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Bioconjug Chem. Author manuscript; available in PMC 2020 February 20.

Published in final edited form as:
Bioconjug Chem. 2019 February 20; 30(2): 273–283. doi:10.1021/acs.bioconjchem.8b00778.

Overcoming Endosomal Entrapment in Drug Delivery

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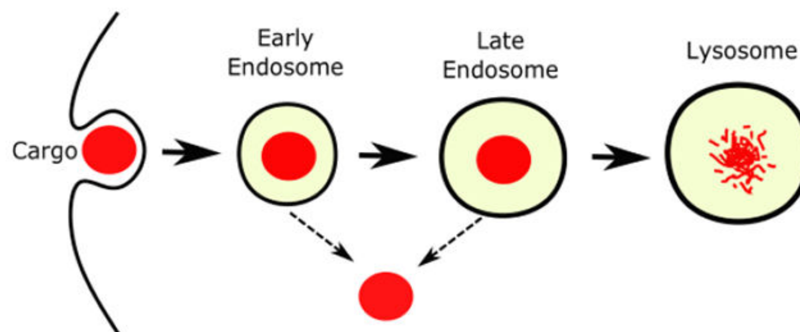
Abstract

Intracellular delivery of biological agents such as peptides, proteins, and nucleic acids generally rely on the endocytic pathway as the major uptake mechanism, resulting in their entrapment inside the endosome and lysosome. The recent discovery of cell-penetrating molecules of exceptionally high endosomal escape and cytosolic delivery efficiencies and elucidation of their mechanism of action represent major breakthroughs in this field. In this *Topical Review*, we provide an overview of the recent progress in understanding and enhancing the endosomal escape process and the new opportunities opened up by these recent findings.

Graphical Abstract



1. INTRODUCTION

Current drugs target only ~20% of disease relevant human proteins.[1] The greater majority of these drugs are small molecules (MW<500), which have the ability to modulate either extra- or intracellular targets, but are generally limited to targeting proteins with deep binding pockets/grooves (e.g., enzymes, receptors, and ion channels). Macromolecular drugs (MW>5000) such as monoclonal antibodies are capable of binding to essentially any target, but are too large to cross the cell membrane and thus limited to extracellular targets. This leaves ~80% of disease relevant human proteins currently undruggable, including some of the most important oncology targets (e.g., Ras and Myc).[2] Among the "undruggable" targets

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are two prototypical protein families – defective/missing proteins caused by genetic mutations[3] and those involved in intracellular protein-protein interactions (PPIs).[4] Targeting these "undruggable" proteins will likely require alternative modalities such as proteins (e.g., antibodies), peptides (including macrocyclic peptides), nucleic acids, and protein-nucleic acid complexes (e.g., CRISPR/Cas9[5]). In principle, diseases caused by loss of protein function can be treated by supplying the patient's cells with a recombinant form of the protein or the coding gene for that protein. Some of the genetic defects may also be permanently corrected by CRISPR/Cas9 or other gene editing therapies. Antibodies, other protein drugs, and more recently macrocyclic peptides have demonstrated remarkable success for modulating extracellular targets including PPIs; if introduced into the cell, they should be effective against intracellular PPIs as well. However, these modalities are generally impermeable to the cell membrane on their own and require proper delivery vectors to reach the diseased tissue and the intracellular space.

2. DRUG DELIVERY SYSTEMS AND THE ENDOSOMAL ENTRAPMENT PROBLEM

The delivery systems that have been explored so far can be divided into two main categories —viral and non-viral delivery. This *Topical Review* will only cover non-viral systems, including receptor-mediated endocytosis,[6] cell-penetrating peptides (CPPs),[7–10] bacterial endotoxins,[11] and the various liposome- and polyplex-based delivery vectors.[12–15] A common feature of these delivery systems is that the vector/cargo complex is initially taken up by cells via one or more endocytic mechanisms and brought into the early endosome (Figure 1). The early endosome (which has slightly acidic pH of ~6.3) gradually matures into late endosome, during which the luminal pH is progressively lowered to ~5.5 by vacuolar ATPase proton pumps that actively transport protons into the endosome. Finally, the late endosome fuses with lysosome (pH 4-5) and the intraluminal contents are degraded by lysosomal enzymes. For the vector and/or cargo to reach the cytosol, they must escape from the endosomal/lysosomal pathway before being degraded inside the lysosome. Unfortunately, for most of the non-viral delivery systems reported to date, their endosomal escape process is very inefficient (generally thought to be a few percent or less[16,17]), resulting in entrapment of most of the vector and/or cargo inside the endosome/lysosome.[18–22] Moreover, lack of mechanistic understanding of the endosomal escape process has prevented any rational approach to improving the escape efficiency. Endocytic recycling (i.e., exocytosis through egress from the late endosome/lysosome) can also reduce the cytosolic delivery efficiency.[23]

Drug delivery by receptor-mediated endocytosis was initially developed to limit the distribution of toxic drugs to only the pathologic cells, thus minimizing collateral damage to normal cells.[24] This method was later used to enable otherwise membrane-impermeable drugs to enter target cells by receptor-mediated endocytosis.[6] For example, chemically stabilized small interference RNAs (siRNAs) have been directly conjugated to N-acetylgalactosamine (GalNAc) to improve their uptake efficiency and specificity for hepatocytes.[25,26] In this case, the 3′ terminus of the siRNA sense strand is attached to three GalNAc molecules by means of a triantennary spacer. The trimeric GalNAc structure

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ensures high-affinity and specific binding to the asialoglycoprotein receptor on hepatocytes and efficient endocytic uptake by the liver tissue. How the GalNAc-siRNA conjugates escape from the endosome is currently unknown, as they contain no moiety aimed at achieving endosomal escape. It was thought that the active transport system results in a very high siRNA loading inside the endosomes/lysosomes of hepatocytes, such that even a tiny fraction of the siRNA leaked into the cytosol is sufficient to generate therapeutic benefits. Liposomes and nanoparticles have also been decorated with specific ligands (e.g., folate) to increase their uptake into cancer cells.[27,28]

CPPs are short peptides (usually 5-30 aa) that are capable of translocating across the eukaryotic cell membrane without causing significant damage to the membrane. CPPs have been used to deliver a wide range of cargoes into mammalian cells, including small molecules, peptides, proteins, nucleic acids and their analogs, nanoparticles, and liposomes.[7–10] It is generally accepted that at low concentrations and especially when conjugated to macromolecular cargoes (e.g., proteins), CPPs enter cells by endocytic mechanisms.[29] For most CPPs, subsequent escape from the endosomal/lysosomal pathway is inefficient,[16,18,19] although three types of conformationally constrained cell-penetrating molecules with high endosomal escape efficiencies (e.g., cyclic CPPs[30–32] and miniature proteins[33,34]) have recently been discovered (vide infra). At high concentrations, CPPs can directly translocate across the plasma membrane by a yet poorly defined mechanism,[29,35] thus bypassing the endosomal/lysosomal pathway. However, therapeutic utility of the direct translocation mechanism may be limited due to the high concentrations required for direct translocation by most CPPs (typically   10 μM). In addition, direct translocation of CPPs is accompanied by the rapid influx of Ca^{2+} ions into the cytosol, resulting in cell injury and death.[36,37]

Many polyplex- and liposome-based systems have been developed for intracellular delivery of biological agents.[12–15] In the case of polyplexes, polycationic vectors are usually mixed with nucleic acid cargos to form nanometer-sized noncovalent complexes, which are taken up by cells through endocytic mechanisms. Again, the vast majority of the nanoparticles remain entrapped inside the endosomal/lysosomal compartments.[20–22] Lipid vesicles have been designed with protein and nucleic acid cargos loaded inside.[12,13] It was thought that when encountering the plasma membrane, the lipid vesicles fuse with the plasma membrane, releasing the cargoes directly into the cytosol or, after endocytic uptake, the vesicles fuse with the endosomal membrane to release the luminal contents into the cytosol. Polyplex- and liposome-based systems are often limited to distribution into organs/tissues with good blood access and/or relatively large blood vessel fenestrations, such as liver, spleen, or kidney.[38,39]

3. MECHANISM OF ENDOSOMAL ESCAPE

Viruses and bacterial toxins have evolved efficient endosomal escape strategies. Many of the non-viral delivery vectors described above also have the capacity to exit the endosome and achieve cytosolic delivery of the attached/associated cargoes, albeit usually at low levels of efficiency. The mechanism(s) by which these natural and synthetic systems escape from the endosome has been the subject of intense investigation over the past decades but, until very recently, has remained elusive. A number of different hypotheses have been put forward in

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the literature. Below we briefly discuss the proposed mechanisms and, when available, the key evidence for and against each of them.

3.1. Proton Sponge Effect and Osmotic Lysis

The "proton sponge" hypothesis[40–42] is often invoked to explain the cytosolic delivery of nucleic acids by polycations such as polyethyleneimine (PEI) and poly(amidoamine) dendrimers, which have large buffering capacity near the physiological pH. When polycation/cargo complexes are internalized by endocytosis, they are first brought into the early endosomes. It is hypothesized that the polyamines prevent endosomal acidification by acting as "proton sponges" and, as a result, the ATPase proton pumps continue to transport protons into the endosomes. To balance the charge inside the vesicles, chloride ions are also transported into the endosomes, causing osmotic swelling of the endosomes leading to eventual rupture of their membrane and release of the endosomal contents into the cytosol (Figure 2a). The "proton sponge" hypothesis has been challenged by several experimental observations (Table 1). First, not all polymers that buffer within the pH 5-7 range are capable of inducing endosomal escape.[43] Conversely, cationic CPPs (e.g., Tat and R_9) do not undergo protonation in the pH 5-7 range but can nonetheless escape from the endosome. Second, Benjaminsen et al. measured the lysosomal pH as a function of PEI content and found that PEI does not affect the lysosomal pH as previously suggested.[44] Third, Wang et al. developed ultra-pH-sensitive nanoparticles that enter cells through endocytosis and clamp the endosomal/lysosomal pH at any desired value (pH 4.0-7.4).[45] Clamping the endosomal pH at three different values for up to 90 min did not cause any damage to the plasma or endosomal membrane. Fourth, recent studies showed that endosomal escape of siRNA-containing polyplexes and lipoplexes does not lead to complete endosome rupture as the "proton sponge" hypothesis postulates.[46,47] Rather, a significant fraction of cargo stayed within endosomes as well as the lipid part of lipoplex. Finally, lysis of the late endosome/lysosome would cause significant cytotoxicity and yet, many of the CPPs and lipoplex systems are relatively nontoxic to the cell.

3.2. Membrane Fusion

When enveloped viruses are brought into the endosome, their viral membrane can fuse with the endosomal membrane, allowing the viral capsid to enter the cytosol.[48] Similarly, it was proposed that the lipid bilayer of a liposome can fuse with the plasma or endosomal membrane and release the vesicular contents into the cytosol (Figure 2b).[49] However, this mechanism is inconsistent with a recent observation that only ~50% of the siRNA inside each lipid nanoparticle (LNP) is released from the endosome into the cytosol,[47] as membrane fusion is expected to result in complete release of the liposomal contents. Obviously, the fusion mechanism does not apply to any delivery system that does not involve liposomes (e.g., CPPs and polyplexes).

3.3. Pore Formation

Bacteria can introduce their proteins, known as effectors, into host cells to modify the behavior of the latter.[11] Bacterial toxins typically consist of three functional domains: a receptor-binding domain for recognition of specific receptors on the host cell surface, a transmembrane domain for inducing endosomal escape, and an effector domain that is the

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actual toxin. It is generally thought that the toxin proteins are brought into the early endosome by receptor-mediated endocytosis. As the endosomal pH decreases, the transmembrane domain undergoes a conformational change and inserts into the endosomal membrane to form a pore, through which the effector domain translocates from the endosome to the cytosol.[50,51] Peptides (e.g., CPPs) have been proposed to induce endosomal escape by forming similar pores on the endosomal membrane.[52] Interaction between cationic amphipathic peptides and lipid bilayers may lead to the formation of two different types of pores: barrel-stave vs toroidal pores.[53,54] In the barrel-stave pore model, peptides are aligned parallel with the phospholipids to serve as staves which together form a barrel-shaped pore, with the hydrophobic side facing the lipids and the hydrophilic side facing the channel (Figure 2c). Formation of toroidal pores is thought to be facilitated by peptides binding to the membrane surface in a perpendicular orientation followed by membrane bending inward to form a toroidal pore (Figure 2d).

A major limitation of the pore formation hypothesis is its inability to explain how macromolecular cargoes such as proteins and plasmid DNA are translocated across the endosomal membrane (Table 1). An average-sized globular protein (50 kDa) has a diameter of ~50 Å, which is roughly the same dimension as the thickness of a lipid bilayer. Formation of a 50-Å barrel-stave pore would require at least 15-20 α-helices (and more for β-strands) at the channel surface. It is inconceivable how 15-20 50-Å proteins could be accommodated around a 50-Å pore without physically blocking the pore. To reconcile this, it has been proposed that bacterial toxins undergo denaturation under the acidic endosomal pH and the unfolded polypeptide threads through small pores.[55] However, some bacterial toxins (e.g., Diphtheria toxin) can deliver cargo proteins of varying sizes and thermal stabilities (including hyper-stable proteins) across the endosomal membrane with similar efficiencies.[56] Furthermore, it was recently shown that Diphtheria toxin is capable of delivering non-covalently associated nucleic acid cargoes across the endosomal membrane.[57] Similarly, CPPs have been shown to deliver non-covalently associated protein cargoes into the cytosol.[58] These observations suggest that the bacterial toxin and/or cargo molecules are transported across the endosomal membrane in their folded states. Pore formation also cannot reconcile the observation that endosomal release of siRNA/polyplex or siRNA/lipoplex occurs instantly and is followed by rapid (10–20 s) diffusion of siRNA throughout the cytosol.[46,47] Thus, pore formation is unlikely the mechanism for endosomal escape of CPPs, polyplexes, lipoplexes, or at least some of the bacterial toxins.

3.4. Membrane Destabilization/Disruption

Given the inadequacy of the hypotheses described above, membrane destabilization and/or disruption is increasingly being used to describe the endosomal escape of biological cargos, especially the polymer based delivery systems.[18,59] In brief, either the vector alone (e.g., a CPP or a cationic polymer) or the vector-cargo conjugate/complex interacts directly with the endosomal luminal membrane through charge-charge and/or hydrophobic interactions, leading to local membrane destabilization and permeability (Figure 2e). Since membrane destabilization/disruption is local, most of the endolysosomal compartment is kept intact during and after escape, as opposed to a complete lysis of the endosome/lysosome suggested by the “proton sponge” hypothesis. However, because this mechanism also requires the

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cargo (and the vector in most cases) to physically move across a lipid bilayer, it has some of the same limitations of the pore formation hypothesis, e.g., inability to explain how macromolecular cargos (e.g., proteins, plasmid DNA, and viral particles) exit the endosome without compromising the integrity of the endosome.

3.5. Vesicle Budding and Collapse

Pei and co-workers recently proposed a vesicle budding and collapse mechanism for endosomal escape of CPPs and CPP-cargo conjugates.[32] Initially demonstrated with cyclic CPPs on model membranes (giant unilamellar vesicles), vesicle budding and collapse were subsequently observed in live cells (A. Sahni and D. Pei, manuscript in preparation). In this mechanism, CPPs bind to the luminal leaflet of the endosomal membrane, inducing membrane curvature and budding of CPP-enriched lipid domains as small vesicles (Figure 2f). The budded vesicles then disintegrate into amorphous lipid/peptide aggregates, either as they bud off the endosomal membrane or shortly after budding off the membrane, resulting in the release of vesicular contents into the cytosol. Immediately prior to budding off, CPPs are concentrated at the budding neck, which has high potential energy due to membrane distortion (i.e., formation of acute negative Gaussian curvature). The budding neck resembles the "transition state" of a chemical reaction; selective binding of CPPs to the budding neck likely stabilizes the "transition state" and reduces the "activation" energy barrier, thereby accelerating the rate of the budding event. Endosomal acidification likely facilitates the budding event by increasing the binding affinity of arginine-rich CPPs for the endosomal membrane.[32] Note that a key distinction from the above mechanisms is that during and after each vesicle budding/collapse event, the endosome stays intact. Multiple budding/collapse events may occur on the same endosome, either simultaneously or sequentially, until the vesicle is largely depleted of the CPP.[32]

The vesicle budding/collapse mechanism resolves some of the long-standing enigmas in the CPP field (Table 1). For example, it readily explains how large protein cargos attached to CPPs exit the endosome in their folded states, since they do not physically cross the lipid bilayer at all. It provides a simple explanation for the effect of cargo on CPP activity. The cargo (e.g., a negatively charged one) may physically interact with the CPP and prevent it from binding to the membrane phospholipids[60] or, in the case of hydrophobic dyes,[61] may enhance the performance of a CPP by directly binding to membrane phospholipids during endocytic uptake and/or endosomal escape. The model predicts that peptides with greater binding affinity for the endosomal membrane should have higher endosomal release efficiency and exit the endosomal/lysosomal pathway at an earlier time point. This is indeed the case - for a panel of 10 CPPs examined (including Tat and R_9), their endosomal escape efficiencies are linearly correlated with the endosomal membrane-binding affinities.[32] Conformationally constrained CPPs bind to the endosomal membrane with greater affinity and exit the endosome more efficiently and earlier than linear CPPs (vide infra).[32,34] In contrast, Tat, which does not bind to the endosomal membrane with sufficient affinity at neutral pH, cannot escape from the early endosome.[31,34] It appears that the highly acidic pH (4.5-5.5) and the negatively charged phospholipids of the late endosome are required for Tat to gain sufficient membrane-binding affinity and induce vesicle budding and collapse. It is worth noting that, although vesicle budding and collapse have so far been experimentally

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demonstrated for CPPs only,[32] the mechanism appears applicable to bacterial toxins, polyplexes, lipoplexes/LNPs, and non-enveloped viruses (Table 1). For example, the instantaneous, partial release of cargo without complete disruption of the endosome, as observed during lipoplex- and polyplex-mediated siRNA delivery,[46,47] is readily explained by the sudden collapse of a budded vesicle. Unlike other endosomal escape hypotheses, vesicle budding and collapse offer a potential mechanism by which an intact viral particle exits the endosome. A 24-residue amphipathic α-helical peptide of capsid protein VI of adenovirus was shown to be primarily responsible for the endosomal escape of the 90-nm virus during host infection.[62] This peptide binds with high affinity (apparent K_D ~3 μM) to vesicles of endosomal membrane composition, induces curvature on the vesicles, and causes the latter to "fragment" into smaller ones (and peptide/lipid aggregates). We posit that upon endosomal entry, an adenovirus releases up to ~360 copies of protein VI, which subsequently bind to the endosomal membrane and induce budding and collapse of a virus-loaded vesicle. Similarly, it is conceivable that the transmembrane domain of a bacterial toxin binds to the endosomal membrane and induces vesicle budding and collapse, in the presence or absence of membrane insertion of an amphipathic α-helix.[50,51]

4. STRATEGIES FOR IMPROVING ENDOSOMAL ESCAPE

Inspired by the efficient endosomal escape of viruses and bacterial toxins, researchers have explored numerous strategies to improve the endosomal escape efficiency of the non-viral delivery systems described above or, in some cases, to bypass the endosomal/lysosomal pathway altogether. We describe below some of the more widely pursued and clinically relevant methods for enhancing endosomal release. Other methods, such as photochemical internalization (PCI) technology[63] and iTOP for directly delivering proteins across the plasma membrane,[64] will not be discussed here. It must be emphasized that, although each strategy was based on a certain mechanistic hypothesis, the actual mechanism of action is generally unknown and may well be different from what was originally envisioned.

4.1. "Endosomal Buffering"

The putative "proton sponge" effect has been widely explored for intracellular delivery of biological agents, especially nucleic acids. In addition to PEI, a variety of other polyamines and polyamidoamines, both linear and dendritic, were developed.[15] Common features include pKa values in the physiological range and high buffering capacity when inside the acidic endosome/lysosome. They are typically mixed with a nucleic acid cargo to form noncovalent nanoparticles or polyplexes, which are taken up by cells via endocytosis. Once inside the endosome, it is thought that they cause osmotic lysis of the endosome/lysosome and release of the polyplex into the cytosol. Polymeric materials containing the imidazole ring (which has a pKa value of 6.5-7.1) such as polyhisitidines,[65] histidylated polylysines,[66] and histidylated lipids[67] have been used as transfectants. These pH-sensitive cationic polymers are effective for nucleic acid delivery in vitro and their membrane lytic activity is largely limited to the endosomal membrane. However, large excess of the polymers (N/P ratio >10) is required for effective transfection, often resulting in dose-limiting toxicity during in vivo applications. Given the fact that the "proton sponge" hypothesis is now being challenged, it may be fruitful to reexamine the vast literature on "endosomal buffering" in

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the context of new mechanistic insights (e.g., the budding and collapse mechanism) and use any improved mechanistic understanding to guide the design of the next generation of cationic polymers.

4.2. Fusion with Bacterial Toxins

Researchers have taken advantage of the modular structures of bacterial toxins to design fusion proteins for two different purposes. First, the receptor-binding domains of bacterial toxins were replaced with ligands that bind selectively to the surface of cancer cells.[68] The resulting fusion proteins are selectively taken up by cancer cells via receptor-mediated endocytosis. Once inside the endosome, the transmembrane domain of the toxin mediates efficient endosomal escape and delivery of the effector domain into the cytosol of cancer cells, resulting in selective killing of the cancer cells. Several immunotoxin drugs based on diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A have already been approved by the FDA for treating various cancers.[68] Second, the transmembrane domains of bacterial toxins may be used to deliver exogenous cargoes into mammalian cells. Pentelute and co-workers employed the protective antigen (PA) of Anthrax toxin as a transporter and delivered a variety of proteins and peptides into the cytosol of mammalian cells by fusing them to an N-terminal fragment of the anthrax lethal factor (LFN), the second component of the Anthrax toxin.[69] Kakimoto et al. improved the transfection efficiency of PEI for plasmid DNA by fusing the transmembrane domain of diphtheria toxin to PEI (via a biotin-streptavidin pair), apparently due to enhanced endosomal escape.[57] A limitation of this approach is that bacterial toxins often cause immune responses in vivo.

4.3. Membrane Fusogenic and Endolytic Peptides

Inspired by the fusion of viral envelopes with the endosomal membrane of a host cell, researchers have prepared synthetic fusogenic peptides based on the fusion domain of the influenza virus and used them to improve endosomal release of non-viral delivery vectors. For example, influenza-derived fusogenic peptide diINF-7 was employed to enhance endosomal release of siRNA carriers for silencing the genes encoding the epidermal growth factor receptor (EGFR) and K-Ras oncogenes.[70] The addition of diINF-7 improved the transfection efficiency of Lipofectamine and knockdown of EGFR expression by >2-fold in human epidermoid carcinoma A431 cells relative to Lipofectamine alone. Similarly, a 3.5-fold improvement in knockdown of the K-Ras oncogene was observed in C26 murine colon carcinoma cells in the presence of diINF-7. Harashima and coworkers constructed a multifunctional envelope-type nano device (MEND) for the efficient delivery of nucleic acids to cells.[71] To improve the efficiency of siRNA delivery, the researchers modified the surface of MEND with a pH-sensitive fusogenic peptide, GALA. The resulting system was highly efficient in siRNA-mediated gene knockdown of luciferase expression in a human fibrosarcoma cell line stably expressing the luciferase (HT1080-luc) and in a xenograft mouse model with subcutaneously implanted HT1080-luc tumor cells. Alipour et al. fused an influenza-derived fusogenic peptide (H5WYG) to the N-terminus of histone protein H1 and employed the fusion protein to deliver plasmid DNA into mammalian cells.[72]

The pore-forming ability of viroporins was also exploited for gene-delivery purposes. The physiological function of these highly hydrophobic proteins is to create channels and

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facilitate ion flow across biological membranes.[73] Peptides derived from the endodomain of the HIV envelope glycoprotein gp41 (corresponding to residues 783-806 of gp160) form pores in the cell membrane by adopting an amphipathic α-helical structure.[74] Modification of cationic polymer PEI with a lytic peptide derived from gp41 enhanced PEI-mediated siRNA delivery in HeLa cells compared to unmodified polyplexes.[75] Confocal microscopy imaging confirmed that addition of the gp41 peptide increases the endosomal escape of the delivery vehicle.

Peptides of non-viral origin have also been used for endosomal disruption. For example, melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) is a membrane-disruptive peptide from the venom of European honey bee *Apis mellifera*. Unfortunately, melittin is active at neutral as well as acidic pH and therefore highly toxic. Bozema et al. masked key lysine residues in melittin with dimethylmaleic anhydride.[76] The masked peptide has no lytic activity at the plasma membrane, but becomes unmasked and activated once inside the acidic endosomal lumen. Masked melittin has successfully been used to deliver siRNA and other nucleic acids into cells. Finally, Chen et al. reported comb-like polymers synthesized by grafting hydrophobic decylamine (NDA) onto the carboxylic acid groups along the backbone of poly(L-lysine iso-phthalamide) at different densities.[77] The resulting amphipathic, pH-responsive polymers greatly enhanced the release of endocytosed materials from endosomes into the cytoplasm of different types of cancerous and noncancerous mammalian cells.

4.4. Chemical Agents

Lysosomotropic agents such as chloroquine and its analogs have been used to enhance the endosomal release of nucleic acids and proteins.[78–80] These agents are uncharged under neutral pH and cross cell membranes by passive diffusion. Upon entry into the acidic endosomes, they become protonated and accumulate inside the endosomes. Proposed roles of chloroquine in facilitating endosomal release include 1) inhibition of endosomal acidification; 2) induction of osmotic swelling of endosomal vesicles; 3) inhibition of the degradation of nucleic acid and protein cargos; and 4) disruption of the endosomal membrane. Interestingly, the addition of 4–6 mM Ca^{2+}, which does not perform any of the above roles of chloroquine, also dramatically increases the endosomal release of DNA formulated as polyplexes or lipoplexes[81,82] and cationic peptide-conjugated peptide nucleic acids.[83] Ca^{2+} is known to bind to negatively charged membranes to generate spontaneous membrane curvatures and trigger the formation of tubular protrusions.[84] Presumably, both chloroquine and Ca^{2+} enhance endosomal release by inducing membrane curvature and vesicle budding from the endosomal membrane. Note that although chloroquine and Ca^{2+} have high in vitro efficacy (up to 50-fold enhancement of endosomal escape efficiency), in vivo translation of this approach is limited because of high systemic toxicity.

4.5. CPP-EED Hybrid Peptides

The endosomal escape of CPPs can be enhanced by the addition of endosomal escape domains (EEDs). Early on, researchers employed fusogenic or lytic peptides derived from viruses as EEDs. The challenge is to find peptides that are able to disrupt endosomal membranes without also damaging the plasma membrane or membranes of other organelles. A common strategy involved pH-sensitive peptides that are inactive at neutral pH but shift to

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an active, membrane-disruptive state under the acidic endosomal pH. One such peptide is the HA2 peptide (GLFGAIAGFIENGWEGMIDGWYG) derived from the N-terminus of influenza virus hemagglutinin protein. Upon protonation of its glutamate and aspartate residues, HA2 becomes more hydrophobic and is able to fuse and lyse lipid bilayers. Dowdy and co-workers conjugated the HA2 peptide to D-Tat and found that the addition of dTat-HA2 in trans markedly enhanced the escape of a Tat-Cre recombinase fusion protein from macropinosomes.[85] Co-treatment of reporter cells with dTat-HA2 and Tat-Cre increased the fraction of recombination-positive cells from <5% (Tat-Cre alone) to >60%. A fusion peptide between Tat and fusogenic peptide CM18 (KWKLFKKIGAVLKVLTTG-YGRKKRRQRRR) was also found to "permeabilize" endosomes and enhance the release of a variety of co-incubated macromolecules into the cytosol.[86] The exact mechanism by which these fusogenic peptides mediate the endosomal release of macromolecular cargoes has not yet been elucidated, but probably does not involve membrane fusion. On the other hand, vesicle budding is the reverse process of membrane fusion; it is conceivable that the fusogenic peptides improve endosomal escape by inducing vesicle budding and collapse.

More recently, Liu and co-workers screened peptides previously reported to disrupt microbial membranes without known mammalian cell toxicity and discovered a 13-residue peptide, aurein 1.2 (GLFDIIKKIAESF), which improved cytosolic delivery of proteins by up to ~5-fold.[87] It was shown that aurein 1.2 enhanced endosomal escape of the associated endocytosed protein cargo. When applied to the delivery of +36 GFP–Cre recombinase fusions into the inner ear of live mice, fusion with aurein 1.2 dramatically improved the cytosolic delivery efficiency. Finally, Dowdy and colleagues covalently attached various hydrophobic peptides to Tat and systematically tested their EED activities by using a quantitative live-cell split-GFP complementation assay.[88] They found that EEDs containing two indole rings or one indole ring and two phenyl groups significantly enhanced cytosolic delivery efficiency of Tat.

Oligomerization of CPPs (or "self-fusion") represents yet another effective strategy to enhance endosomal escape. By introducing a single cysteine residue to the C-terminus of penetratin and analogs (PenArg and EB1), Amand et al. showed that the transfection capacity for plasmid DNA in HEK293T cells was greatly increased.[89] Dimerization of EB1 (via disulfide formation) improved its endosomal escape efficiency while reducing cytotoxicity. Similarly, Erazo-Oliveras et al. observed that dimerization of tetramethylrhodamine-labeled Tat through a disulfide bond (dfTAT) vastly improved its endosomal escape efficiency as well as the overall cytosolic delivery efficiency.[90] According to the vesicle budding and collapse mechanism (Figure 2f), dimerization of a CPP or the addition of a hydrophobic EED would increase its binding affinity for the endosomal membrane and therefore its endosomal escape efficiency.[32] Insertion of hydrophobic EED group(s) into the lipid bilayer would also generate positive membrane curvature, an essential feature of the budding neck (vide infra).

4.6. Conformationally Constrained CPPs

Two new types of CPPs and a family of non-peptidic cell-penetrating motifs (CPMs) were recently found to possess unusually high endosomal escape efficiencies. Schepartz and co-

workers designed a penta-arginine motif across three turns of an α-helix (Figure 3a).[33,34] Proteins incorporating this motif (e.g., miniature proteins 5.3 and ZF5.3) effectively overcome endosomal entrapment and are released from the early ($Rab5^{+}$) endosome into the cytosol of mammalian cells.[34] Meanwhile, Pei and colleagues discovered a family of small, amphipathic cyclic peptides as highly active CPPs.[30–32] The total cellular uptake, endosomal escape, and cytosolic delivery efficiencies of these two CPP families were quantitated by flow cytometry analysis of HeLa cells treated with the CPPs that were labeled with a pair of fluorescent dyes, tetramethylrhodamine (TMR) and naphthofluorescein (NF).[32,91] TMR is pH insensitive and reports the total amount of CPP internalized by the cell, while the pH-sensitive NF fluorescence (NF fluoresces in the neutral cytosol/nucleus but not inside the acidic endosomes/lysosomes) reflects the amount of CPP inside the cytosol and nucleus. The NF/TMR fluorescence ratio indicates the efficiency of endosomal release. Remarkably, the most active cyclic CPP (CPP12; Figure 3b) and miniature protein 5.3 exhibited cytosolic delivery efficiencies (defined as the ratio of cytosolic over extracellular cargo concentration) of 121% and 156%, respectively, compared to 2.0% for Tat.[32] Efficient cytosolic entry of the CPPs was also confirmed by fluorescence correlation spectroscopy[92] and a chloroalkane penetration assay.[93] The superior cytosolic entry efficiencies of CPP12 and miniprotein 5.3 are primarily the result of vastly improved endosomal escape efficiencies (17- and 28-fold higher than that of Tat, respectively). Cyclic CPPs and protein 5.3 are conformationally constrained and their structural rigidity (and the entropic advantage associated with it) endows them with greater binding affinity for the plasma and endosomal membranes than conventional linear CPPs.[32] Improved binding to the plasma and endosomal membranes in turn facilitates their endocytic uptake and endosomal escape, respectively. The cyclic CPPs also differ from classical CPPs (e.g., Tat and R_9) by containing both arginine and hydrophobic residues, a structural feature critical for high CPP activity. Vesicle budding requires the formation of negative Gaussian curvatures (i.e., simultaneous positive and negative curvatures in orthogonal directions) at the budding neck. It was hypothesized that insertion of hydrophobic groups in between phospholipid molecules generates positive curvature, whereas the bidentate hydrogen-bond interaction between the guanidinium groups of arginine residues and the lipid phosphates induces negative curvature.[94]

Armed with mechanistic insights gleaned from cyclic CPPs, Pei and co-workers recently designed a family of highly efficient non-peptidic CPMs.[95] The CPMs consist of 4 guanidinium groups and one or two aromatic hydrophobic amino acids (e.g., Nal) assembled around a rigid scaffold (a benzene ring), resembling the structures of cyclic CPPs (Figure 3c). The CPMs exhibit ~5-fold higher cytosolic delivery efficiency than cyclic CPP9, one of the most active cyclic CPPs.[32] Interestingly, following cytosolic entry, the CPMs and CPM-cargo conjugates are localized almost exclusively inside the mitochondrial matrix, suggesting that their endosomal escape is nearly quantitative. Compared to previous mitochondrion transporters, triphenylphosphonium cation (TPP)[96] and mitochondrion-penetrating peptides (MPPs)[97], the CPMs exhibited both markedly improved specificity for the mitochondrion matrix and 170-fold higher cellular entry efficiency (when conjugated to a membrane-impermeable peptidyl cargo). The CPMs should be useful for specific delivery of therapeutic agents and chemical probes into the mitochondrion.

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The cyclic CPPs have been used to deliver a variety of cargos including small molecules,[30–32] linear peptides,[31,98] cyclic peptides,[31,99–104] proteins,[31] and nucleic acids (M.B. and D.P., unpublished results) into mammalian cells in vitro and in vivo. Their ability to exit the early endosome[31] makes them ideal vectors for delivering biological agents such as peptides, proteins, and nucleic acids, which are susceptible to acid denaturation and/or enzymatic degradation if brought into the late endosome/lysosome. Cyclic CPPs are highly stable against proteolytic degradation, are essentially nontoxic in cell culture, and exhibit good biodistribution and bioavailability profiles following major routes of administration (including oral bioavailability[32]). Macrocyclic peptides incorporating cyclic CPPs have been developed to modulate previously challenging intracellular targets including calcineurin,[98] CAL-PDZ,[100] K-Ras,[101] NEMO,[102,103] Pin1,[99] and protein tyrosine phosphatases.[99,104] Intranasal administration (1 mg/kg) of a peptidyl inhibitor against the calcineurin-NFAT interaction has demonstrated in vivo efficacy for treatment of acute lung injury/acute respiratory distress syndrome (ARDS) in murine models.[98]

5. CONCLUSIONS, NEW OPPORTUNITIES, AND FUTURE DIRECTIONS

For decades, endosomal entrapment has been a major bottleneck in drug delivery, due to lack of mechanistic understanding of the endosomal escape process or a delivery vehicle that effectively overcomes the endosomal membrane. The recent discovery of three non-viral delivery systems with high endosomal escape/cytosolic delivery efficiencies (miniature proteins, cyclic CPPs, and non-peptidic CPMs) and a novel endosomal escape mechanism (vesicle budding and collapse) represents a major step forward towards overcoming the endosomal entrapment problem. Cyclic CPPs possess not only high cargo delivery efficiencies, but also excellent “drug-like” properties including metabolic stability, bioavailability, and biodistribution. The latter properties are of particular importance, as lack of biodistribution/bioavailability was one of the key challenges during the clinical development of first-generation CPPs. The availability of highly effective transporters should open up new opportunities for both basic biomedical research and drug discovery. An exciting application is the delivery of proteins into the cytosol of mammalian cells. Patients with genetic diseases caused by missing or defective genes may be treated with recombinant proteins chemically modified with a cyclic CPP[31] or genetically fused to a miniature protein.[105] Monoclonal antibodies (and their fragments/surrogates) may be delivered into cells to specifically block the function of a target protein, as therapeutic agents or mechanistic probes. As discussed above, cyclic CPPs may be integrated into macrocyclic peptide design to generate cell-permeable, metabolically stable peptide therapeutics to target challenging proteins such as those involved in intracellular protein-protein interactions.[98–104] Several powerful combinatorial library technologies are now available for generating macrocyclic peptides with antibody-like affinity and specificity against essentially any protein of interest.[106,107] We have shown that cyclic CPPs effectively deliver siRNA as well as Argonaute/siRNA complexes into mammalian cells with minimal cytotoxicity (M.B. and D.P., unpublished results). Delivery of other forms of nucleic acids and protein/nucleic acid complexes (e.g., CRISPR/Cas9) should also be feasible. Finally, few therapeutic options are currently available for mitochondrial diseases. The discovery of CPMs as mitochondrion-specific transporters may pave the way for developing novel drugs against mitochondrial

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diseases. After three decades of persistent efforts and numerous failures, the time for CPPs (and CPMs) to make a major impact on drug discovery has perhaps finally arrived.

The improved mechanistic understanding of CPPs may stimulate new research directions in several different fields. First, it should now be feasible to design CPPs and CPMs of desired properties. For example, an amphipathic molecule that binds with high affinity to and induces negative Gaussian curvature at the endosomal membrane, but does not do so at the plasma membrane, is expected to function as an EED. Integration of an EED into existing delivery systems should improve their cytosolic delivery efficiency and/or tissue selectivity. Conversely, a CPP or CPM that binds robustly to the plasma membrane but does not induce endosomal escape may be able to cross biological membranes by transcytosis and useful for oral drug delivery or delivery of drugs across the blood-brain barrier.[108] Second, non-enveloped viruses and bacterial toxins can efficiently exit the endosome, but their molecular mechanism(s) is currently unknown. We hypothesize that they may escape the endosome by the vesicle budding and collapse mechanism. Obviously, additional experiments are required to test this hypothesis.

With regard to future directions of CPPs, the following key questions still need to be addressed. Does the vesicle budding and collapse mechanism, initially demonstrated with cyclic CPPs, apply to all CPPs and the co-called membrane fusogenic and lytic peptides? How do cyclic CPPs induce negative Gaussian curvature on lipid bilayers and endosomal escape, on the molecular level? What causes the budded vesicles to collapse? How does the membrane lipid composition from different cell types or different membranes within the same cell affect the budding/collapsing event? Do cellular proteins play a role in the process? Finally, is the budding and collapse mechanism universal for all endosomal escape events, or two or more endosomal escape mechanisms operative simultaneously? Understanding these mechanistic details would guide the design of additional CPPs (or other non-viral delivery vectors) as well as inform how to apply these vectors for best results in cargo delivery. On the translational side, the efficacy, toxicity, and ADME (Absorption, Distribution, Metabolism, and Excretion) properties of CPPs need to be further evaluated. Strategies need to be developed to selectively deliver CPPs and CPP-cargo conjugates into target tissues or cells (e.g., cancer cells). It also remains to be determined whether the highly efficient CPPs and CPMs may be leveraged to enhance the delivery efficiency of other non-viral delivery systems, such as the liposome-, nanoparticle-, and polyplex-based systems.

ACKNOWLEDGMENTS

We thank Drs. Yizhou Dong and Ziqing Qian (Ohio State University) for their helpful comments. Work in the Pei group is supported by a grant from the National Institutes of Health (GM122459).

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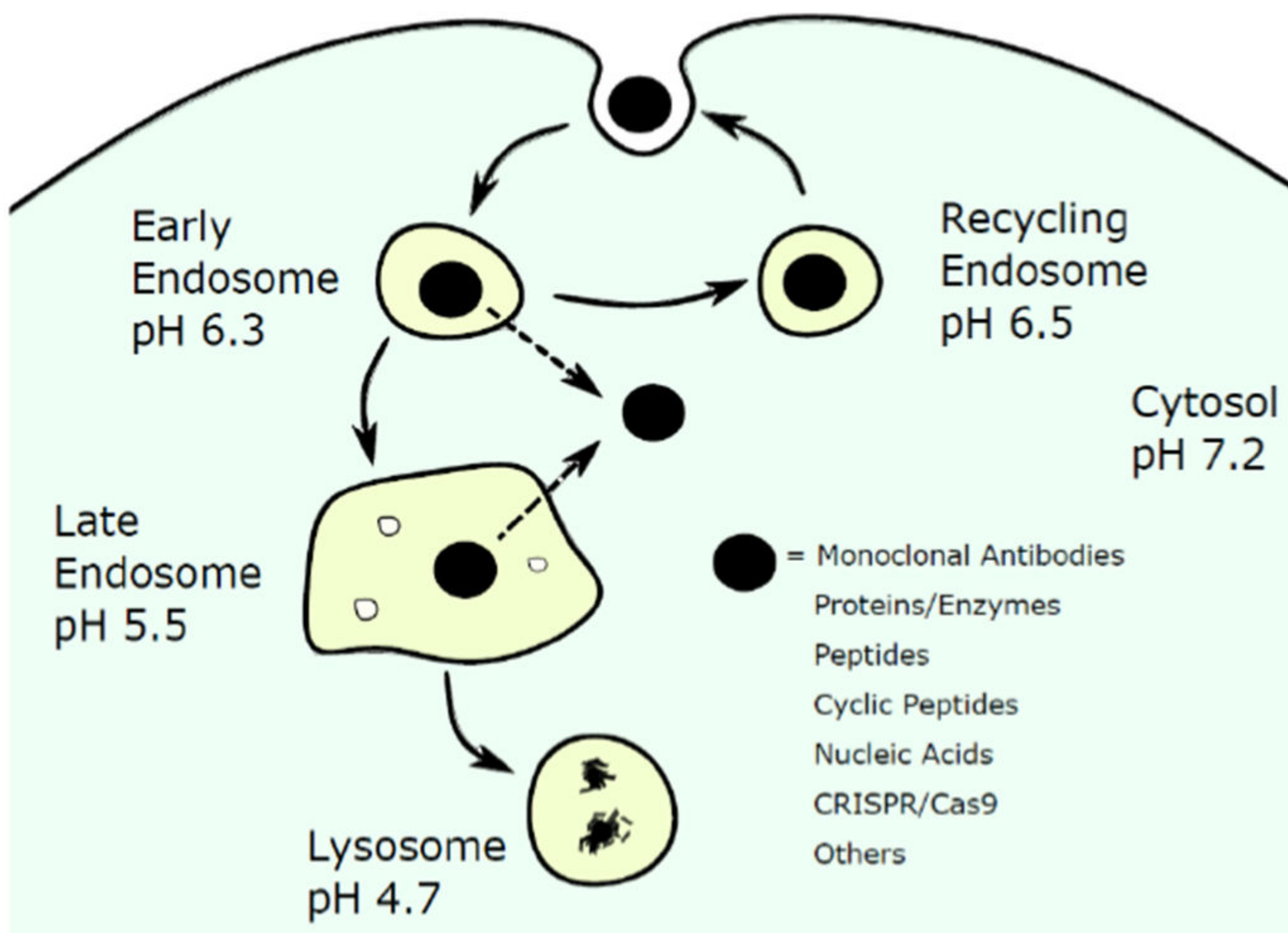

Figure 1.

Endocytic uptake and intracellular trafficking of biological cargoes by mammalian cells. Biological cargoes internalized by different endocytic mechanisms are initially all transported to the early endosome, where the luminal pH is slightly acidic (~6.3). Maturation of the early endosome into the late endosome is accompanied by further acidification (to pH~5.5). Finally, the late endosome fuses with lysosome (pH~4.7) and the cargoes are degraded by hydrolytic enzymes. A fraction of the cargoes may recycle back to the cell surface via the recycling endosome. For most of the non-viral delivery systems, cargo escape from the endo-/lysosomal pathway into the cytosol is inefficient to nonexistent.

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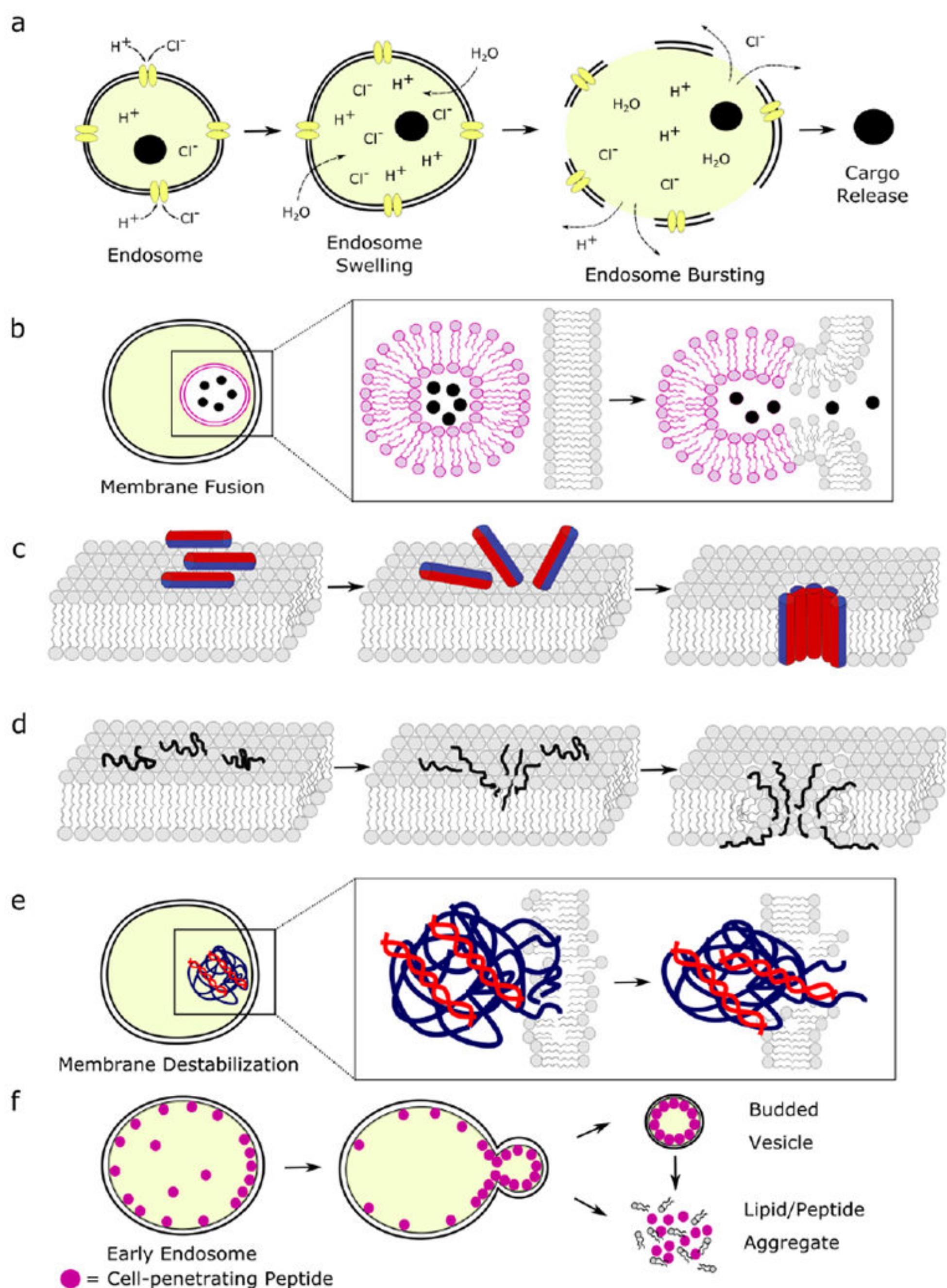

Figure 2.

Proposed mechanisms of endosomal escape. a) Proton sponge effect and osmotic lysis of the endosome/lysosome; b) Membrane fusion mechanism for liposome-based delivery systems; c) Barrel-stave pore formation; d) Toroidal pore formation; e) Membrane destabilization mechanism for polymer-based delivery systems; and e) Vesicle budding and collapse mechanism.

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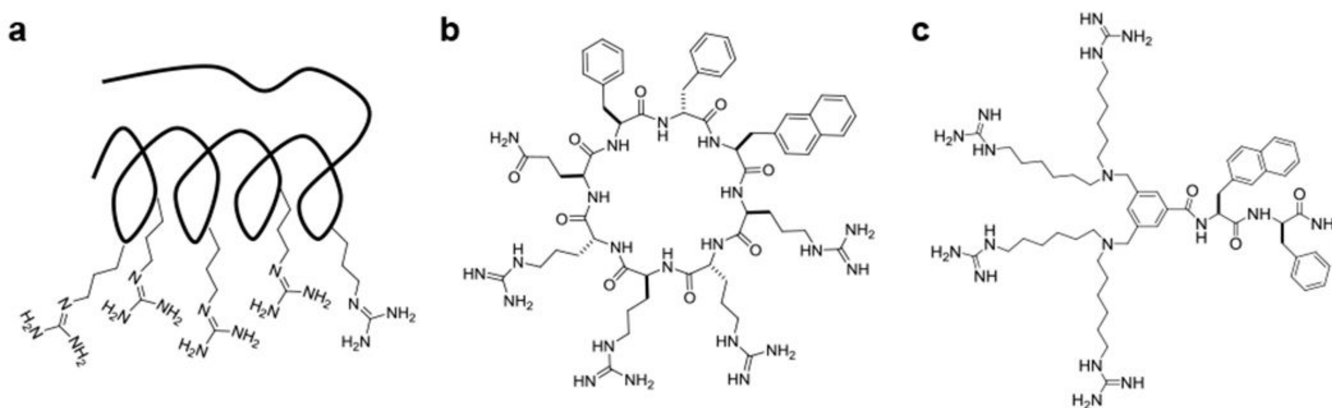

Figure 3.
Conformationally constrained CPPs and CPMs. a) Scheme showing the structure of miniature protein 5.3. b) Structure of cyclic CPP12. c) Structure of CPM2.

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Table 1.

Experimental evidence for ( ), consistent with (○), or against (×) the proposed endosomal escape mechanisms

Experimental Observation	Proton Sponge	Membrane Fusion	Pore Formation	Membrane Disruption	Vesicle Budding and Collapse
Endosomal release of CPPs (e.g., cyclic CPPs)[31,32,34]	×	×	○	○	
Endosomal release of nucleic acids in lipoplexes[46,47]	×	○	×	○	○
Endosomal release of nucleic acids in polyplexes[46,47]	○	×	×	○	○
Endosomal release of folded proteins[31,56]	○	○	×	×	
Endosomal release of non-covalently attached cargos[57,58]	○	○	×	○	○
Endosomal release of non-enveloped viral particles	×	×	×	×	○
Instantaneous release of cargos followed by diffusion[46,47]	○	○	×	×	○
Partial release of cargos without complete disruption of the endosome[46,47]	×	×	○	○	○
Role of endosomal acidification[32]	○	○	○	○	
Conformational constraints enhance CPP activity[32]	×	×	×	○	