Promises and Pitfalls of Intracellular Delivery of Proteins

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ABSTRACT: The direct delivery of functional proteins into the cell cytosol is a key issue for protein therapy, with many current strategies resulting in endosomal entrapment. Protein delivery to the cytosol is challenging due to the high molecular weight and the polarity of therapeutic proteins. Here we review strategies for the delivery of proteins into cells, including cell-penetrating peptides, virus-like particles, supercharged proteins, nanocarriers, polymers, and nanoparticle-stabilized nanocapsules. The advantages and disadvantages of these approaches including cytosolar delivery are compared and contrasted, with promising pathways forward identified.

INTRODUCTION

Protein delivery has important applications both in vitro and in vivo. In vitro, delivery of proteins into cells provides a potentially game-changing approach to manipulate signaling pathways,1 stimulate potent antitumor immune cells,2 and induce pluripotency for tissue engineering and wound healing.3 Effective in vivo protein delivery would provide therapeutic replacement of missing, dysfunctional, or poorly expressed proteins. Delivery of key functional proteins provides strategies to agonize or antagonize key intracellular pathways for both chronic and acute conditions, such as cancer, inflammatory diseases, oxidative stress-related disorders, diabetes, and brain injury. To date, around 100 proteins with different functions have been transported into cells in various animal models, with some protein systems making it to clinical trials.4,5

Protein delivery into cells has two major challenges. First, the protein must be transported into the cell. Proteins can be modified or conjugated to take advantage of normal cell uptake processes including endocytosis and phagocytosis.6 Once inside the cell, however, there is a more vexing problem: delivered proteins are trapped in vesicular structures (e.g., endosomes) after internalization, which prevents their access to the cytosol.7 The encapsulated proteins therefore do not achieve their desired biological activity because they do not reach their cytosolic targets. While progress has been made on both delivery and endosomal escape, effective delivery of proteins to the cytosol remains a challenge. In this Topical Review we will highlight current approaches to protein delivery, focusing on the strengths and challenges of three broad categories: mechanical methods, covalent protein modification, and supramolecular delivery systems.

STRATEGIES OF CURRENT PROTEIN DELIVERY

Mechanical Delivery Methods. Mechanical/physical methods such as microinjection and electroporation are the most traditional methods for protein delivery. These approaches provide the delivered proteins with direct access to the cytosol, which makes them very useful for in vitro investigations. These methods, however, are low-throughput, invasive, and require specialized equipment to mechanically/physically puncture membranes: important issues for in vivo applications.6,9 In vivo use of these methods is complicated by the need for direct physical access to the targeted cells, which limits the volume of tissue that can be locally treated. Additionally, the transient cell permeabilization provided by these approaches allows influx of other proteins and biomolecules into the cell, generating potential side effects.10

Carrier-Based Delivery. The inherent advantages of carrier-based delivery systems makes them attractive alternatives to mechanical methods for transporting proteins into cells. There are two categories of delivery strategies that have been broadly employed: covalent protein modifications and supramolecular delivery systems. These categories can be further divided into several subgroups, including cell-penetrating peptides (CPPs), virus-like particles, supercharged proteins, nanocarriers, supramolecular carrier-based delivery systems, and nanoparticle-stabilized nanocapsules (Figure 1).

Covalent Protein Modifications. Cell-Penetrating Peptides. Protein modification with cell-penetrating peptides (CPPs) and other modifying group proteins provide promising vectors for protein delivery. While these functionalization strategies commonly use proteins modified with short cationic...
peptides at one of their termini during expression, post-
modification of the protein provides a complementary means of
accessing proteins that are functionalized at other positions
(Figure 2). CPPs have been quite effective at bringing a wide
range of synthetic and biological components into cells,
including enzymes, cytokines, apoptotic molecules, protein
hormones, molecular chaperones, and cell-signaling proteins.
There is, however, some controversy as to the mechanism of
cell uptake of CPP-tagged materials. The original
hypothesis was that CPPs worked by penetrating the cell
membrane through a translocation or transduction process,
which would provide cytosolic access. Further research
indicates that uptake often occurs through endocytic
mechanisms that result in endocytic entrapment.
Regardless of mechanism, there have been numerous
eXamples of CPP-mediated delivery of proteins in vitro.
CPPs used in these studies have ranged from classical ones,
such as TAT peptide, polyarginine peptide, Pep-1, and
penetratin, to newly discovered ones. However, the efficiency
of cytosolic access of these protein conjugates is debatable.
selective α-PKC inhibitor composed of a fragment of the α-
PKC C2 domain (dV1−1), where TAT peptide did not show
significant decrease in heart tissue damage from artery-opening
surgery in a phase IIb clinical trial, although KAI-9803 reduced
cardiac damage in a rat model of acute myocardial infarction
caused by ischemia-reperfusion.

Virus-Like Particles. Virus-like particles (VLPs) are another
eXisting category of protein delivery vehicle. VLPs are formed
by self-assembly of virus capsid proteins, which are similar in
size and conformation to intact infectious virions, but possess
nonviral properties, including being nonreplicating, non-
pathogenic, and genomeless. Recent reports show that
VLPs can be used to deliver biologically active proteins into
cultured cells as heterologous protein fused with the anchoring
protein of VLPs. However, the intercellular location of
proteins that results from VLPs delivery requires evaluation of
the efficiency of cytosolic access, and the immunogenicity and
safety of viral proteins in VLPs needs to be further determined
by in vivo studies.

Supercharged Proteins. Supercharged proteins are a class of
engineered or naturally occurring proteins with unusually high
positive or negative net theoretical charge (typically >1 net
charge unit per kilo-Dalton of molecular weight). Previous
reports suggest that engineered GFP variants with very high net
bioconjugate chemistry
positive charge can penetrate and deliver other macromolecules into mammalian cells.\textsuperscript{30} Recent studies indicate that a large, diverse class of naturally occurring human proteins with unusually high net positive charge (possibly >2\% of the human proteome) have the ability to deliver functional proteins into mammalian cells both \textit{in vitro} and in retinal, pancreatic, and white adipose tissues \textit{in vivo}.\textsuperscript{31} However, the internalization mechanism of naturally occurring proteins with high net positive charge for protein delivery remains unclear, and supercharged modification of proteins may alter the protein properties and activity. Nevertheless, there are examples of local protein delivery by using supercharged proteins, including intramuscular injection of Cre fused to a supercharged protein, which leads to functional delivery only near the injection site, as most of the protein fusion precipitated.\textsuperscript{31}

\textbf{Covalent Nanoconjugates}. Nanocarriers provide an alternative strategy to direct protein delivery, offering increased options for control of size and surface properties.\textsuperscript{32,33} Two strategies have been used for nanocarrier-based protein delivery.\textsuperscript{34} Covalent attachment provides a stable linkage between carrier and protein, an important issue for \textit{in vivo} applications. These covalent conjugates can, however, interfere in protein folding and function. One successful application of a covalent bioconjugate is the use of magnetic nanoparticles (MNPs) as nanocarriers for delivery of serratiopeptidase to targeted cells, in which chitosan amino-functionalized MNPs conjugated to serratiopeptidase through a glutaraldehyde linker increased the anti-inflammatory activity of the therapeutic protein \textit{in vitro} and \textit{in vivo}.\textsuperscript{35} Another example of nanocarrier-mediated protein delivery is the covalent conjugation of mesoporous silica nanoparticles (MSN) to a small mitochondrial heme protein, cytochrome C (Cyt C), for induction of cancer cell death (Figure 3).\textsuperscript{36}

\textbf{Supramolecular Delivery Systems}. \textit{Carrier Based Delivery Systems}. Supramolecular carrier-based delivery systems are modular and operate through reversible associations with target proteins. In noncovalent strategies, proteins and delivery vectors self-assemble, which allows the transport of unmodified proteins into the cell. These strategies can use native protein, and overcome some of the limitations of covalent protein modification strategies, including protein misfolding and denaturation. One example of protein delivery is by Kim and co-workers, in which self-assembled nano-particles composed of glycol chitosan (GC)-bearing β-cyclo-dextrin (GC-βCD) were used as a protein carrier to deliver human growth hormone (hGH) into cells; as a result hGH was released from the nanoparticles in a sustained manner for 9 days.\textsuperscript{37} Another example is the targeted delivery of proteins into the brain by using a chitosan and rabies virus glycoprotein (RVG) peptide-conjugated, pluronic-based complex nanocarrier.\textsuperscript{38}

\textbf{Liposomes}. Liposomes are one of the more traditional nanocarriers, with strengths that include modularity and ease of preparation. Liposomal carriers have been used to effectively deliver a wide variety of proteins into cells, including albumin, antibodies, enzymes, and cytokines.\textsuperscript{39,40} For example, lysine-based cationic liposomes effectively delivered albumin and antibodies into cytoplasm via caveolae-mediated endocytosis, and the delivery efficiency of the liposome/albumin complexes can reach 99\% at 37 °C.\textsuperscript{41} Although encapsulation of protein in liposomes has high cellular transport efficiency, the amount of encapsulated protein remains a challenge. A recent report showed that a freeze–thaw cycling process can be used to encapsulate a large amount of hydrophilic proteins into unilamellar liposomes.\textsuperscript{42} However, it is risky with regard to loss of protein activity during the freeze–thaw process.\textsuperscript{43}

\textbf{Lipoplexes}. Lipoplexes are composed of surfactants, proteins, lipids, polymers, or a combination of these materials, and include solid lipid particles, oily suspensions, submicron lipid emulsions, lipid implants, lipid microbubbles, inverse lipid micelles, lipid microtubules, lipospheres, and lipid micro- cylinders.\textsuperscript{44} One of the examples for lipoplex-mediated protein delivery is to use a mixture of a Wr-T peptide and a commercially available cationic lipid reagent (BioPORTER) to efficiently deliver a variety of proteins into the cytoplasm of living cells.\textsuperscript{45} Additionally, the advantage of lipoplex-mediated delivery is the flexibility to produce different types of protein delivery vehicles based upon the molecular structure of the lipids used in the composition.\textsuperscript{46} For example, solid lipid particles composed of four different types of lipids and two triglycerides with different chain-lengths of fatty acyl groups can be used as efficient vehicles for oral delivery of peptide and protein drugs, and the drug release mechanism (lipase-mediated degradation-based release or, alternatively, lipase-degradation-independent release) from solid lipid particles is

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\caption{MNP\textsuperscript{s} deliver serratiopeptidase into cells. (A) Scheme of the immobilization of Cyt-c Lac into MSN-SH via redox-sensitive smart bonds followed by its intracellular delivery into cancer cells. (B) Internalization of the MSN-SPDP-Cyt c-Lac bioconjugate by HeLa cells observed by confocal microscopy. The left image is the autofluorescence image of the cells, the lower left shows the FITC labeled MSN internalized by the cells, the lower right shows the FM4-64 labeled endosomes, and the upper right micrograph is the merged image. FITC, fluorescein isothiocyanate. Reprinted with permission from ref 36. Copyright 2014 American Chemical Society.}
\end{figure}
dependent on the selection of lipid, which can be used in the design of lipid excipients for oral delivery of protein drugs.47

Polymers. Polymers have the advantage of controllable multivalency, providing the capability to tune both the strength and structure of polyplexes that result from supramolecular coordination of the polymer to the protein. Polyethylenimine (PEI) has been widely used for nucleic acid delivery,48 providing a cationic carrier to facilitate endocytosis and the capability to disrupt endosomes through the “proton sponge” effect.49 PEI is somewhat toxic, so a variety of other polymer backbones including linear, branched, and dendritic architectures have been also tested. A particularly interesting example of dendrimer-based delivery was shown by Yao and colleagues, who used carboxymethyl (CM) chitosan-poly(amidoamine) dendrimer core–shell nanoparticles to load and release lysozyme.50 These new dendrimer nanoparticles had better loading capacity and pH sensitivity than previously generated CM-chitosan polyion micelles. Examples of linear and branched polymer-based protein delivery include delivery of bovine serum albumin (BSA) and lysozyme to human breast carcinoma cells through complexation to polymer polyelectrolytes produced from PEI (Figure 4).51 Many more examples of different polymer structures have been used for protein delivery.52−56

Nanoplexes. Nanoplexes exploit the structural diversity of nanoparticles, which is composed of chemically modified nanoparticle, proteins, polymer, or other components that can incorporate unique imaging properties from the core in nanosize scale. One of the simplest examples of protein delivery through a nanoplex was by Rotello and co-workers,57,58 in which a cationic nanoparticle transported a large anionic protein β-galactosidase (473 kDa) into cells. The delivered protein retained activity inside the cell. When fluorescently tagged protein was used, punctate fluorescence was observed, indicative of vesicular localization. This fluorescence did not colocalize with endosomal tracking agents, which suggests post-uptake release (Figure 5). Another example of nanoplex-based delivery was by Palivan and colleagues,59 in which self-assembled polyethylene glycol (PEG) modified chitosan Bombyx mori nanoparticles (PEG-O-ChsBm) were used to deliver BSA into HeLa and THP-1 cells. More examples of nanoplex structures were also reported for protein delivery.60,61

Figure 4. PEI-based polyelectrolytes deliver protein into MCF7 cells. (A) Confocal images for intracellular tracking of the cationic polyelectrolyte/BSA complexes (WR1) and BSA (10 μg/mL) and (B) the intracellular endolysosome amount of the BSA- and polycation/BSA-treated cells. Free BSA was labeled with RITC (red). Acidic compartments and nuclei were stained with LysoTracker Green (green) and Hoechst 33342 (blue), respectively. RITC, Rhodamine Bisothiocyanate. Reprinted with permission from ref 51. Copyright 2013 American Chemical Society.

Figure 5. Nanoplex delivery of a large anionic protein β-galactosidase (473 kDa) into cells. (A) Intracellular delivery of functional protein using gold nanoparticles. (B) Co-localization study using confocal microscopy after protein transfection (NP_Pep/FITC-gal: 100 nM/50 nM) of HeLa cells in the presence of FM4-64: (a) green fluorescence from FITC-gal, (b) red fluorescence from FM4-64, an endosome-specific marker, and (c) overlap of the green and the red channels. In the merged image, green spots (shown with green arrowheads) indicate proteins outside endosomes, while entrapped proteins inside endosomes appear as yellow dots (shown with yellow arrowheads). Reprinted with permission from ref 57. Copyright 2010 American Chemical Society.
Inactivation during the delivery process. CASP3 was especially stringent test of the efficacy of this approach, as caspases are delicate enzymes that would be susceptible to inactivation during the delivery process. NPSCs were promising vehicles for in vitro applications; however, their usefulness in vivo has not been demonstrated.

CONCLUSIONS

Protein delivery has the potential to revolutionize therapeutics, allowing us to treat currently untreatable diseases and minimize side effects from off-target interactions. Tremendous progress has been made, and there are many promising leads in the effort to deliver proteins. All of the strategies described in this Topical Review have successful applications, and many are working their way toward translation. Each of these approaches, however, currently has limitations and challenges that will need to be overcome for optimal application. There are many ways in which these roadblocks can be addressed both synthetically and biologically through engineering of either protein or delivery vehicle. Perhaps the most promising route, however, would be the synergistic coengineering of proteins and carriers to provide integrated vectors to provide enhanced delivery of active proteins to their desired intracellular locations.

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