PEI and TP10 individually [188]. Some CPPs can even provide cell targeting benefits. Lee et al. created the CPP BR2 (RAGLPFQVGRLLRRLLR) that showed similar transfection efficiency to PEI and R9, another popular CPP, but BR2 demonstrated selective

Table 2
Summary of non-viral vehicle modifications to promote endosomal escape.

Endosomal Escape Strategy	Class of Delivery Vehicle	Vehicle Composition	Cargo	Method(s) To Assess Endosomal Escape	Ref.
Membrane permeabilization by small molecule	LNP	cholesterol conjugated siRNA co-delivered with chloroquine	siRNA	confocal microscopy with a fluorescently labeled Galectin-9 as a membrane disruption sensor	[186]
	Polymer NP	methoxy poly(ethylene glycol)-poly(L-lactic acid)	chloroquine and chemotherapeutic agents	siRNA-induced knockdowns of lysosomal factors and co-localization analysis with confocal microscopy	[206]
Membrane permeabilization by CPP	LNP	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-polyethylene glycol-2000-RFKH	miRNA and irinotecan	co-localization analysis with confocal microscopy	[207]
	Polymeric Dendrimer	peptide-modified PEG-PAMAM dendrimer	methotrexate	co-localization analysis with confocal microscopy	[208]
Proton sponge-mediated lysis	Polyplex	poly(amino-co-ester) (PACE)	pDNA	pH-sensing assay with fluorescent probes	[195]
	Nanomicelle	PEG-PTTMA-P(GMA-S-DNA) (PTMS)	siRNA	pharmacological inhibitors and co-localization analysis with confocal microscopy	[192]
Membrane fusion	Liposome	pH-sensitive cationic lipid, DOPC, DOPE, cholesterol, DSPE-PEG amine	curcumin and paclitaxel	FRET fusion assay	[202]
Photochemical disruption	Polyplex micelle	PEG-PAsp(DET)-PLys	pDNA	co-localization analysis with super-resolution and confocal microscopy	[204]
	Liposome	Lipofectamine	siRNA and $TPPS_{2a}$ (photosensitizer)	co-localization analysis with confocal microscopy	[209]

delivery to cancer cells [189]. While certain CPPs have demonstrated high transfection efficiency and favorable cytotoxicity, they are still limited in terms of cell specificity and by their susceptibility to inactivation by proteases [190]. In some cases, the addition of TAT peptides to NPs also does not increase delivery to targeted cells after intravenous injection [191].

4.2. *Proton sponge effect via pH buffering*

Following the logic of the proton sponge hypothesis, improving the buffering capability of delivery vehicles should promote endosomal escape. Du et al. created a pH-sensitive nanomicelle, PEG-PTTMA-P (GMA-S-DMA) (PTMS), which relies on dimethylamino groups to trigger an increase in osmotic pressure [192]. When loaded with siRNA, these vehicles achieved better gene silencing efficiency than Lipofectamine 2000 in A549 and HeLa cells. These encouraging results were also seen *in vivo*, as injections into tumor sites were able to inhibit further growth with 45% gene knockdown [192]. Other attempts using a similar strategy of introducing secondary and tertiary amines onto chitosan NPs have led to a 100-fold increase in transfection of pDNA when compared to unmodified NPs [193]. As described above, PEI can attain high transfection efficiency due to its ionizable amine groups, but the polymer has high cytotoxicity. We have developed a suite of poly(amino-co-ester)(PACE)-based polymers, which are also synthesized with amine groups to promote escape, but have minimal cytotoxicity due to reduced cationic charge [194]. PACE polymers can be further modified with added orthoester groups into the polymer backbone to increase the acidic sensitivity. These alterations indeed caused a higher rate of endosomal escape as measured by a FITC/Cy5 ratiometric assay [195]. Another modification that has recently been explored is the conjugation of different amine-containing end groups onto the PACE backbone. The polymers in this library have widely differing endosomal escape and mRNA transfection efficiencies, indicating that enhancing buffering capacities is a promising strategy for improving delivery [43]. This end group modification approach has also been explored for other polymers, most notably PBAE. From studying a library of over 2000 unique PBAE structures, one group has demonstrated that the structure of amine end groups impacts almost every step of delivery—from DNA complexation to cellular uptake to the final protein expression [196]. With regards to endosomal escape in particular, incorporating an imidazole group seemed to help the polymer complex avoid trafficking to acidic lysosomes [197].

4.3. *Membrane fusion*

For LNPs, many groups have used the incorporation of fusogenic lipids, like DOPE, in order to promote membrane fusion [198,199]. One group formulated a liposome with DOPE, cholesteryl hemisuccinate (CHEMS), and distearoylphosphatidylethanolamine-PEG_{2000} (DSPE-PEG_{2000}) to deliver a chemotherapeutic drug. This carrier was successful in inhibiting tumor growth in an A549 lung tumor-bearing mice model at an extremely low dose of 1.0 mg/kg [200]. Incorporation of pH-sensitive elements, such as dioleoylphosphatidylcholine (DOPC) or N-succinyl-DOPE, can further increase delivery by allowing for release in response to the acidic endosome [201]. Moku and co-workers used an endosomal pH-sensitive histidinylated lipid in combination with DOPC, DOPE, cholesterol, and DSPE-PEG_{2000} to deliver the drugs curcumin and paclitaxel. FRET experiments confirmed that the LNPs demonstrated enhanced fusion profiles at endosomal pH ranges compared to the cytosolic pH. Furthermore, in a melanoma mouse model, these LNPs could inhibit mouse tumor growth and improve survival [202].

4.4. *Photochemical disruption*

This unique method relies on light activation to rupture endocytic compartments. It often employs a small light-sensitive molecule that produces reactive oxidative species upon irradiation and disrupts the endosomal membrane. Jayakumar et al. created a silica core-shell NP activated by near-infrared light. Once activated, these NPs emit both UV and visible light to active TPPS2A, the photosensitizer, and a photomorpholino, which enhances gene knockdown. Using these modifications, the researchers were able to enhance gene knockdown by 30% *in vitro* and successfully demonstrated their relevance *in vivo* using a murine melanoma model [203]. Another group was able to achieve 100-fold enhanced gene expression using a light-responsive polyplex micelle that incorporated a photosensitizer [204]. But, the benefits of photochemical disruption are not applicable for all vehicles, as it was shown to decrease the delivery efficiency of electrotransfected pDNA, possibly due to increased exposure to endonucleases after endosomal membrane rupture [205].

5. Conclusions and future outlook

The endolysosomal pathway provides a formidable barrier to efficient non-viral therapeutic delivery in cells. To realize the full potential

of nanomedicines, a more comprehensive understanding of the mechanisms of endosomal escape is necessary, alongside more research on the structure-function relationships between vehicle properties and trafficking pathways. The first step in achieving this goal is the development of high-throughput and straightforward techniques to measure endosomal escape. Current methods are largely qualitative and insufficient for distinguishing between different proposed endosomal escape mechanisms. Most techniques require the addition of probes or dyes, which could potentially interfere with trafficking. Progress is further complicated by the fact that the trafficking and escape mechanisms are most likely dependent on both vehicle characteristics and cell type. Additionally, the relevance of *in vitro* measurements to *in vivo* delivery needs to be elucidated, as physiological environments may alter delivery pathways. The present strategies for promoting endosomal escape have already shown promise in increasing the efficiency of delivery vehicles, but future vehicle designs need to also consider toxicity, immunogenicity, stability, and specific tissue and subcellular-targeting concerns. Once a concrete understanding of delivery vehicle trafficking is established, it will lead the way for the rational design of smarter nanomedicines.

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgements

This work was supported by a grant from the National Institutes of Health (NIH; UG3 HL147352). A.S.P was supported by two NIH National Research Service Awards (NRSAs: a T32 GM86287 training grant and an F32 HL142144 individual postdoctoral fellowship), as well as a postdoctoral research fellowship award (PIOTRO20F0) from the Cystic Fibrosis Foundation (CFF).

References

[1] C.E. Thomas, A. Ehrhardt, M.A. Kay, Progress and problems with the use of viral vectors for gene therapy, Nat. Rev. Genet. 4 (2003) 346–358.
[2] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, Nat. Rev. Genet. 15 (2014) 541–555.
[3] R.S. Riley, C.H. June, R. Langer, M.J. Mitchell, Delivery technologies for cancer immunotherapy, Nat. Rev. Drug Discov. 18 (2019) 175–196.
[4] G. Tosi, J.T. Duskey, J. Kreuter, Nanoparticles as carriers for drug delivery of macromolecules across the blood-brain barrier, Expert Opin. Drug Deliv. 17 (2020) 23–32.
[5] M. Gagliardi, C. Borri, Polymer Nanoparticles as Smart Carriers for the Enhanced Release of Therapeutic Agents to the CNS, Curr. Pharm. Des. 23 (2017) 393–410.
[6] R. Ferrari, M. Sponchioni, M. Morbidelli, D. Moscatelli, Polymer nanoparticles for the intravenous delivery of anticancer drugs: the checkpoints on the road from the synthesis to clinical translation, Nanoscale 10 (2018) 22701–22719.
[7] D. Landesman-Milo, S. Ramishetti, D. Peer, Nanomedicine as an emerging platform for metastatic lung cancer therapy, Cancer Metastasis Rev. 34 (2015) 291–301.
[8] J.A. Kulkarni, P.R. Cullis, R. van der Meel, Lipid nanoparticles enabling gene therapies: from concepts to clinical utility, Nucleic Acid Therapeut. 28 (2018) 146–157.
[9] G. Cavallaro, C. Sardo, E.F. Craparo, B. Porsio, G. Giammona, Polymeric nanoparticles for siRNA delivery: Production and applications, Int. J. Pharm. 525 (2017) 313–333.
[10] X. Xu, T. Wan, H. Xin, D. Li, H. Pan, J. Wu, Y. Ping, Delivery of CRISPR/Cas9 for therapeutic genome editing, J. Gene Med. 21 (2019), e3107.
[11] A.S. Piotrowski-Daspit, P.M. Glazer, W.M. Saltzman, Debugging the genetic code: Non-viral in vivo delivery of therapeutic genome editing technologies, Curr. Opin. Biomed. Eng. 7 (2018) 24–32.
[12] S. Al-Halifa, L. Gauthier, D. Arpin, S. Bourgault, D. Archambault, Nanoparticle-based vaccines against respiratory viruses, Front. Immunol. 10 (2019) 22.
[13] L.A. Jackson, E.J. Anderson, N.G. Rouphael, P.C. Roberts, M. Makhene, R. N. Coler, M.P. McCullough, J.D. Chappell, M.R. Denison, L.J. Stevens, A. J. Pruijssers, A. McDermott, B. Flach, N.A. Doria-Rose, K.S. Corbett, K. M. Morabito, S. O'Dell, S.D. Schmidt, P.A. Swanson, M. Padilla, J.R. Mascola, K. M. Neuzil, H. Bennett, W. Sun, E. Peters, M. Makowski, J. Albert, K. Cross, W. Buchanan, R. Pikaart-Tautges, J.E. Ledgerwood, B.S. Graham, J.H. Beigel, An mRNA Vaccine against SARS-CoV-2 — Preliminary Report, N. Engl. J. Med. 383 (2020) 1920–1931.
[14] M.J. Mulligan, K.E. Lyke, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, K. Neuzil, V. Raabe, R. Bailey, K.A. Swanson, P. Li, K. Koury, W. Kalina, D. Cooper, C. Fontes-Garfias, P.-Y. Shi, Ö. Türeci, K.R. Tompkins, E.E. Walsh, R. Frenck, A.R. Falsey, P.R. Dormitzer, W.C. Gruber, U. Şahin, K.U. Jansen, Phase I/II study of COVID-19 RNA vaccine BNT162b1 in adults, Nature 586 (2020) 589–593.
[15] L.R. Baden, H.M. El Sahly, B. Essink, K. Kotloff, S. Frey, R. Novak, D. Diemert, S. A. Spector, N. Rouphael, C.B. Creech, J. McGettigan, S. Khetan, N. Segall, J. Solis, A. Brosz, C. Fierro, H. Schwartz, K. Neuzil, L. Corey, P. Gilbert, H. Janes, D. Follmann, M. Marovich, J. Mascola, L. Polakowski, J. Ledgerwood, B. S. Graham, H. Bennett, R. Pajon, C. Knightly, B. Leav, W. Deng, H. Zhou, S. Han, M. Ivarsson, J. Miller, T. Zaks, Efficacy and Safety of the mRNA-1273 SARS-CoV-2 Vaccine, N. Engl. J. Med. 384 (2020) 403–416.
[16] F.P. Polack, S.J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J. L. Perez, G. Pérez Marc, E.D. Moreira, C. Zerbini, R. Bailey, K.A. Swanson, S. Roychoudhury, K. Koury, P. Li, W.V. Kalina, D. Cooper, R.W. Frenck, L. L. Hammitt, Ö. Türeci, H. Nell, A. Schaefer, S. Ünal, D.B. Tresnan, S. Mather, P. R. Dormitzer, U. Şahin, K.U. Jansen, W.C. Gruber, Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine, N. Engl. J. Med. 383 (2020) 2603–2615.
[17] A.S. Piotrowski-Daspit, A.C. Kauffman, L.G. Bracaglia, W.M. Saltzman, Polymeric vehicles for nucleic acid delivery, Adv. Drug Deliv. Rev. 156 (2020) 119–132.
[18] O.S. Thomas, W. Weber, Overcoming physiological barriers to nanoparticle delivery—are we there yet? Front. Bioeng. Biotechnol. 7 (2019).
[19] N.D. Donahue, H. Acar, S. Wilhelm, Concepts of nanoparticle cellular uptake, intracellular trafficking, and kinetics in nanomedicine, Adv. Drug Deliv. Rev. 143 (2019) 68–96.
[20] M.P. Stewart, A. Lorenz, J. Dahlman, G. Sahay, Challenges in carrier-mediated intracellular delivery: moving beyond endosomal barriers, WIREs Nanomed. Nanobiotechnol. 8 (2016) 465–478.
[21] L.I. Selby, C.M. Cortez-Jugo, G.K. Such, A.P.R. Johnston, Nanoescapology: progress toward understanding the endosomal escape of polymeric nanoparticles, WIREs Nanomed. Nanobiotechnol. 9 (2017), e1452.
[22] K. Sandvig, S. Kavaliauskiene, T. Skotland, Clathrin-independent endocytosis: an increasing degree of complexity, Histochem. Cell Biol. 150 (2018) 107–118.
[23] C. Hinze, E. Boucrot, Endocytosis in proliferating, quiescent and terminally differentiated cells, J. Cell Sci. 131 (2018) jcs216804.
[24] M. Kaksonen, A. Roux, Mechanisms of clathrin-mediated endocytosis, Nat. Rev. Mol. Cell Biol. 19 (2018) 313–326.
[25] J. Xu, R. Camfield, S.M. Gorski, The interplay between exosomes and autophagy - partners in crime, J. Cell Sci. 131 (2018).
[26] M.J. Manganiello, C. Cheng, A.J. Convertine, J.D. Bryers, P.S. Stayton, Diblock copolymers with tunable pH transitions for gene delivery, Biomaterials 33 (2012) 2301–2309.
[27] S.R. Clark, K.Y. Lee, H. Lee, J. Khetan, H.C. Kim, Y.H. Choi, K. Shin, Y.-Y. Won, Determining the effects of PEI adsorption on the permeability of 1,2-dipalmitoylphosphatidylcholine/bis(monoacylglycero)phosphate membranes under osmotic stress, Acta Biomater. 65 (2018) 317–326.
[28] X. Su, J. Fricke, D.G. Kavanagh, D.J. Irvine, In vitro and in vivo mrna delivery using lipid-enveloped pH-responsive polymer nanoparticles, Mol. Pharm. 8 (2011) 774–787.
[29] Z. Liu, D. Xing, Q.P. Su, Y. Zhu, J. Zhang, X. Kong, B. Xue, S. Wang, H. Sun, Y. Tao, Y. Sun, Super-resolution imaging and tracking of protein-protein interactions in sub-diffraction cellular space, Nat. Commun. 5 (2014) 4443.
[30] A. Nickerson, T. Huang, L.J. Lin, X. Nan, Photoactivated localization microscopy with bimolecular fluorescence complementation (BiFC-PALM) for nanoscale imaging of protein-protein interactions in cells, PLoS One 9 (2014), e100589.
[31] D. Kamiyama, S. Sekine, B. Barsi-Rhyne, J. Hu, B. Chen, L.A. Gilbert, H. Ishikawa, M.D. Leonetti, W.F. Marshall, J.S. Weissman, B. Huang, Versatile protein tagging in cells with split fluorescent protein, Nat. Commun. 7 (2016) 11046.
[32] Y.E. Kim, Y.N. Kim, J.A. Kim, H.M. Kim, Y. Jung, Green fluorescent protein nanopolygons as monodisperse supramolecular assemblies of functional proteins with defined valency, Nat. Commun. 6 (2015) 7134.
[33] N. Milech, B.A.C. Longville, P.T. Cunningham, M.N. Scobie, H.M. Bogdawa, S. Winslow, M. Anastasas, T. Connor, F. Ong, S.R. Stone, M. Kerfoot, T. Heinrich, K.M. Kroeger, Y.-F. Tan, K. Hoffmann, W.R. Thomas, P.M. Watt, R.M. Hopkins, GFP-complementation assay to detect functional CPP and protein delivery into living cells, Sci. Rep. 5 (2015) 18329.
[34] P. Lönn, A.D. Kacsinta, X.S. Cui, A.S. Hamil, M. Kaulich, K. Gogoi, S.F. Dowdy, Enhancing endosomal escape for intracellular delivery of macromolecular biologic therapeutics, Sci. Rep. 6 (2016) 32301.
[35] H.H. Wang, A. Tsourkas, Cytosolic delivery of inhibitory antibodies with cationic lipids, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 22132–22139.
[36] B.C. Evans, R.B. Fletcher, K.V. Kilchrist, E.A. Dailing, A.J. Mukalel, J.M. Colazo, M. Oliver, J. Cheung-Flynn, C.M. Brophy, J.W. Tierney, J.S. Isenberg, K. D. Hankenson, K. Ghimire, C. Lander, C.A. Gersbach, C.L. Duvall, An anionic, endosome-escaping polymer to potentiate intracellular delivery of cationic peptides, biomacromolecules, and nanoparticles, Nat. Commun. 10 (2019) 5012.
[37] T.L.M. Thurston, M.P. Wandel, N. von Muhlinen, Á. Foeglein, F. Randow, Galectin 8 targets damaged vesicles for autophagy to defend cells against bacterial invasion, Nature 482 (2012) 414–418.
[38] K.V. Kilchrist, J.W. Tierney, C.L. Duvall, Genetically encoded split-luciferase biosensors to measure endosome disruption rapidly in live cells, ACS Sensors 5 (2020) 1929–1936.
[39] Z.J. Deng, S.W. Morton, D.K. Bonner, L. Gu, H. Ow, P.T. Hammond, A plug-and-play ratiometric pH-sensing nanoprobe for high-throughput investigation of endosomal escape, Biomaterials 51 (2015) 250–256.

[40] S. Lerch, S. Ritz, K. Bley, C. Messerschmidt, C. Weiss, A. Musyanovych, K. Landfester, V. Mailänder, Nanoprobing the acidification process during intracellular uptake and trafficking, Nanomedicine 11 (2015).
[41] Y. Jiang, G. Liu, X. Wang, J. Hu, G. Zhang, S. Liu, Cytosol-specific fluorogenic reactions for visualizing intracellular disintegration of responsive polymeric nanocarriers and triggered drug release, Macromolecules 48 (2015) 764–774.
[42] J.M. Holub, J.R. Larochelle, J.S. Appelbaum, A. Schepartz, Improved assays for determining the cytosolic access of peptides, proteins, and their mimetics, Biochemistry 52 (2013) 9036–9046.
[43] Y. Jiang, Q. Lu, Y. Wang, E. Xu, A. Ho, P. Singh, Y. Wang, Z. Jiang, F. Yang, G. T. Tietjen, P. Cresswell, W.M. Saltzman, Quantitating endosomal escape of a library of polymers for mRNA delivery, Nano Lett. 20 (2020) 1117–1123.
[44] P. Guterstam, F. Madani, H. Hirose, T. Takeuchi, S. Futaki, S. El Andaloussi, A. Gräslund, U. Langel, Elucidating cell-penetrating peptide mechanisms of action for membrane interaction, cellular uptake, and translocation utilizing the hydrophobic counter-anion pyrenebutyrate, Biochim. Biophys. Acta 1788 (2009) 2509–2517.
[45] A.I. Ivanov, Pharmacological Inhibition of Endocytic Pathways: Is It Specific Enough to Be Useful? in: A.I. Ivanov (Ed.), Exocytosis and Endocytosis Humana Press, Totowa, NJ, 2008, pp. 15–33.
[46] A. Kichler, C. Leborgne, E. Coeytaux, O. Danos, Polyethylenimine-mediated gene delivery: a mechanistic study, J. Gene Med. 3 (2001) 135–144.
[47] G. Sahay, W. Querbes, C. Alabi, A. Eltoukhy, S. Sarkar, C. Zurenko, E. Karagiannis, K. Love, D. Chen, R. Zoncu, Y. Buganim, A. Schroeder, R. Langer, D.G. Anderson, Efficiency of siRNA delivery by lipid nanoparticles is limited by endocytic recycling, Nat. Biotechnol. 31 (2013) 653–658.
[48] G. Sahay, D.Y. Alakhova, A.V. Kabanov, Endocytosis of nanomedicines, J. Control. Release 145 (2010) 182–195.
[49] Y. Yan, K. Jiang, P. Liu, X. Zhang, X. Dong, J. Gao, Q. Liu, M.P. Barr, Q. Zhang, X. Hou, S. Meng, P. Gong, Bafilomycin A1 induces caspase-independent cell death in hepatocellular carcinoma cells via targeting of autophagy and MAPK pathways, Sci. Rep. 6 (2016) 37052.
[50] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls, Mol. Ther. 18 (2010) 561–569.
[51] A. Panarella, M.G. Bexiga, G. Galea, E.D. Neill, A. Salvati, K.A. Dawson, J. C. Simpson, A systematic High-Content Screening microscopy approach reveals key roles for Rab33b, OATL1 and Myo6 in nanoparticle trafficking in HeLa cells, Sci. Rep. 6 (2016) 28865.
[52] D. Ross-Thriepland, A. Bornot, L. Butler, A. Desai, H. Jaiswal, S. Peel, M. R. Hunter, U. Odunze, B. Isherwood, D. Gianni, Arrayed CRISPR screening identifies novel targets that enhance the productive delivery of mRNA by MC3-based lipid nanoparticles, SLAS Discov. 25 (2020) 605–617.
[53] P. Navarro Negredo, J.R. Edgar, A.G. Wrobel, N.R. Zaccai, R. Antrobus, D. J. Owen, M.S. Robinson, Contribution of the clathrin adaptor AP-1 subunit µ1 to acidic cluster protein sorting, J. Cell Biol. 216 (2017) 2927–2943.
[54] A. Tanaka, U. Tumkosit, S. Nakamura, D. Motooka, N. Kishishita, T. Priengprom, A. Sa-Ngasang, T. Kinoshita, N. Takeda, Y. Maeda, Genome-wide screening uncovers the significance of N-sulfation of heparan sulfate as a host cell factor for chikungunya virus infection, J. Virol. 91 (2017) (e00432-00417).
[55] M.S. Cartiera, K.M. Johnson, V. Rajendran, M.J. Caplan, W.M. Saltzman, The uptake and intracellular fate of PLGA nanoparticles in epithelial cells, Biomaterials 30 (2009) 2790–2798.
[56] J. Zhang, D. Chang, Y. Yang, X. Zhang, W. Tao, L. Jiang, X. Liang, H. Tsai, L. Huang, L. Mei, Systematic investigation on the intracellular trafficking network of polymeric nanoparticles, Nanoscale 9 (2017) 3269–3282.
[57] H.Y. Nam, S.M. Kwon, H. Chung, S.Y. Lee, S.H. Kwon, H. Jeon, Y. Kim, J.H. Park, J. Kim, S. Her, Y.K. Oh, I.C. Kwon, K. Kim, S.Y. Jeong, Cellular uptake mechanism and intracellular fate of hydrophobically modified glycol chitosan nanoparticles, J. Control. Release 135 (2009) 259–267.
[58] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stöter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, Image-based analysis of lipid nanoparticle–mediated siRNA delivery, intracellular trafficking and endosomal escape, Nat. Biotechnol. 31 (2013) 638–646.
[59] M.T.D.P. Favaro, U. Unzueta, M. de Cabo, A. Villaverde, N. Ferrer-Miralles, A. R. Azzoni, Intracellular trafficking of a dynein-based nanoparticle designed for gene delivery, Eur. J. Pharm. Sci. 112 (2018) 71–78.
[60] P. Sandin, L.W. Fitzpatrick, J.C. Simpson, K.A. Dawson, High-speed imaging of rab family small GTPases reveals rare events in nanoparticle trafficking in living cells, ACS Nano 6 (2012) 1513–1521.
[61] E.A. Jares-Erijman, T.M. Jovin, FRET imaging, Nat. Biotechnol. 21 (2003) 1387–1395.
[62] T. Wang, S. Yang, V.A. Petrenko, V.P. Torchilin, Cytoplasmic delivery of liposomes into MCF-7 breast cancer cells mediated by cell-specific phage fusion coat protein, Mol. Pharm. 7 (2010) 1149–1158.
[63] J. Tang, B. Kong, H. Wu, M. Xu, Y. Wang, Y. Wang, D. Zhao, G. Zheng, Carbon nanodots featuring efficient FRET for real-time monitoring of drug delivery and two-photon imaging, Adv. Mater. 25 (2013) 6569–6574.
[64] S.N. Tammam, H.M.E. Azzazy, A. Lamprecht, The effect of nanoparticle size and NLS density on nuclear targeting in cancer and normal cells; impaired nuclear import and aberrant nanoparticle intracellular trafficking in glioma, J. Control. Release 253 (2017) 30–36.
[65] R.N. Majzoub, C.-L. Chan, K.K. Ewert, B.F.B. Silva, K.S. Liang, C.R. Safinya, Fluorescence microscopy colocalization of lipid-nucleic acid nanoparticles with wildtype and mutant Rab5-GFP: A platform for investigating early endosomal events, Biochim. Biophys. Acta 1848 (2015) 1308–1318.
[66] E. Hinde, K. Thammasiraphop, H.T.T. Duong, J. Yeow, B. Karagoz, C. Boyer, J. J. Gooding, K. Gaus, Pair correlation microscopy reveals the role of nanoparticle shape in intracellular transport and site of drug release, Nat. Nanotechnol. 12 (2017) 81–89.
[67] P. Säälik, K. Padari, A. Niinep, A. Lorents, M. Hansen, E. Jokitalo, Ü. Langel, M. Pooga, Protein delivery with transportans is mediated by caveolae rather than flotillin-dependent pathways, Bioconjug. Chem. 20 (2009) 877–887.
[68] P.M. Tiwari, E. Eroglu, S.S. Bawage, K. Vig, M.E. Miller, S. Pillai, V.A. Dennis, S. R. Singh, Enhanced intracellular translocation and biodistribution of gold nanoparticles functionalized with a cell-penetrating peptide (VG-21) from vesicular stomatitis virus, Biomaterials 35 (2014) 9484–9494.
[69] C.H.J. Choi, L. Hao, S.P. Narayan, E. Auyeung, C.A. Mirkin, Mechanism for the endocytosis of spherical nucleic acid nanoparticle conjugates, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 7625–7630.
[70] S. Akhtar, F.A. Khan, A. Buhaimed, Functionalized magnetic nanoparticles attenuate cancer cells proliferation: Transmission electron microscopy analysis, Microsc. Res. Tech. 82 (2019) 983–992.
[71] A.R. King, C.D. Corso, E.M. Chen, E. Song, P. Bongiorni, Z. Chen, R.K. Sundaram, R.S. Bindra, W.M. Saltzman, Local DNA repair inhibition for sustained radiosensitization of high-grade gliomas, Mol. Cancer Ther. 16 (2017) 1456–1469.
[72] J. Pi, J. Cai, Cell topography and its quantitative imaging by AFM, Methods Mol. Biol. 1886 (2019) 99–113.
[73] M.S. Shim, X. Wang, R. Ragan, Y.J. Kwon, Dynamics of nucleic acid/cationic polymer complexation and disassembly under biologically simulated conditions using in situ atomic force microscopy, Microsc. Res. Tech. 73 (2010) 845–856.
[74] A. Mecke, S. Uppuluri, T.M. Sassanella, D.-K. Lee, A. Ramamoorthy, J.R. Baker, B. G. Orr, M.M. Banaszak Holl, Direct observation of lipid bilayer disruption by poly (amidoamine) dendrimers, Chem. Phys. Lipids 132 (2004) 3–14.
[75] D. Hofmann, S. Tenzer, M.B. Bannwarth, C. Messerschmidt, S.-F. Glaser, H. Schild, K. Landfester, V. Mailänder, Mass spectrometry and imaging analysis of nanoparticle-containing vesicles provide a mechanistic insight into cellular trafficking, ACS Nano 8 (2014) 10077–10088.
[76] D.Y. Öztaş, M. Altunbek, D. Uzunoglu, H. Yılmaz, D. Çetin, Z. Suludere, M. Çulha, Tracing size and surface chemistry-dependent endosomal uptake of gold nanoparticles using surface-enhanced raman scattering, Langmuir 35 (2019) 4020–4028.
[77] T. Chernenko, C. Matthäus, L. Milane, L. Quintero, M. Amiji, M. Diem, Label-free raman spectral imaging of intracellular delivery and degradation of polymeric nanoparticle systems, ACS Nano 3 (2009) 3552–3559.
[78] J. Saarinen, F. Gütter, M. Lindman, M. Agopov, S.J. Fraser-Miller, R. Scherließ, E. Jokitalo, H.A. Santos, L. Peltonen, A. Isomäki, C.J. Strachan, Cell-nanoparticle interactions at (sub)-nanometer resolution analyzed by electron microscopy and correlative coherent anti-stokes raman scattering, Biotechnol. J. 14 (2019), e1800413.
[79] B.Y. Moghadam, W.-C. Hou, C. Corredor, P. Westerhoff, J.D. Posner, Role of nanoparticle surface functionality in the disruption of model cell membranes, Langmuir 28 (2012) 16318–16326.
[80] W.P.R. Verdurmen, M. Mazlami, A. Plückthun, A quantitative comparison of cytosolic delivery via different protein uptake systems, Sci. Rep. 7 (2017) 13194.
[81] S. Cabantous, T.C. Terwilliger, G.S. Waldo, Protein tagging and detection with engineered self-assembling fragments of green fluorescent protein, Nat. Biotechnol. 23 (2005) 102–107.
[82] S. Ben Djemaa, K. Hervé-Aubert, L. Lajoie, A. Falanga, S. Galdiero, S. Nedellec, M. Soucé, E. Munnier, I. Chourpa, S. David, E. Allard-Vannier, gH625 cell-penetrating peptide promotes the endosomal escape of nanovectorized siRNA in a triple-negative breast cancer cell line, Biomacromolecules 20 (2019) 3076–3086.
[83] N.L. Ross, E.V. Munsell, C. Sabanayagam, M.O. Sullivan, Histone-targeted polyplexes avoid endosomal escape and enter the nucleus during postmitotic redistribution of ER membranes, Mol. Ther. Nucleic Acids 4 (2015), e226.
[84] H.R. Kim, S. Gil, K. Andrieux, V. Nicolas, M. Appel, H. Chacun, D. Desmaële, F. Taran, D. Georgin, P. Couvreur, Low-density lipoprotein receptor-mediated endocytosis of PEGylated nanoparticles in rat brain endothelial cells, Cell Mol Life Sci 64 (2007) 356–364.
[85] J. Singh, D. Michel, J.M. Chitanda, R.E. Verrall, I. Badea, Evaluation of cellular uptake and intracellular trafficking as determining factors of gene expression for amino acid-substituted gemini surfactant-based DNA nanoparticles, J. Nanobiotechnol. 10 (2012) 7.
[86] D. Dutta, J.G. Donaldson, Search for inhibitors of endocytosis: Intended specificity and unintended consequences, Cell Logist 2 (2012) 203–208.
[87] A. Akinc, M. Thomas, A.M. Klibanov, R. Langer, Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis, J. Gene Med. 7 (2005) 657–663.
[88] S. Sabnis, E.S. Kumarasinghe, T. Salerno, C. Mihai, T. Ketova, J.J. Senn, A. Lynn, A. Bulychev, I. McFadyen, J. Chan, Ö. Almarsson, M.G. Stanton, K.E. Benenato, A novel amino lipid series for mRNA delivery: improved endosomal escape and sustained pharmacology and safety in non-human primates, Mol. Ther. 26 (2018) 1509–1519.
[89] S.N. Tammam, H.M.E. Azzazy, H.G. Breitinger, A. Lamprecht, Chitosan nanoparticles for nuclear targeting: the effect of nanoparticle size and nuclear localization sequence density, Mol. Pharm. 12 (2015) 4277–4289.

[90] R. Zhang, J. Yang, D.C. Radford, Y. Fang, J. Kopeček, FRET imaging of enzyme-responsive HPMA copolymer conjugate, Macromol. Biosci. 17 (2017) 1600125.
[91] J.K. Vasir, V. Labhasetwar, Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles, Biomaterials 29 (2008) 4244–4252.
[92] V.P. Torchilin, Recent advances with liposomes as pharmaceutical carriers, Nat. Rev. Drug Discov. 4 (2005) 145–160.
[93] M.A. Fridrik, U. Jaeger, A. Petzer, W. Willenbacher, F. Keil, A. Lang, J. Andel, S. Burgstaller, O. Krieger, W. Oberaigner, K. Sihorsch, R. Greil, Cardiotoxicity with rituximab, cyclophosphamide, non-pegylated liposomal doxorubicin, vincristine and prednisolone compared to rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisolone in frontline treatment of patients with diffuse large B-cell lymphoma: A randomised phase-III study from the Austrian Cancer Drug Therapy Working Group [Arbeitsgemeinschaft Medikamentöse Tumortherapie AGMT](NHL-14), Eur. J. Cancer 58 (2016) 112–121.
[94] P.S. Gill, J. Wernz, D.T. Scadden, P. Cohen, G.M. Mukwaya, J.H. von Roenn, M. Jacobs, S. Kempin, I. Silverberg, G. Gonzales, M.U. Rarick, A.M. Myers, F. Shepherd, C. Sawka, M.C. Pike, M.E. Ross, Randomized phase III trial of liposomal daunorubicin versus doxorubicin, bleomycin, and vincristine in AIDS-related Kaposi's sarcoma, J. Clin. Oncol. 14 (1996) 2353–2364.
[95] I.S. Zuhorn, D. Hoekstra, On the mechanism of cationic amphiphile-mediated transfection. to fuse or not to fuse: is that the question? J. Membr. Biol. 189 (2002) 167–179.
[96] O. Zelphati, F.C. Szoka Jr., Mechanism of oligonucleotide release from cationic liposomes, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11493–11498.
[97] J.H. Kang, W.Y. Jang, Y.T. Ko, The effect of surface charges on the cellular uptake of liposomes investigated by live cell imaging, Pharm. Res. 34 (2017) 704–717.
[98] S. Honary, F. Zahir, Effect of zeta potential on the properties of nano-drug delivery systems - a review (Part 1), Trop. J. Pharm. Res. 12 (2013).
[99] Y. Tomori, N. Iijima, S. Hinuma, H. Ishii, K. Takumi, S. Takai, H. Ozawa, Morphological analysis of trafficking and processing of anionic and cationic liposomes in cultured cells, Acta Histochem. Cytochem. 51 (2018) 81–92.
[100] R.M. Straubinger, K. Hong, D.S. Friend, D. Papahadjopoulos, Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles, Cell 32 (1983) 1069–1079.
[101] K. Ewert, H.M. Evans, A. Ahmad, N.L. Slack, A.J. Lin, A. Martin-Herranz, C. R. Safinya, Lipoplex structures and their distinct cellular pathways, Adv. Genet. 53 (2005) 119–155.
[102] J. Smisterová, A. Wagenaar, M.C. Stuart, E. Polushkin, G. ten Brinke, R. Hulst, J. B. Engberts, D. Hoekstra, Molecular shape of the cationic lipid controls the structure of cationic lipid/dioleylphosphatidylethanolamine-DNA complexes and the efficiency of gene delivery, J. Biol. Chem. 276 (2001) 47615–47622.
[103] F. Shi, L. Wasungu, A. Nomden, M.C.A. Stuart, E. Polushkin, J.B.F.N. Engberts, D. Hoekstra, Interference of poly(ethylene glycol)–lipid analogues with cationic-lipid-mediated delivery of oligonucleotides; role of lipid exchangeability and non-lamellar transitions, Biochem. J. 366 (2002) 333–341.
[104] M. Scarzello, V. Chupin, A. Wagenaar, M.C.A. Stuart, J.B.F.N. Engberts, R. Hulst, Polymorphism of pyridinium amphiphiles for gene delivery: influence of ionic strength, helper lipid content, and plasmid DNA complexation, Biophys. J. 88 (2005) 2104–2113.
[105] H. Farhood, N. Serbina, L. Huang, The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer, Biochim. Biophys. Acta Biomembr. 1235 (1995) 289–295.
[106] I. Koltover, T. Salditt, J.O. Rädler, C.R. Safinya, An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery, Science 281 (1998) 78–81.
[107] A. Fasbender, J. Marshall, T.O. Moninger, T. Grunst, S. Cheng, M.J. Welsh, Effect of co-lipids in enhancing cationic lipid-mediated gene transfer in vitro and in vivo, Gene Ther. 4 (1997) 716–725.
[108] M. Nakanishi, A. Noguchi, Confocal and probe microscopy to study gene transfection mediated by cationic liposomes with a cationic cholesterol derivative, Adv. Drug Deliv. Rev. 52 (2001) 197–207.
[109] X. Zhou, L. Huang, DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action, Biochim. Biophys. Acta Biomembr. 1189 (1994) 195–203.
[110] Z.U. Rehman, D. Hoekstra, I.S. Zuhorn, Mechanism of polyplex- and lipoplex-mediated delivery of nucleic acids: real-time visualization of transient membrane destabilization without endosomal lysis, ACS Nano 7 (2013) 3767–3777.
[111] A. Wittrup, A. Ai, X. Liu, P. Hamar, R. Trifonova, K. Charisse, M. Manoharan, T. Kirchhausen, J. Lieberman, Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown, Nat. Biotechnol. 33 (2015) 870–876.
[112] R. Roberts, W.T. Al-Jamal, M. Whelband, P. Thomas, M. Jefferson, J. van den Bossche, P.P. Powell, K. Kostarelos, T. Wileman, Autophagy and formation of tubulovesicular autophagosomes provide a barrier against nonviral gene delivery, Autophagy 9 (2013) 667–682.
[113] J.J. Lu, R. Langer, J. Chen, A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery, Mol. Pharm. 6 (2009) 763–771.
[114] I.S. Zuhorn, W.H. Visser, U. Bakowsky, J.B.F.N. Engberts, D. Hoekstra, Interference of serum with lipoplex–cell interaction: modulation of intracellular processing, Biochim. Biophys. Acta Biomembr. 1560 (2002) 25–36.
[115] S. Li, W.C. Tseng, D.B. Stolz, S.P. Wu, S.C. Watkins, L. Huang, Dynamic changes in the characteristics of cationic lipidic vectors after exposure to mouse serum: implications for intravenous lipofection, Gene Ther. 6 (1999) 585–594.
[116] S. Audouy, G. Molema, L. de Leij, D. Hoekstra, Serum as a modulator of lipoplex-mediated gene transfection: dependence of amphiphile, cell type and complex stability, J. Gene Med. 2 (2000) 465–476.
[117] B.M. Tandia, C. Lonez, M. Vandenbranden, J.M. Ruysschaert, A. Elouahabi, Lipid mixing between lipoplexes and plasma lipoproteins is a major barrier for intravenous transfection mediated by cationic lipids, J. Biol. Chem. 280 (2005) 12255–12261.
[118] K. Kubota, K. Onishi, K. Sawaki, T. Li, K. Mitsuoka, T. Sato, S. Takeoka, Effect of the nanoformulation of siRNA-lipid assemblies on their cellular uptake and immune stimulation, Int. J. Nanomedicine 12 (2017) 5121–5133.
[119] S. Patel, N. Ashwanikumar, E. Robinson, Y. Xia, C. Mihai, J.P. Griffith, S. Hou, A. A. Esposito, T. Ketova, K. Welsher, J.L. Joyal, Ö. Almarsson, G. Sahay, Naturally-occurring cholesterol analogues in lipid nanoparticles induce polymorphic shape and enhance intracellular delivery of mRNA, Nat. Commun. 11 (2020) 983.
[120] M. Yanez Arteta, T. Kjellman, S. Bartesaghi, S. Wallin, X. Wu, A.J. Kvist, A. Dabkowska, N. Székely, A. Radulescu, J. Bergenholtz, L. Lindfors, Successful reprogramming of cellular protein production through mRNA delivered by functionalized lipid nanoparticles, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E3351–e3360.
[121] M. Schlich, R. Palomba, G. Costabile, S. Mizrahy, M. Pannuzzo, D. Peer, P. Decuzzi, Cytosolic delivery of nucleic acids: The case of ionizable lipid nanoparticles, Bioeng. Transl. Med. (2021) e10213.
[122] S. Patel, N. Ashwanikumar, E. Robinson, A. DuRoss, C. Sun, K.E. Murphy-Benenato, C. Mihai, Ö. Almarsson, G. Sahay, 0, Nano Lett. 17 (2017) 5711–5718.
[123] S.C. Allgood, M.R. Neunuebel, The recycling endosome and bacterial pathogens, Cell. Microbiol. 20 (2018), e12857.
[124] M. Maugeri, M. Nawaz, A. Papadimitriou, A. Angerfors, A. Camponeschi, M. Na, M. Hölttä, P. Skantze, S. Johansson, M. Sundqvist, J. Lindquist, T. Kjellman, I.-L. Mårtensson, T. Jin, P. Sunnerhagen, S. Östman, L. Lindfors, H. Valadi, Linkage between endosomal escape of LNP-mRNA and loading into EVs for transport to other cells, Nat. Commun. 10 (2019) 4333.
[125] E.J. Sayers, S.E. Peel, A. Schantz, R.M. England, M. Beano, S.M. Bates, A.S. Desai, S. Puri, M.B. Ashford, A.T. Jones, Endocytic profiling of cancer cell models reveals critical factors influencing LNP-mediated mrna delivery and protein expression, Mol. Ther. 27 (2019) 1950–1962.
[126] M.J. Neill, J. Guo, C. Byrne, R. Darcy, C.M. Driscoll, Mechanistic studies on the uptake and intracellular trafficking of novel cyclodextrin transfection complexes by intestinal epithelial cells, Int. J. Pharm. 413 (2011) 174–183.
[127] H. Matsui, L.G. Johnson, S.H. Randell, R.C. Boucher, Loss of binding and entry of liposome-DNA complexes decreases transfection efficiency in differentiated airway epithelial cells, J. Biol. Chem. 272 (1997) 1117–1126.
[128] C. Foerg, U. Ziegler, J. Fernandez-Carneado, E. Giralt, H.P. Merkle, Differentiation Restricted Endocytosis of Cell Penetrating Peptides in MDCK Cells Corresponds with Activities of Rho-GTPases, Pharm. Res. 24 (2007) 628–642.
[129] E. Ramsay, M. Raviña, S. Sarkhel, S. Hehir, N.R. Cameron, T. Ilmarinen, H. Skottman, J. Kjems, A. Urtti, M. Ruponen, A. Subrizi, Avoiding the pitfalls of siRNA delivery to the retinal pigment epithelium with physiologically relevant cell models, Pharmaceutics 12 (2020).
[130] P. Ilina, S. Partti, J. Niklander, M. Ruponen, Y.-R. Lou, M. Yliperttula, Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models, Exp. Cell Res. 332 (2015) 89–101.
[131] N.D. Sonawane, F.C. Szoka Jr., A.S. Verkman, Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes, J. Biol. Chem. 278 (2003) 44826–44831.
[132] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J. P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 7297–7301.
[133] J.-P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, CHIMIA Int. J. Chem. 51 (1997) 34–36.
[134] M. Wojnilowicz, A. Glab, A. Bertucci, F. Caruso, F. Cavalieri, Super-resolution Imaging of proton sponge-triggered rupture of endosomes and cytosolic release of small interfering RNA, ACS Nano 13 (2019) 187–202.
[135] L.M.P. Vermeulen, T. Brans, S.K. Samal, P. Dubruel, J. Demeester, S.C. De Smedt, K. Remaut, K. Braeckmans, Endosomal size and membrane leakiness influence proton sponge-based rupture of endosomal vesicles, ACS Nano 12 (2018) 2332–2345.
[136] R.V. Benjaminsen, M.A. Mattebjerg, J.R. Henriksen, S.M. Moghimi, T.L. Andresen, The possible "proton sponge " effect of polyethylenimine (PEI) does not include change in lysosomal pH, Mol. Ther. 21 (2013) 149–157.
[137] R. Gref, Y. Minamitake, M. Peracchia, V. Trubetskoy, V. Torchilin, R. Langer, Biodegradable long-circulating polymeric nanospheres, Science 263 (1994) 1600–1603.
[138] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, Adv. Drug Deliv. Rev. 99 (2016) 28–51.
[139] S. Mishra, P. Webster, M.E. Davis, PEGylation significantly affects cellular uptake and intracellular trafficking of non-viral gene delivery particles, Eur. J. Cell Biol. 83 (2004) 97–111.
[140] L. Kong, F. Campbell, A. Kros, DePEGylation strategies to increase cancer nanomedicine efficacy, Nanoscale Horizons 4 (2019) 378–387.
[141] Y. Guan, H. Lu, W. Li, Y. Zheng, Z. Jiang, J. Zou, H. Gao, Near-infrared triggered upconversion polymeric nanoparticles based on aggregation-induced emission and mitochondria targeting for photodynamic cancer therapy, ACS Appl. Mater. Interfaces 9 (2017) 26731–26739.

[142] I.M. Helander, H.L. Alakomi, K. Latva-Kala, P. Koski, Polyethyleneimine is an effective permeabilizer of gram-negative bacteria, Microbiology 143 (Pt 10) (1997) 3193–3199.
[143] C.K. Choudhury, A. Kumar, S. Roy, Characterization of conformation and interaction of gene delivery vector polyethylenimine with phospholipid bilayer at different protonation state, Biomacromolecules 14 (2013) 3759–3768.
[144] A. Mecke, I.J. Majoros, A.K. Patri, J.R. Baker, M.M. Banaszak Holl, B.G. Orr, Lipid bilayer disruption by polycationic polymers: the roles of size and chemical functional group, Langmuir 21 (2005) 10348–10354.
[145] A.A. Eltoukhy, G. Sahay, J.M. Cunningham, D.G. Anderson, Niemann-Pick C1 affects the gene delivery efficacy of degradable polymeric nanoparticles, ACS Nano 8 (2014) 7905–7913.
[146] J. Panyam, W.-Z. Zhou, S. Prabha, S.K. Sahoo, V. Labhasetwar, Rapid endo-lysosomal escape of poly(DL-lactide-coglycolide) nanoparticles: implications for drug and gene delivery, FASEB J. 16 (2002) 1217–1226.
[147] S.K. Lai, K. Hida, S.T. Man, C. Chen, C. Machamer, T.A. Schroer, J. Hanes, Privileged delivery of polymer nanoparticles to the perinuclear region of live cells via a non-clathrin, non-degradative pathway, Biomaterials 28 (2007) 2876–2884.
[148] X. Sun, C. Cheng, J. Zhang, X. Jin, S. Sun, L. Mei, L. Huang, Intracellular trafficking network and autophagy of PHBHHx nanoparticles and their implications for drug delivery, Sci. Rep. 9 (2019) 9585.
[149] J. Panyam, V. Labhasetwar, Dynamics of endocytosis and exocytosis of poly(D,L-lactide-co-glycolide) nanoparticles in vascular smooth muscle cells, Pharm. Res. 20 (2003) 212–220.
[150] S.K. Sahoo, V. Labhasetwar, Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention, Mol. Pharm. 2 (2005) 373–383.
[151] L. Palmerston Mendes, J. Pan, V.P. Torchilin, Dendrimers as nanocarriers for nucleic acid and drug delivery in cancer therapy, Molecules 22 (2017) 1401.
[152] J.F. Kukowska-Latallo, A.U. Bielinska, J. Johnson, R. Spindler, D.A. Tomalia, J. R. Baker Jr., Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 4897–4902.
[153] J. Haensler, F.C. Szoka Jr., Polyamidoamine cascade polymers mediate efficient transfection of cells in culture, Bioconjug. Chem. 4 (1993) 372–379.
[154] M.L. Patil, M. Zhang, O. Taratula, O.B. Garbuzenko, H. He, T. Minko, Internally cationic polyamidoamine PAMAM-OH dendrimers for siRNA delivery: effect of the degree of quaternization and cancer targeting, Biomacromolecules 10 (2009) 258–266.
[155] Y. Wen, Z. Guo, Z. Du, R. Fang, H. Wu, X. Zeng, C. Wang, M. Feng, S. Pan, Serum tolerance and endosomal escape capacity of histidine-modified pDNA-loaded complexes based on polyamidoamine dendrimer derivatives, Biomaterials 33 (2012) 8111–8121.
[156] C.L. Waite, S.M. Sparks, K.E. Uhrich, C.M. Roth, Acetylation of PAMAM dendrimers for cellular delivery of siRNA, BMC Biotechnol. 9 (2009) 38.
[157] Z.-Y. Zhang, B.D. Smith, High-generation polycationic dendrimers are unusually effective at disrupting anionic vesicles: membrane bending model, Bioconjug. Chem. 11 (2000) 805–814.
[158] S. Hong, A.U. Bielinska, A. Mecke, B. Keszler, J.L. Beals, X. Shi, L. Balogh, B. G. Orr, J.R. Baker Jr., M.M. Banaszak Holl, Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport, Bioconjug. Chem. 15 (2004) 774–782.
[159] S. Vaidyanathan, K.B. Anderson, R.L. Merzel, B. Jacobovitz, M.P. Kaushik, C. N. Kelly, M.A. van Dongen, C.A. Dougherty, B.G. Orr, M.M. Banaszak Holl, Quantitative Measurement of Cationic Polymer Vector and Polymer-pDNA Polyplex Intercalation into the Cell Plasma Membrane, ACS Nano 9 (2015) 6097–6109.
[160] W.-d. Tian, Y.-q. Ma, Insights into the endosomal escape mechanism via investigation of dendrimer–membrane interactions, Soft Matter 8 (2012) 6378–6384.
[161] M.-L. Ainalem, R.A. Campbell, S. Khalid, R.J. Gillams, A.R. Rennie, T. Nylander, On the ability of PAMAM dendrimers and dendrimer/DNA aggregates to penetrate POPC model biomembranes, J. Phys. Chem. B 114 (2010) 7229–7244.
[162] S. Gao, M.J. Simon, B. Morrison 3rd, S. Banta, Bifunctional chimeric fusion proteins engineered for DNA delivery: optimization of the protein to DNA ratio, Biochim. Biophys. Acta 1790 (2009) 198–207.
[163] M.P. Gillmeister, M.J. Betenbaugh, P.S. Fishman, Cellular trafficking and photochemical internalization of cell penetrating peptide linked cargo proteins: a dual fluorescent labeling study, Bioconjug. Chem. 22 (2011) 556–566.
[164] Y.J. Lee, A. Erazo-Oliveras, J.P. Pellois, Delivery of macromolecules into live cells by simple co-incubation with a peptide, Chembiochem 11 (2010) 325–330.
[165] S. Al-Taei, N.A. Penning, J.C. Simpson, S. Futaki, T. Takeuchi, I. Nakase, A. T. Jones, Intracellular traffic and fate of protein transduction domains HIV-1 TAT peptide and octaarginine. Implications for their utilization as drug delivery vectors, Bioconjug. Chem. 17 (2006) 90–100.
[166] J.P. Richard, K. Melikov, E. Vives, C. Ramos, B. Verbeure, M.J. Gait, L. V. Chernomordik, B. Lebleu, Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake, J. Biol. Chem. 278 (2003) 585–590.
[167] T. Hitz, R. Iten, J. Gardiner, K. Namoto, P. Walde, D. Seebach, Interaction of α-and β-oligoarginine-acids and amides with anionic lipid vesicles: a mechanistic and thermodynamic study, Biochemistry 45 (2006) 5817–5829.
[168] S.-T. Yang, E. Zaitseva, L.V. Chernomordik, K. Melikov, Cell-penetrating peptide induces leaky fusion of liposomes containing late endosome-specific anionic lipid, Biophys. J. 99 (2010) 2525–2533.
[169] A. Erazo-Oliveras, K. Najjar, D. Truong, T.-Y. Wang, D.J. Brock, A.R. Prater, J.-P. Pellois, The late endosome and its lipid BMP act as gateways for efficient cytosolic access of the delivery agent dfTAT and its macromolecular cargos, Cell Chem Biol 23 (2016) 598–607.
[170] D.J. Brock, L. Kustigian, M. Jiang, K. Graham, T.-Y. Wang, A. Erazo-Oliveras, K. Najjar, J. Zhang, H. Rye, J.-P. Pellois, Efficient cell delivery mediated by lipid-specific endosomal escape of supercharged branched peptides, Traffic 19 (2018) 421–435.
[171] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim. Biophys. Acta Biomembr. 1462 (1999) 55–70.
[172] K.A. Brogden, Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. 3 (2005) 238–250.
[173] F. Salomone, F. Cardarelli, M. Di Luca, C. Boccardi, R. Nifosì, G. Bardi, L. Di Bari, M. Serresi, F. Beltram, A novel chimeric cell-penetrating peptide with membrane-disruptive properties for efficient endosomal escape, J. Control. Release 163 (2012) 293–303.
[174] A. Fasoli, F. Salomone, M. Benedusi, C. Boccardi, G. Rispoli, F. Beltram, F. Cardarelli, Mechanistic insight into CM18-Tat11 peptide membrane-perturbing action by whole-cell patch-clamp recording, Molecules 19 (2014) 9228–9239.
[175] M. Meyer, A. Philipp, R. Oskuee, C. Schmidt, E. Wagner, Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery, J. Am. Chem. Soc. 130 (2008) 3272–3273.
[176] S.A. Wharton, S.R. Martin, R.W. Ruigrok, J.J. Skehel, D.C. Wiley, Membrane fusion by peptide analogues of influenza virus haemagglutinin, J. Gen. Virol. 69 (Pt 8) (1988) 1847–1857.
[177] D.V. Zhelev, N. Stoicheva, P. Scherrer, D. Needham, Interaction of synthetic HA2 influenza fusion peptide analog with model membranes, Biophys. J. 81 (2001) 285–304.
[178] T. Korte, K. Ludwig, A. Herrmann, ph-dependent hydrophobicity profile of hemagglutinin of influenza virus and its possible relevance in virus fusion, Biosci. Rep. 12 (1992) 397–406.
[179] L.C. Wyatt, J.S. Lewis, O.A. Andreev, Y.K. Reshetnyak, D.M. Engelman, Applications of pHLIP technology for cancer imaging and therapy, Trends Biotechnol. 35 (2017) 653–664.
[180] Y.K. Reshetnyak, M. Segala, O.A. Andreev, D.M. Engelman, A Monomeric Membrane Peptide that Lives in Three Worlds: In Solution, Attached to, and Inserted across Lipid Bilayers, Biophys. J. 93 (2007) 2363–2372.
[181] C.J. Cheng, R. Bahal, I.A. Babar, Z. Pincus, F. Barrera, C. Liu, A. Svoronos, D. T. Braddock, P.M. Glazer, D.M. Engelman, W.M. Saltzman, F.J. Slack, MicroRNA silencing for cancer therapy targeted to the tumour microenvironment, Nature 518 (2015) 107–110.
[182] A. El-Sayed, I.A. Khalil, K. Kogure, S. Futaki, H. Harashima, Octaarginine- and octalysine-modified nanoparticles have different modes of endosomal escape, J. Biol. Chem. 283 (2008) 23450–23461.
[183] L. Gomes dos Reis, W.-H. Lee, M. Svolos, L.M. Moir, R. Jaber, A. Engel, N. Windhab, P.M. Young, D. Traini, Delivery of pDNA to lung epithelial cells using PLGA nanoparticles formulated with a cell-penetrating peptide: understanding the intracellular fate, Drug Dev. Ind. Pharm. 46 (2020) 427–442.
[184] A.M. Villamil Giraldo, H. Appelqvist, T. Ederth, K. Öllinger, Lysosomotropic agents: Impact on lysosomal membrane permeabilization and cell death, Biochem. Soc. Trans. 42 (2014) 1460–1464.
[185] F. Joris, L. De Backer, T. Van de Vyver, C. Bastiancich, S.C. De Smedt, K. Raemdonck, Repurposing cationic amphiphilic drugs as adjuvants to induce lysosomal siRNA escape in nanogel transfected cells, J. Control. Release 269 (2018) 266–276.
[186] H. Du Rietz, H. Hedlund, S. Wilhelmson, P. Nordenfelt, A. Wittrup, Imaging small molecule-induced endosomal escape of siRNA, Nat. Commun. 11 (2020) 1809.
[187] K. Tanaka, T. Kanazawa, Y. Shibata, Y. Suda, T. Fukuda, Y. Takashima, H. Okada, Development of cell-penetrating peptide-modified MPEG-PCL diblock copolymeric nanoparticles for systemic gene delivery, Int. J. Pharm. 396 (2010) 229–238.
[188] K. Kilk, S. El-Andaloussi, P. Järver, A. Meikas, A. Valkna, T. Bartfai, P. Kogerman, M. Metsis, Ü. Langel, Evaluation of transportan 10 in PEI mediated plasmid delivery assay, J. Control. Release 103 (2005) 511–523.
[189] Y.W. Lee, Y.E. Hwang, J.Y. Lee, J.H. Sohn, B.H. Sung, S.C. Kim, VEGF siRNA delivery by a cancer-specific cell-penetrating peptide, J. Microbiol. Biotechnol. 28 (2018) 367–374.
[190] H. Derakhshankhah, S. Jafari, Cell penetrating peptides: a concise review with emphasis on biomedical applications, Biomed. Pharmacother. 108 (2018) 1090–1096.
[191] N.A. McNeer, E.B. Schleifman, A. Cuthbert, M. Brehm, A. Jackson, C. Cheng, K. Anandalingam, P. Kumar, L.D. Shultz, D.L. Greiner, W. Mark Saltzman, P. M. Glazer, Systemic delivery of triplex-forming PNA and donor DNA by nanoparticles mediates site-specific genome editing of human hematopoietic cells in vivo, Gene Ther. 20 (2013) 658–669.
[192] L. Du, J. Zhou, L. Meng, X. Wang, C. Wang, Y. Huang, S. Zheng, L. Deng, H. Cao, Z. Liang, A. Dong, Q. Cheng, The pH-triggered triblock nanocarrier enabled highly efficient siRNA delivery for cancer therapy, Theranostics 7 (2017) 3432–3445.
[193] B. Ghosn, S.P. Kasturi, K. Roy, Enhancing polysaccharide-mediated delivery of nucleic acids through functionalization with secondary and tertiary amines, Curr. Top. Med. Chem. 8 (2008) 331–340.
[194] A.C. Kauffman, A.S. Piotrowski-Daspit, K.H. Nakazawa, Y. Jiang, A. Datye, W. M. Saltzman, Tunability of biodegradable poly(amine-co-ester) polymers for customized nucleic acid delivery and other biomedical applications, Biomacromolecules 19 (2018) 3861–3873.

[195] J. Zhang, J. Cui, Y. Deng, Z. Jiang, W.M. Saltzman, Multifunctional poly(amine-co-ester-co-ortho ester) for efficient and safe gene delivery, ACS Biomater. Sci. Eng. 2 (2016) 2080–2089.
[196] J.J. Green, R. Langer, D.G. Anderson, A combinatorial polymer library approach yields insight into nonviral gene delivery, Acc. Chem. Res. 41 (2008) 749–759.
[197] A. Akinc, D.M. Lynn, D.G. Anderson, R. Langer, Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery, J. Am. Chem. Soc. 125 (2003) 5316–5323.
[198] J. Heyes, L. Palmer, K. Bremner, I. MacLachlan, Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids, J. Control. Release 107 (2005) 276–287.
[199] Z. Du, M.M. Munye, A.D. Tagalakis, M.D.I. Manunta, S.L. Hart, The role of the helper lipid on the DNA transfection efficiency of lipopolyplex formulations, Sci. Rep. 4 (2014) 7107.
[200] E.R. Gomes, M.V.M. Novais, I.T. Silva, A.L.B. Barros, E.A. Leite, J. Munkert, A.C. M. Frade, G.D. Cassali, F.C. Braga, R.M. Pádua, M.C. Oliveira, Long-circulating and fusogenic liposomes loaded with a glucoevatromonoside derivative induce potent antitumor response, Biomed. Pharmacother. 108 (2018) 1152–1161.
[201] S.R. Paliwal, R. Paliwal, S.P. Vyas, A review of mechanistic insight and application of pH-sensitive liposomes in drug delivery, Drug Deliv. 22 (2015) 231–242.
[202] G. Moku, S.K. Gulla, N.V. Nimmu, S. Khalid, A. Chaudhuri, Delivering anti-cancer drugs with endosomal pH-sensitive anti-cancer liposomes, Biomater. Sci. 4 (2016) 627–638.
[203] M.K.G. Jayakumar, A. Bansal, K. Huang, R. Yao, B.N. Li, Y. Zhang, Near-Infrared-light-based nano-platform boosts endosomal escape and controls gene knockdown in vivo, ACS Nano 8 (2014) 4848–4858.
[204] T. Nomoto, S. Fukushima, M. Kumagai, K. Machitani, Y. Matsumoto Arnida, M. Oba, K. Miyata, K. Osada, N. Nishiyama, K. Kataoka, Three-layered polyplex micelle as a multifunctional nanocarrier platform for light-induced systemic gene transfer, Nat. Commun. 5 (2014) 3545.
[205] L.D. Cervia, C.-C. Chang, L. Wang, F. Yuan, Distinct effects of endosomal escape and inhibition of endosomal trafficking on gene delivery via electrotransfection, PLoS One 12 (2017) e0171699.
[206] M. Shao, W. Zhu, X. Lv, Q. Yang, X. Liu, Y. Xie, P. Tang, L. Sun, Encapsulation of chloroquine and doxorubicin by MPEG-PLA to enhance anticancer effects by lysosomes inhibition in ovarian cancer, Int. J. Nanomedicine 13 (2018) 8231–8245.
[207] V. Juang, C.-H. Chang, C.-S. Wang, H.-E. Wang, Y.-L. Lo, pH-responsive PEG-shedding and targeting peptide-modified nanoparticles for dual-delivery of irinotecan and microrna to enhance tumor-specific therapy, Small 15 (2019) 1903296.
[208] P. Ma, H. Yu, X. Zhang, H. Mu, Y. Chu, L. Ni, P. Xing, Y. Wang, K. Sun, Increased active tumor targeting by an αvβ3-targeting and cell-penetrating bifunctional peptide-mediated dendrimer-based conjugate, Pharm. Res. 34 (2017) 121–135.
[209] S. Oliveira, M.M. Fretz, A. Høgset, G. Storm, R.M. Schiffelers, Photochemical internalization enhances silencing of epidermal growth factor receptor through improved endosomal escape of siRNA, Biochim. Biophys. Acta Biomembr. 1768 (2007) 1211–1217.
[210] M. Takikawa, M. Fujisawa, K. Yoshino, S. Takeoka, Intracellular Distribution of Lipids and Encapsulated Model Drugs from Cationic Liposomes with Different Uptake Pathways, Int J Nanomedicine 15 (2020) 8401–8409.