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Accessing intracellular targets through nanocarrier-mediated cytosolic protein delivery

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Abstract

Protein-based therapeutics have unique therapeutic potential due to their specificity, potency, and low toxicity. The vast majority of intracellular applications of proteins require access to the cytosol. Direct entry to the cytosol is challenging due to the impermeability of the cell membrane to proteins. As a result, multiple strategies have focused on endocytic uptake of proteins. Endosomally entrapped cargo, however, can have very low escape efficiency, with protein degradation occurring in acidic endo-lysosomal compartments. In this review, we briefly discuss endosomal escape strategies and review the strategy of cell membrane fusion, a recent strategy for direct delivery of proteins into the cell cytoplasm.

Keywords

intracellular protein delivery; endosomal entrapment; nanocarrier; endosomal escape; membrane fusion

Effective intracellular protein delivery in protein therapeutics

Proteins play dynamic and diverse roles in virtually all aspects of cellular function, making proteins potential therapeutics [1, 2]. Recent developments in protein technology have enabled proteins to be engineered to perform complex functions. These engineered proteins, such as CRISPR/Cas9 (see Glossary), have created new possibilities in the field of protein therapeutics to treat several diseases (for example muscular dystrophy, cystic fibrosis) that

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are ‘undruggable’ by conventional small molecule drugs [1]. Current protein therapeutics mainly focus on extracellular targets and a number of them such as Matuzumab [3], Nimotuzumab [4] and more have been successfully translated to the clinic [5]. Effective intracellular protein delivery would dramatically increase the potential for protein therapeutics, accessing the multitude of processes that occur inside of cells. However, cytosolic protein delivery is challenging, mainly due to the inherent impermeability of the cell-membrane to large, highly polar proteins and poor escape from vesicular compartments (endosomes) formed during endocytosis, the most common mode of biomacromolecule uptake by cells [1, 2].

Numerous approaches have been undertaken to transport proteins into cells, [6] including physical techniques such as electroporation, microinjection and acoustics [7], decoration of proteins with cell penetrating peptides (CPPs) [8–13] or other ligands, and using nanocarriers [14, 15] such as lipids [16], inorganic nanoparticles (NPs) [17–19], and polymers [20–24]. Although mechanical methods provide direct access of the protein into the cytosol of the cells, they require specialized equipment that punctures the cell membrane and are not suitable for clinical applications. While CPPs have also gained substantial attention recently due to their direct penetration abilities, their mechanisms of uptake have been debated [25, 26] with endocytosis postulated as being the predominant pathway of uptake [27–30]. Among these approaches, nanocarriers have been widely used for intracellular protein delivery due to their easier tunability and variability.

Nanocarriers carrying protein therapeutics are predominantly taken up by the cells via several endocytic mechanisms including phagocytosis, macropinocytosis, clathrin- and caveolae-mediated endocytosis [31]. The protein delivery vehicles enter into the cells via endocytosis but then the cargo becomes entrapped in the endosomes (endosomal entrapment). Endosomallyentrapped [32, 33] proteins are eventually degraded by proteases in the acidic endo-lysosomal compartments without being able to access the cytosol (Figure 1, Key Figure) [1, 2, 6]. This sequestration and degradation limits the efficacy of protein delivery systems that are internalized into the cells by endocytic pathways.

Access of delivered proteins to the cytosol is critical for intracellular activity, either directly for activity or as a gateway to the nucleus or subcellular organelles [6]. Despite continued efforts, the maximum reported efficiency of endosomal escape is < 10% [34], necessitating the need to develop new strategies for cytosolic protein delivery. Direct delivery of proteins into the cytosol has emerged as viable alternative and hold potential for future therapeutic applications. By circumventing the problems associated with inefficient endosomal escape, this route enables direct transport of proteins to the cytosol with highly increased therapeutic efficacies.

In this review, we will discuss the use of nanocarriers that employ endosomal escape strategies as well as vehicles that access cell membrane fusion, a recently discovered non-endosomal and hence direct cytosolic delivery pathway (Figure 1). We will highlight key examples of efforts to effect cytosolic delivery and discuss how these protein delivery systems are moving towards clinical translatability.

Endosomal escape strategies

The relative ease of inducing endosomal uptake of protein delivery vehicles has generated numerous approaches for endosomal uptake and escape [35, 36]. In this section we will focus on the three predominant strategies for endosomal escape: osmotic lysis using the 'proton sponge' effect, endosomal membrane destabilization and endosomal membrane fusion (Figure 2).

Proton sponge effect (pH-buffering) with osmotic lysis

Endosomal escape induced by the proton sponge effect is mediated by agents with a high buffering capacity that can rupture the endosomal membrane through osmotic pressure (Figure 2A). This strategy is hypothesized to operate through influx of H^+ into endosomes followed by counterions$^-$ and water molecules. This influx increases the internal pressure of the endosome, eventually lysing the endosome and releasing entrapped contents to the cytosol [6]. Agents that employ the proton sponge effect typically feature secondary and tertiary amines groups that become protonated at endosomal/lysosomal pH. Poly(ethylenimine) (PEI) and poly(amidoamine) (PAMAM) are commonly used as proton sponges [37, 38]. In 2015, Pitard and co-workers developed a liposome-based formulation that employs a proton sponge mechanism to deliver β-galactosidase (β-gal) enzyme and the anti-cytokeratin8 (K8) antibody into cytosol of HeLa cells [39]. Several other strategies have been developed for triggering endosomal escape by protonation, swelling and rupture of the membrane [38] but the efficiency of escape is generally relatively low, and subsequent cytosolic release is limited. Moreover, recent reports have raised concerns about the mechanistic aspect of this escape strategy [40]. A study by Braeckmans and co-workers [41] investigated mechanistic factors that impact on the endosomal escape, revealing that endosomal escape frequency varied with different cell lines. Furthermore, based on a mathematical model, they demonstrated that both endosomal size and endosomal membrane leakiness play a significant role in endosomal escape.

Endosomal escape through membrane destabilization

Endosomal membrane destabilization can allow cargo to escape from endosomal entrapment through membrane disruption (Figure 2B). This destabilization occurs via interaction of nanocarriers either by themselves or together with membrane-destabilizing agents, such as proteins/peptides [e.g. saporin [42], polyhistidine [43]) and chemicals (e.g. chloroquine [44], methylamine [45])] with the endosomal membrane, resulting in the release of cargo into the cytosol. Below we will discuss nanocarriers including inorganic NPs [46], lipids [47], and polymers [48–50] that effect endosomal escape by membrane destabilization.

Inorganic NP-based strategies for endosomal escape—Intracellular protein delivery using inorganic NPs including gold [51] and silica [52, 53] offer several useful qualities, including control over size and surface functionalization, long circulation time, efficient cellular uptake, targeting ability and minimal toxicity [54]. Notably, the choice of the inorganic core, size and surface functionality of the NP play a critical role to control the protein loading and uptake efficiency [8].

Yu *et al.* reported the use of octadecyl-functionalized 'rough' silica NPs (C18-RSN) to deliver therapeutic protein ribonuclease A (RNase A), causing significant apoptosis in both human breast cancer (MCF-7) and squamous cell carcinoma (SCC-25) cell lines [55]. The hydrophobic modification with octadecyl group helped achieve endosomal escape by disrupting the endo-lysosomal membranes as compared to RSN without the hydrophobic modification. Murthy and co-workers employed gold NPs to develop a system they called 'CRISPR-Gold' which delivers Cas9 ribonucleoprotein (RNP, protein and guide RNA) along with a donor DNA into the nucleus to induce a double stranded break in the targeted genomic region which is then repaired via homology directed repair (HDR). In this work, [51] 15 nm gold NPs were conjugated with donor DNA by thiol linkage and complexed with Cas9 RNP, and an endosomal disrupting polymer, poly(N-(N-(2-aminoethyl)-2-aminoethyl) aspartamide) (PAsp(DET)), which was crucial for triggering endosomal disruption and causing the release of CRISPR–Gold into the cytoplasm. Once in the cytoplasm, glutathione released the thiol-linked DNA from the gold core of CRISPR–Gold, which causes the rapid release of Cas9 RNP and donor DNA. This system achieved about 10% gene editing efficiency *in vitro* in the fluorescent reporter BFP-mGFP-HEK cells that expressed 11.3% of the BFP-HEK cells to express GFP via HDR. Sequencing studies further confirmed that the GFP sequence in the edited cells exactly matched the donor DNA sequence. *In vivo*, the system achieved 5.4% gene editing in Duchenne muscular dystrophy murine model.

Lipid-based strategies for endosomal escape—Endosomal membranes are phospholipid bilayers, enabling lipid-based strategies for intracellular protein delivery. In one example, Liu, Xu and co-workers used bioreducible lipid NPs to facilitate intracellular delivery of functional proteins such as Cre recombinase (a topoisomerase I like enzyme that carries out sit- specific DNA recombination) and CRISPR/Cas9 [16]. In this work, twelve bioreducible lipids molecules featuring disulfide functional groups and long hydrophobic alkyl carbon chains were synthesized. Cationic protein Cre recombinase was fused to GFP variants with very high net negative charge to impart overall negative charge and anionic Cas9 protein and single guide RNA (sgRNA) were complexed with the positively charged lipids. Electrostatic attraction facilitated self-assembly as well as efficient cargo loading. The disulfide groups of the bioreducible lipids facilitated the escape of proteins from endosomes into the cytosol in response to the reductive acidic environment of end/lysosomes, enabling gene recombination and genome editing with efficiencies greater than 70% *in vitro*. Additionally, the strategy demonstrated functional protein delivery into the mouse brain for gene recombination *in vivo* to treat neurological diseases.

In a recent study, Jiang and co-workers developed a vehicle based on lipid/gold NPs to deliver Cas9 protein and sgRNA for cancer gene therapy where the sgRNA was designed to target Plk-1(polo-like kinase 1) gene, a master regulator of mitosis that is overexpressed in cancer cells. [56]. Cationic gold nanoclusters (GNs) were modified with HIV-1-transactivator of transcription peptide (TAT peptide), collectively called (TAT-GNs). The positively charged TAT-GNs were co-assembled with the negatively charged Cas9 proteins and sgRNA plasmids to form a ternary complex [TAT-GNs/Cas9 protein/sgRNA plasmid (GCP)] through electrostatic interactions. GCP was further encapsulated in an anionic lipid shell (1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP)/dioleoyl-phosphatidylethanol-

amine (DOPE)/cholesterol), followed by post-modification with polyethylene glycol-phospholipids (DSPE-PEG) on the surface of the lipid shell to form lipid-coated GCP, LGCP. The ion pairs formed between the cationic DOTAP and the anionic lipids on the endosome membrane facilitated the formation of the inverted hexagonal phase in the binding lipids, triggering proposed membrane fusion between LGCP and endosome membrane. This interaction eventually led to destabilization of endosome membrane, allowing for the disassembly of LGCP and subsequent release of the complex of TAT-GNs/Cas9 protein/sgPlk1 plasmid from LGCP. Their studies revealed that ~26 % gene editing efficiency was achieved *in vitro* and ~5% inhibition of the melanoma progression was induced in melanoma mice model.

Polymer-based strategies for endosomal escape—Recently, several novel polymers have been designed to respond to environmental changes including pH [57] and the presence of reactive oxygen species (ROS) [58, 59] to induce endosomal escape via membrane destabilization. Chang and co-workers synthesized guanidinobenzoic acid-based polymer consisting of three parts- a dendrimer scaffold, a hydrophobic membrane-disruptive region, and a multivalent protein binding surface [60] to increase the protein binding efficiency, endocytosis, and endosomal membrane disruption respectively, for the delivery of several biomacromolecules such as bovine serum albumin, p53, saporin, R-phycoerythrin, β-galactosidase and peptides *in vitro*. The dendrimer modified with guanidyl and phenyl groups were shown to provide efficient intracellular protein delivery. However, the dendrimer with only phenyl or guanidyl ligand failed to form complexes or provide effective endosomal escape, respectively. They also investigated the delivery efficiency *in vivo* in lung tumor bearing mice and found a decrease in the tumor volume following intratumoral injection of saporin, a ribosome deactivating protein that is membrane impermeable.

Dhal *et al.* recently demonstrated endosomal escape and protein delivery using cross-linked micron- and sub-micron-sized hyaluronic acid (HA) hydrogels [61]. They hypothesized that these hydrogels fuse with the endosomal membrane after protonation in the acidic environment of glucuronic acid units on the HA backbone, leading to membrane destabilization and eventual endosomal disruption. Cheng *et al.* demonstrated relatively efficient endosomal escape and cytosolic delivery of therapeutic proteins using fluoropolymer vectors both *in vitro* and *in vivo* [62]. The hydrophobic and lipophobic fluoroalkyl chains modified on these vectors facilitated endosomal escape through membrane destabilization due to the fluorophilic effect [63]. Higher delivery efficiency was achieved by polymers with longer fluorous chains and higher fluorination degrees. They also showed *in vitro* protein delivery in breast cancer cells, and *in vivo* protein delivery in mammary tumor bearing mice model using a modified complex consisting of a polymer coated with an anionic HA shell and saporin. The HA coating enhanced stability of the system for *in vivo* application by shielding the positive surface charge and the fluoroalkyl chains induced endosomal escape by destabilizing the endosomal membrane.

Thayumanavan *et. al.* recently generated functional polymer-protein conjugate nanoassemblies responsive to reactive oxygen species (ROS), reducing environment and varying pH, using rapid and reversible click reaction between salicylic hydroxamic acid and boronic acid moieties [64]. The use of this approach was demonstrated by successful

cytosolic delivery of several proteins with different isoelectric points and sizes ranging from 14 kDa to 400 kDa, into different cancer cell lines.

Endosomal membrane fusion

Cytosolic protein delivery by endosomal escape can be aided by fusion of a nanocarrier with the endosomal membrane only when the NP itself is enveloped by a fusogenic membrane [65] (Figure 2C). This mechanism has been majorly adopted by liposomal nanocarriers where the lipid bilayer of the vehicle protects complexed proteins from degradation and fuses with the endosomal membrane during endosome maturation. This can enable the efficient endosomal escape of cationic, lipid-delivered cargo into the cytosol. Although this approach has been majorly applied for delivery of nucleic acids, proteins can also serve as a good candidate. Recently, Liu *et* al. reported an endocytotic proteoliposome-mediated membrane fusion approach using cationic lipid nucleic acid transfection reagents to achieve ~90% Cre-mediated recombination and ~20% Cas9-mediated genome modification in hair cells of mouse ear *in vivo*.[18]

Delivery of protein therapeutics via cell membrane fusion

While strategies to escape endosomes have been developed to deliver protein therapeutics that utilize the process of endocytosis to enter the target cells, other strategies have also been developed in parallel that seek to deliver protein therapeutics directly into the cells and do not involve the process of endocytosis. Direct delivery of proteins to the cytosol can greatly enhance the efficacy of protein-based therapeutics. Cell membrane fusion is an emerging strategy that provides direct entry of the proteins to the cytosol, bypassing endocytosis (Figure 1). In this route, the vehicle and cargo assemblies fuse with the cell membrane, allowing direct transfer of the protein cargo to the cytosol [66]. By evading the endosomal pathway entirely, the approach avoids issues associated with degradation of cargo and limited cytosolic release, ultimately enhancing delivery efficiency [67]. Membrane fusion is facilitated by nanocarriers such as fusogenic liposomes, NPs and polymers (Figure 3). Below we discuss each via examples.

Liposome-based membrane fusion

Liposomes are vesicular structures that can encapsulate polar cargo such as proteins cargo in their aqueous core. Fusogenic liposomes constitute a promising carrier system [68] that can fuse with the plasma membrane. [69, 70] These fusogenic liposomes efficiently deliver the encapsulated cargo to the cytosol, circumventing the usual low-efficiency endocytic routes of conventional liposomes [71, 72]. In 2017, Csiszar *et al.* developed fusogenic proteoliposomes (FPLs) to deliver water soluble proteins to the cytosol without degradation [73]. Electrostatic interactions between the negatively charged protein cargos and positively charged carriers induced proteoliposome formation. Fluorescent proteins such as enhanced green fluorescent protein (EGFP), Dendra2, and R-phycoerythrin were successfully delivered into mammalian cells with high efficiency using this approach. Colocalization studies using fluorescence microscopy of plasma membrane and protein-loaded FPLs suggested membrane-fusion as the mechanism of uptake. However, positively charged

proteins cannot be delivered by this system because the repulsion between the proteins and similarly charged liposomes prevent FPL formation.

Recently, Kros *et al.* reported cuboidal mesoporous silica NPs (MSNs) coated outside with a fusogenic lipid bilayer for improved protein loading efficiency and membrane fusion-mediated cytosolic protein delivery (Figure 3A) [74]. A pair of complementary coiled-coil (CC) lipopeptides (CP4K4 and CP4E4) (highlighted in red and blue respectively in Figure 3A) was inserted into the lipid bilayer of both liposomes and live cells resulting in fusion between opposing membranes. Mechanistic studies revealed that the driving force for this fusion is the coiled-coil formation between the complementary peptides E4/K4. The MSNs were generated with disk - shaped cavities having a large pore diameter (10 nm) to accommodate the positively charged CytC that induces apoptosis on cytosolic localization. The protein was loaded at pH 7.4 via electrostatic interactions with the negatively charged silanol groups on the surface of NPs. The fusogenic lipid bilayer further strengthened the colloidal stability of the MSNs and prevented premature release of CytC. Mechanistic studies with endosomal inhibitors suggested a membrane-fusion type mechanism induced by this coiled-coil, resulting in efficient cytosolic delivery of CytC with concomitant apoptosis of cells.

NP-mediated membrane fusion

In recent years, the Rotello group has developed NP-stabilized capsules (NPSCs) that provide direct cytosolic delivery of functional proteins. Tang *et al.* have demonstrated the use of NPSCs featuring gold NPs with 2 nm core, functionalized with a cationic tetrapeptide bearing a guanidinium moiety (HKRK-AuNPs), as efficient, non-toxic and stable delivery vehicles for direct cytosolic delivery of anionic proteins including GFP as well as fully functional caspase-3 (CASP3) [75] to HeLa cells (Figure 3B). The CASP3 induced apoptosis, and in later studies showed synergistic activity on co-delivery with paclitaxel (a known anti-cancer drug and an inhibitor of cell division), resulting in increased cancerous cell death *in vitro*. [76]. These NPSCs are formed and stabilized through hydrogen bonding and electrostatic interaction between guanidinium moieties of the HKRK-AuNPs and the carboxylate groups of the oil at the center of the capsule. Additional lateral stability in this system is provided by the interaction between the cationic HKRK-AuNPs and anionic cargo proteins, allowing for construction of stable NPSCs as small as 130 nm. The utility of the NPSC-based protein delivery system was further demonstrated by intracellular targeting of proteins to different subcellular compartments such as peroxisomes and the nucleus [77, 78]. Another study reported an effective intracellular delivery strategy for proteins of high molecular weight such as like β-Gal (464 kDa) by tuning the supramolecular chemistry of these NPSCs [79]. GIPA (1-guanidino-2-(4-imidazole) propionic acid) instead of guanidinium moiety, was used as the terminal group of the AuNP ligand for optimal cytosolic delivery of payload proteins and their subsequent dissociation from the NPSC. However, the NPSC platform is limited to proteins with pI < 7, and the relative complexity of fabrication makes clinical translation challenging.

In 2017, a novel protein–particle co-engineering strategy [80] was developed where proteins genetically engineered with a peptide chain comprised of different lengths of oligoglutamic

acid tags (E-tag, inserted into proteins to increase the membrane permeability and cytosolic delivery efficiency) at their N or C-terminus were self-assembled with argininefunctionalized gold NPs (ArgNPs) through carboxylate (from the E-tag of the proteins) – guanidinium (from the ArgNPs, highlighted in red in Figure 3B) electrostatic interactions to generate hierarchical superstructures. Self-assembled superstructures (250–350 nm diameter) exhibiting multiple levels of structural hierarchy were generated through co-engineering of NPs and recombinant proteins with distinct charge, size, and function [81]. These supramolecular assemblies were shown by time-lapse confocal microscopy to fuse with cell membrane, presumably through interaction of ArgNPs ligands with the cell membrane. This interaction releases the E-tagged protein directly into the cytosol, bypassing endosomal entrapment. Live cell imaging videos were consistent with a membrane fusion-like mechanism, and endocytosis inhibitors did not significantly alter uptake, all consistent with a direct delivery pathway. Notably, in another study, the authors demonstrated a direct cytosolic delivery (90%) of the Cas9 RNP using ArgNP-based supramolecular delivery vehicles with co-engineered Cas9 containing localized negative charge in the form of the E-tag. A SV40 nuclear localization signal (NLS) was attached at the C-terminus of the Cas9 to provide nuclear localization of the relatively large protein. Cytosolic delivery of the RNP in mammalian cells facilitated efficient gene editing in the phosphatase and tensin homolog (*PTEN*) gene (30%) and the *AAVS1* gene (29%) *in vitro* [82]. *In vivo*, the system achieved >8% gene editing efficiency of the *PTEN* gene in macrophages in the liver and spleen through systemic delivery of ArgNPs, Cas9 engineered with twenty E-tag residues (Cas9E20) and associated sgRNAs nanocomposite into mice [83], establishing this as an effective delivery system.

Polymer-mediated membrane fusion

Building upon the results using particle-protein co-engineering strategy, Rotello *et al.* very recently developed a more tunable and versatile polymeric delivery platform. Poly(oxanorborneneimide) (PONI) polymers featuring cationic guanidinium moieties were synthesized via ring opening metathesis polymerization (ROMP) (Figure 3C) [84]. The ‘semi-arthritic’ (neither too rigid nor too flexible) oxanorbornene backbone feature conformational restrictions, which ensures flexible interaction with the cell membrane, while guanidinium (highlighted in red in Figure 3C) plays a pivotal role in fusing of the carrier with the membrane. The polymers self-assembled with E-tagged proteins such as Cre recombinase and delivered them directly to the cytosol of reporter HEK-loxP-dsRed-loxP-GFP cells *in vitro*, similarly to the ArgNPs [80].

In a different approach, Francis *et al.* reported a bioconjugation-based strategy for intracellular protein delivery by site-selectively attaching amphiphilic polymers to the N-terminal positions of proteins using 2-pyridinecarboxaldehyde (PCA) groups [85].The polymer system delivered GFP and functional protein RNase A, which promotes cell death through cytosolic RNA degradation, to cancer cells. Mechanistic studies demonstrated delivery being unaffected even after using endocytosis inhibitors amiloride and dynasore, suggesting the uptake to be via a membrane fusion-like process. However, the method is incompatible with proteins with proline residues in the second position (which inhibits the

cyclization reaction), proteins that are acylated or otherwise blocked at the N-terminus, and proteins that have N-termini that are not solution exposed.

Concluding Remarks and Future Perspective

Protein-based therapies can address conditions that are difficult or impossible to treat using small molecule pharmaceuticals. Over 200 protein-based therapeutics have been approved by the Food and Drug Administration (FDA) to treat a wide range of diseases including cancer, diabetes, and inflammation [86]. Protein delivery using nanocarriers employing either endosomal escape strategies or cell membrane fusion have progressed immensely over the last decade. Both endosomal escape and cell membrane fusion can be facilitated by nanocarriers such as lipids, polymers, or inorganic NPs.

The major advantage of strategies relying on endocytosis is that it is the most common mode of uptake in cells has been very well studied. Endosomal escape can be effected by proton buffering, endosomal membrane destabilization or endosomal membrane fusion. However, inefficient endosomal escape of proteins remains a challenge for this approach. Cell membrane fusion is a promising alternative for direct cytosolic delivery, ensuring direct delivery of proteins to the cytosol with high efficiency and evading endosomal entrapment altogether. Although fusion-based approaches are new, they have the potential to revolutionize the field of protein delivery. The mechanisms employed in these systems, however, still require deeper understanding.

Clearly, there is enormous scope for further development in terms of protein delivery vehicles that address issues like efficiency, specificity, and toxicity. Equally important, these nanocarrier-based protein-delivery strategies need to be moved into clinical translation (see Outstanding Questions)[51, 84, 87]. One interesting question is how to increase the tissue/organ/cell specificity of these nanocarriers. Nanocarriers can easily be tuned in terms of shape, size, and charge to influence their cell selectivity. Additionally, selectivity can be enhanced by decorating the nanocarriers with specialized ligands and/or targeting moieties to specific receptors present on the surface of the target cell of interest (for example, macrophages, hepatocytes, tumor cells and more) [88–90]. In terms of promise *in vivo*, both endosomal escape and cell membrane fusion strategies have exhibited immense potential. Notably, cytosolic delivery of therapeutic gene editing protein CRISPR-Cas9 [91]has been greatly explored by several research groups demonstrating excellent promise for gene editing *in vivo*, including diseases related to mononuclear phagocyte system [83] and genetic Duchenne's syndrome [51]. Significant progress has also been made to deliver antigens into the cytoplasm of dendritic cells and activate CD8+ T cells [86] using nanocarriers such as polymers [92], dendrimers [60], and lipid NPs [93]. This is an important step forward in the field of intracellular protein delivery, as it provides an avenue for the development of protein-based vaccines. Therefore, based on the rapid progress made in the past few years with these nanocarrier-based intracellular protein delivery strategies, we are optimistic that with more extensive research these platforms will make their way into clinical applications in the coming years.

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GLOSSARY

Cas9
Cas9 is a protein that plays a vital role in the defense of certain bacteria against DNA viruses and plasmids (CRISPR, see below). Cas9 is an RNA-guided nuclease that induces double strand breaks by sequence when repaired by non-homologous end joining, creating insertions and deletions. The modular nature of the CRISPR process has made it an emerging tool for gene editing

Cell-penetrating peptides (CPPs)
Short peptides that facilitate cellular uptake of various molecular equipment

Click reaction
A one pot reaction between an azide and an alkyne, catalyzed by copper to form a five membered hetero-atom ring

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)
A family of DNA sequences found in the genomes of prokaryotic organisms, used to detect and destroy DNA from similar bacteriophages during subsequent infections. The term is used colloquially to refer to gene editing strategies relying on these motifs

Donor DNA
DNA delivered along with Cas9 RNP into the cells to insert or change short sequences of endogenous genome target region

Endocytosis
An energy-dependent cellular uptake processes in which materials are brought into the cell. In this process, the cell membrane invaginates around the material to be internalized followed by budding off inside the cell to form a vesicle containing the ingested material. Endocytic mechanisms including phagocytosis, macropinocytosis, clathrin- and caveolae-mediated endocytosis

Endosomal entrapment
A common bottlenecks in several delivery approaches. The vector/cargo complex is taken up by the cells via an endocytic mechanism. This complex remains trapped in the endosome unable to escape and eventually gets degraded by the lysosome

Endosomal escape
Ability of delivery systems to trigger escape of cargo from the endosome into the cytosol, the site of their action

E-tag

Glutamic acid tag (E-tag) is inserted into proteins to induce self-assembly with cationic carriers that increase the membrane permeability and cytosolic delivery efficiency

Donor DNA
DNA delivered along with Cas9 RNP into the cells to insert or change short sequences of endogenous genome target region

Homology directed repair (HDR)
A mechanism in cells to repair or insert sections of DNA to modify double-strand DNA lesions

Isoelectric point
The pH at which a molecule is electrically neutral or carries no net electric charge

Nuclear Localization Signal (NLS)
An amino acid sequence that tags a protein for import into the cell nucleus by nuclear transport. The signal usually consists of one or more short sequences of positively charged lysines or arginines exposed on the protein surface

Phosphatase and tensin homolog (PTEN)
A protein that is encoded by the PTEN gene. This gene is identified as a tumor suppressor so that mutations of this gene are a step in the development of many cancers

Ring Opening Metathesis Polymerization (ROMP)
A type of olefin-metathesis chain growth polymerization reaction

Single guide RNA (sgRNA)
An sgRNA is a single RNA molecule that contains both the custom-designed short crRNA sequence fused to the scaffold tracrRNA sequence. The CRISPR-Cas9 system is directed by sgRNA

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Outstanding questions

- How can the delivery vehicles be rationally designed for delivery of therapeutic proteins to address delivery issues including efficiency, specificity, and toxicity?
- Can cytosolic delivery strategies be developed for targeting different cell types/tissues?
- How could the protein delivery strategies be pushed towards clinical trials?

Highlights

- Intracellular protein delivery is a powerful tool for development of protein-based therapeutics and is a challenging task due to cell membrane impermeability to large biomolecules and endosomal entrapment.
- Most delivery strategies rely on endosomal uptake of the carrier. Endosomally entrapped cargo generally exhibit low escape efficiency leading to eventual degradation.
- Numerous nanocarriers such as lipid, inorganic and polymeric nanoparticles have been developed to induce endosomal escape and deliver therapeutic proteins intracellularly. However, there remains considerable room for improvement in release efficiency.
- Development of non-endosomal uptake strategies including nanocarrier-mediated cell membrane fusion provides a promising alternative for delivering proteins directly to the cytosol.
- Improvements in protein delivery efficiency have enabled the translation of intracellular protein therapeutics towards the clinic.

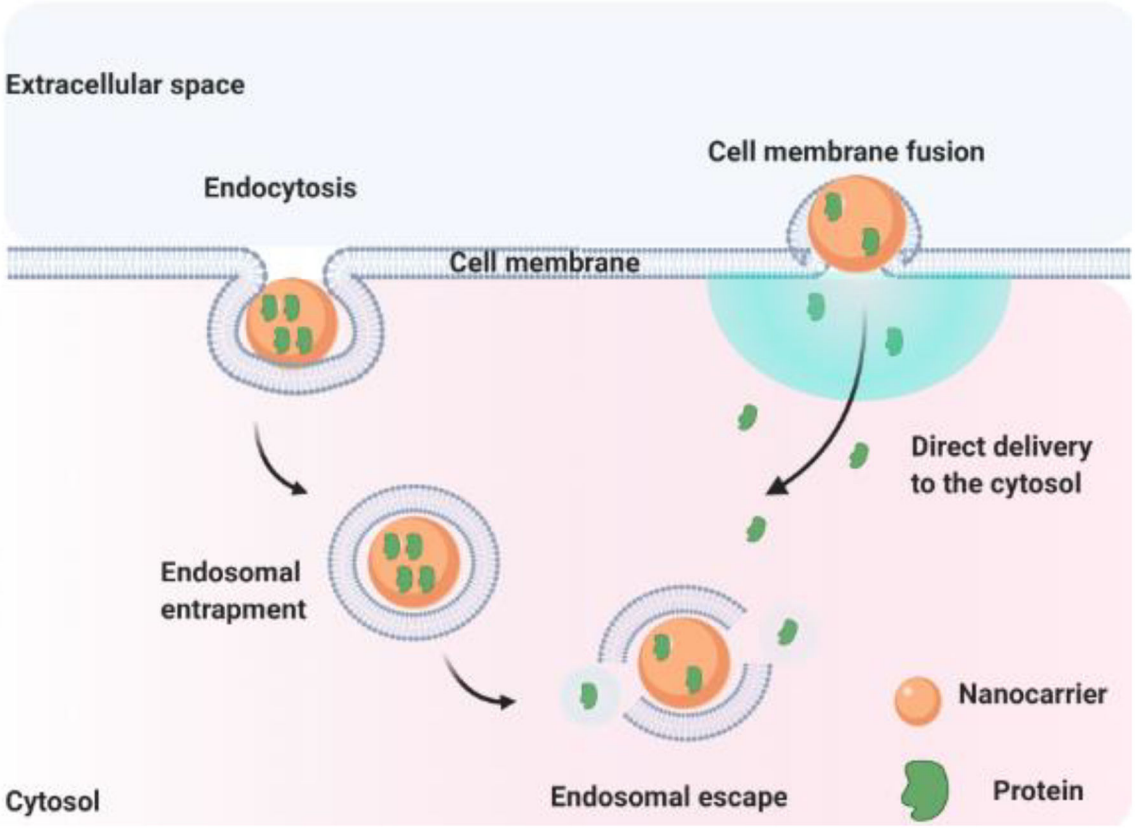

Figure 1, Key Figure. Delivery of proteins using nanocarriers through two different pathways: endocytosis and cell membrane fusion.

In endocytosis, nanocarriers containing cargo are taken up by the cells into endosomes. The cargo must escape out of the endosomes to gain access to the cytosol. Cell membrane fusion is an alternative non-endosomal delivery strategy, where the nanocarrier and cargo assemblies fuse with the cell membrane, allowing direct transfer of the protein cargo to the cytosol.

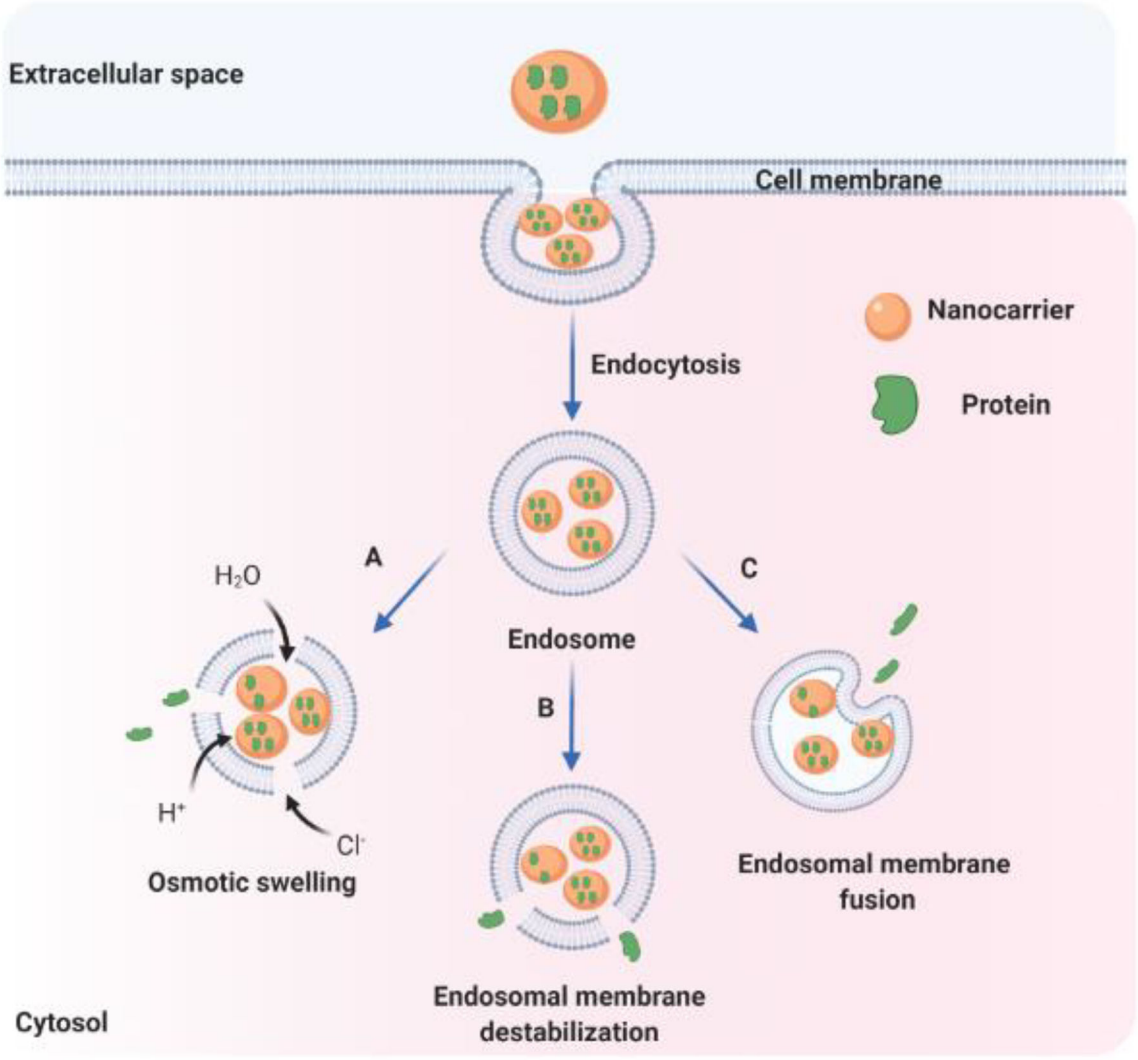

Figure 2. Mechanisms of endosomal escape.

After endocytosis, the entrapped proteins can escape out of the endosomes in three major pathways: (A) osmotic swelling due to the proton sponge effect, (B) endosomal membrane destabilization or (C) endosomal membrane fusion.

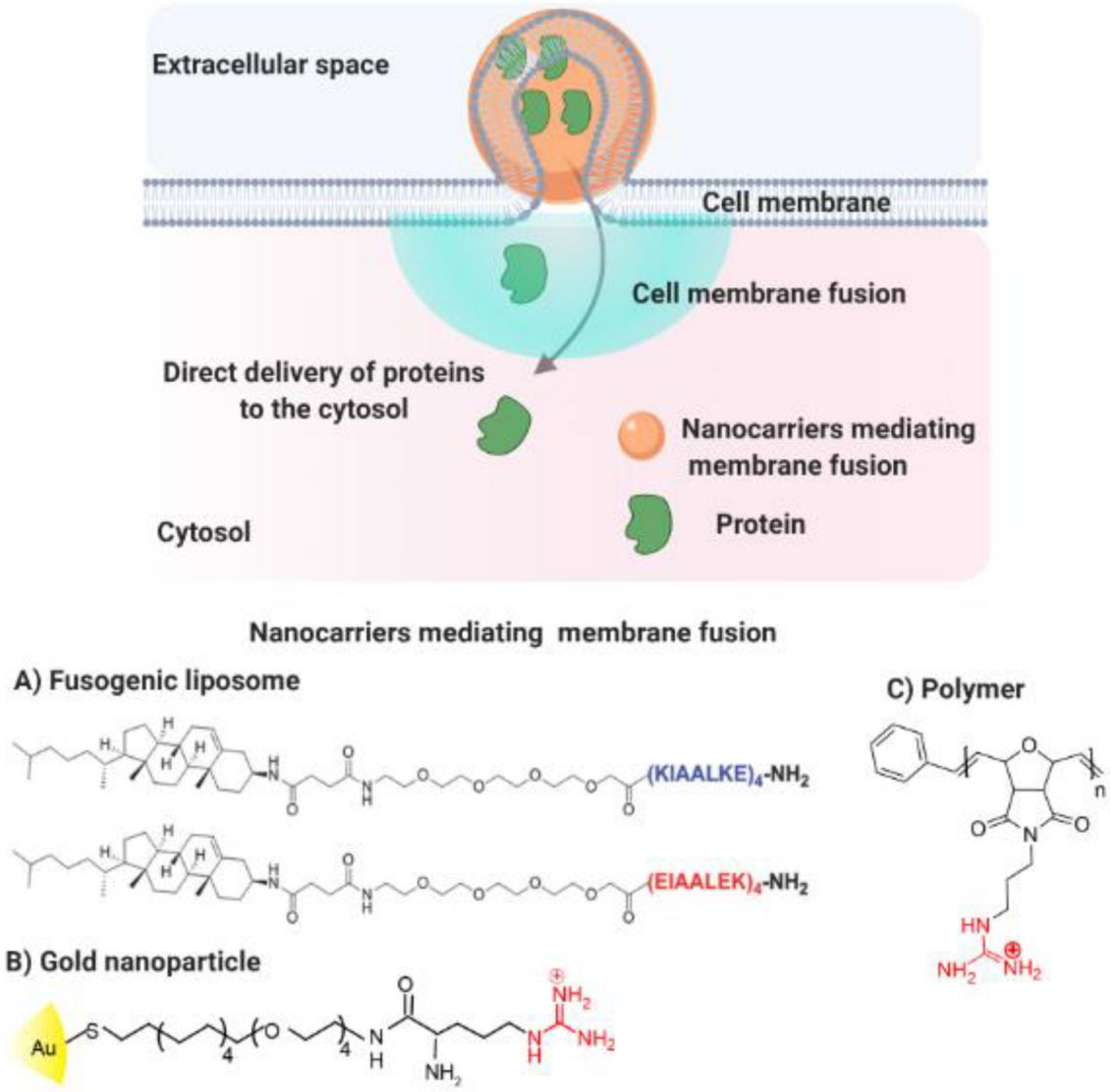

Figure 3. Nanocarriers mediating cell membrane fusion.

Cell membrane fusion is a non-endosomal delivery strategy facilitated by nanocarriers such as (A) fusogenic liposomes, (B) gold nanoparticles and (C) polymers. The highlighted red and blue portion in (A) represents the pair of fusogenic lipopeptides causing membrane fusion. The red bonds in (B) and (C) represent guanidinium moiety, playing a key role in cell membrane fusion.