Recent developments in intracellular protein delivery
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Protein therapeutics based on transcription factors, gene editing enzymes, signaling proteins and protein antigens, have the potential to provide cures for a wide number of untreatable diseases, but cannot be developed into therapeutics due to challenges in delivering them into the cytoplasm. There is therefore great interest in developing strategies that can enable proteins to enter the cytoplasm of cells. In this review article we will discuss recent progress in intracellular protein therapeutics, which are focused on the following four classes of therapeutics, Firstly, vaccine development, secondly, transcription factor therapies, thirdly, gene editing and finally, cancer therapeutics. These exciting new advances raise the prospect of developing cures for several un-treatable diseases.

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Introduction
Protein based therapeutics, such as antibodies, receptor decoys and cell surface ligands have transformed the field of drug development and have the potential to impact all areas of medicine. However, despite their success, the full potential of protein based therapeutics has still not been realized because it has been impossible to develop protein therapeutics that target intracellular biomolecules. For example, therapeutics based on transcription factors, gene editing enzymes, metabolic enzymes and protein antigens, have the potential to provide cures for a wide number of untreatable diseases, but cannot be developed into therapeutics due to challenges in delivering them into the cytoplasm.

Intracellular protein delivery and vaccine development
Vaccines are arguably the most effective class of therapeutics ever developed. However, despite their success, vaccines still need to be developed for many pathogens, ranging from HIV to Hepatitis C. CD8⁺ T cells play an essential role in combating infections generated from viruses and cancer and there is consequently great interest in developing vaccines that can efficiently activate CD8⁺ T cells. A key step in developing vaccines that can activate CD8⁺ T cells is delivering antigen to the cytoplasm of antigen presenting cells (APCs). A large number of studies have now been published demonstrating that nanoparticles can deliver antigens into the cytoplasm of dendritic cells and activate CD8⁺ T cells. This is not surprising because the development of vaccines that can activate CD8⁺ T cells represents perhaps the most straightforward application of intracellular protein delivery [1], and is the most tolerant in terms of design parameters for developing delivery vehicles. For example, although protein antigens need to be delivered to the cytoplasm of dendritic cells to be presented as Class I antigens, the delivered antigens do not need to be folded, and can be denatured, as proteins processed for class I antigen presentation are degraded into peptides. Consequently, all of the problems associated with keeping proteins active and folded during the assembly of the
Protein therapeutics that function inside of cells: an emerging class of therapeutics. Overview of the four major applications of intracellular protein delivery: vaccine development, transcription factor therapy, gene editing and cancer therapy. Schematic diagram describing intracellular protein delivery. Green and blue items represent a cell and a protein therapeutic, respectively.

delivery vehicles are avoided. In addition, APCs and dendritic cells are phagocytic cells and robustly internalize particles between 100 nm-2 μm in size, which is fortuitous, because a variety of strategies exist for developing nanomaterials in this size range. Finally, class I antigen presentation can be quite efficient, and consequently, efficient CD8+ T cell activation can be observed, even if only a small fraction of antigen is delivered into the cytoplasm. A variety of elegant strategies have been developed for delivering antigens and adjuvants into the cytoplasm of dendritic cells [2-10]; Some of these are briefly summarized below.

Numerous microparticle strategies for delivering antigens into the cytoplasm have been developed. For example, Poly(lactic-co-glycolic acid) (PLGA) microparticles containing Ovalbumin, formulated via a double emulsion procedure, were able to efficiently deliver antigens and generate class I antigen presentation [11]. The mechanism by which PLGA can disrupt lysosomes is currently unknown, but presumably is related to hydrolysis of the ester linkage, which presumably causes colloid osmotic disruption of the endosome. A key benefit of the PLGA based systems is that the PLGA polymer itself has FDA approval for a variety of indications. In addition, the hydrolysis products of PLGA can themselves act as TLR ligands and danger signals, and this can further increase the efficiency of antigen presentation [12,13].

However, the hydrolysis kinetics of PLGA are not perfectly tuned for the environment of the phagosome and consequently several other microparticle chemistries have therefore been developed for intracellular delivery of antigens.

Antigen loaded microparticles and microgels, made from acid degradable ketal linkages, can deliver antigens into the cytoplasm of APCs and generate Class I antigen presentation. Ketal linkages are ideal for cytoplasmic delivery because their hydrolysis rate is proportional to the hydronium ion concentration, and hydrolyze 250 times faster at the pH 5.0 environment of the phagosome, versus the pH 7.4 environment of the extracellular space [14,15,16,17]. Microgels made with ketals linkages should disrupt endosomes/phagosomes via a colloid osmotic mechanism because the hydrolysis of the ketal linkage will dramatically increase the solute concentration in the phagosome. In addition, ketal linkage hydrolysis can be exquisitely tuned and microgels with linkages have been developed that hydrolyze with a t1/2 of several minutes at pH 5.0 and several hours at pH 7.4, which is ideal for intracellular drug delivery applications [17].

Transcription factor based therapeutics

Transcription factors (TFs) play a central role in biology, and are master regulators of biological function, through their ability to coordinate the expression of genes [18,19].
Therapeutics based on TFs have the potential to treat a wide number of diseases, as the biology of TFs is fairly well understood and because numerous disease pathologies are related to the down-regulation of specific transcription factors. However delivering TFs is inherently more challenging than delivering antigens because they need to be folded and active to generate a therapeutic effect, and this poses several constraints on delivery vehicle design [20].

Elegant strategies for delivering TFs have been developed that take advantage of the ability of TFs to bind DNA. For example, Liu et al. delivered a model transcription factor composed of the GAL4 DNA binding domain fused to the VP64 transcriptional activation domain, and complexed it to the plasmid DNA that had the GAL4 binding site, upstream of the luciferase gene pGSE4T-Fluc. Adamanetant cyclodextrin-host guest chemistry was then used to assemble a polyelectrolyte delivery vehicle that had RGD and TAT, and this complex was able to efficiently deliver plasmids that had the VP64-Gal4 transcription factor bound to them, and generated a strong bioluminescence in transfected HeLa cells [21].

A DNA based TF delivery strategy was developed, termed DARTs by Lee et al. based on using chemically modified oligonucleotides, which was able to deliver the TF Nrf2 in vivo into the liver. A multifunctional oligonucleotide was synthesized, which contained the promoter sequence that Nrf2 binds, galactose targeting groups, an acetal linker and a hydrophobic endosomal disruptive segment [22]. Nrf2 was assembled with this multifunctional oligonucleotide, because it recognized the Nrf2 promoter sequence, forming the DARTs. The galactose groups in the DART’s played two roles: targeting hepatocytes, and also preventing the endosomal disruptive domain of the DARTs from causing toxicity at pH 7.4 in the blood. After endocytosis, the acid labile acetal hydrolyzed in the endosome and unmasked the hydrophobic domain of DARTs to trigger endosomal disruption and release of Nrf2 into cytosol. The DARTs were able to deliver Nrf2 and increased the expression of Nrf2 dependent genes, such as HO1 and NQO1, and could protect the liver from Tylenol induced liver injury. DARTs have numerous potential applications given the central role of TFs in biology.

Another strategy for the intracellular delivery of TFs is to tether proteins to protein transduction domains (PTDs) such as TAT, ANTP and VP22, which can potentially cross biological membranes efficiently [23]. For example, a fusion protein composed of the TAT peptide fused with a zinc finger protein targeting the SV40 promoter, and the KRAB transcriptional repression domain has been developed. This TAT fused TF was able to downregulate the expression of EGFP and luciferase expression, in plasmids controlled by the SV40 promoter, in mammalian cell lines. However, this system also showed significant cellular toxicity at a concentration higher than 5 μM [24].

Also, Bailus et al. developed a TAT-S1 ATF fusion protein that can block the Ube3a-ATS transcript, and reactivate the expression of Ube3a, and offers a potential treatment for Angelman Syndrome. The TAT-S1 fusion protein was able to cross the blood-brain-barrier after an intraperitoneal injection, and suggests that TAT may facilitate the delivery of large proteins across the blood–brain barrier [20]. Also, high subcutaneous doses of TAT-S1 partially restored Ube3a expression in both the hippocampus and cerebellum in a mouse model of Angelman Syndrome.

**Protein based therapeutics that edit genomes**

Proteins that can perform gene editing have tremendous potential as therapeutics. Of the numerous proteins that can do gene editing, the clustered regularly interspaced short palindromic repeat-associated nuclease Cas9 (CRISPR/Cas9) has emerged as the most heavily investigated gene editing enzyme because it employs a guide RNA to target DNA sequences [25–29]. CRISPR/Cas9 is remarkably versatile and has a variety of different genome editing applications [30]. There is consequently great interest in delivering CRISPR/Cas9 in vivo. Even though viral based delivery strategies with adeno-associated viruses are the most effective methods, viral methods have several limitations and alternatives are being intensely considered. Kim et al. demonstrated that Cas 9 protein with gRNA delivery using electroporation efficiently induced gene editing [31]. Ramakrishna et al. demonstrated that Cas9 protein conjugated to cell penetrating peptides could perform gene editing in human embryonic stem cells, dermal fibroblasts, and several other cell-lines [32]. Zaris et al. demonstrated that the cationic lipid, Lipofectamine, could deliver Cas9 RNP in vivo and demonstrated that Cas9 RNP/Lipofectamine complexes could knock out genes in vivo after direct injection into the mouse inner ear [33]. Bioreducible lipid nanoparticles have been used to deliver Cas9 RNP in vitro and edit cells in culture [34]. Cas9 RNP has also been delivered using a DNA based scaffold, termed DNA nanoclews. The DNA nanoclews are partially complementary to the sgRNA, and this causes them to bind Cas9 RNP. DNA nanoclews complexed with Cas9 RNP were able to efficiently transfect cells after complexation with PEI [35].

A key challenge with developing Cas9 based therapeutics is synthesizing delivery vehicles that can deliver Cas9 RNP and correct gene mutations via HDR. Correcting gene mutations via HDR requires delivering both Cas9 RNP and donor DNA into the same cell, and this is challenging because of the different charge densities of the Cas9 RNP and donor DNA. In this regard, Lee et al. demonstrated that Cas9 RNP and donor DNA could...
be delivered in vivo using a gold nanoparticle based delivery vehicle, termed CRISPR-Gold [36**]. The core of CRISPR-Gold is composed of gold nanoparticles conjugated to thiol modified oligonucleotides (DNA-Thiol), and then assembled with donor DNA, Cas9 RNP, and an endosomal disruptive polymer. CRISPR-Gold was able to efficiently deliver Cas9 RNP and donor DNA into various cell types and into muscle tissue in vivo in a mouse model of Duchenne muscular dystrophy (DMD). In the DMD mouse model, CRISPR-Gold demonstrated an HDR efficiency of 5.5% and regenerated the expression of dystrophin (Figure 2).

**Cancer therapeutics**

Delivery vehicles that can efficiently deliver proteins intracellularly have the potential to revolutionize cancer therapy, as therapies based on peptides and proteins are frequently less toxic than traditional anticancer agents. In this section, we discuss recent advances in developing intracellular protein therapeutics targeted towards treating cancer.

Cell penetrating peptides (CPPs) have been widely used to deliver proteins intracellularly, and have been extensively reviewed [37,38]. We will therefore only highlight a recent development in CPPs. A recent study published in Nature Chemistry modified M-lycotoxin, a naturally occurring CPP, to selectively disrupt endosomal membranes by incorporating a glutamic acid residue in its hydrophobic domain [39]. Upon macropinocytosis the glutamic acid residue is protonated in the acidic environment of the endosome, leading to a membrane disruptive conformation and cytosolic release of co-delivered macromolecules (Figure 3a).

The Baker and Stayton labs developed a copolymer micelle for the intracellular delivery of the engineered protein BINDI, which is a potential therapeutic for Epstein-Barr virus (EBV)-positive B cell lymphoma [40]. EBV encodes BHRF1, which prevents lymphocyte apoptosis by sequestering BIM. BINDI binds BHRF1 with high affinity by recognizing its hydrophobic cleft, and prevents the sequestration of BIM. The intracellular delivery of BINDI should therefore cause apoptosis in EBV infected cancers and suppress tumor growth. BINDI was delivered using a micelle composed of an endosomolytic block, and a hydrophilic block. The hydrophilic segment contained pyridyl disulfide groups for conjugation to BINDI and biotin tags for conjugation to streptavidin-anti CD19. The micelles were able to slow tumor growth and increase survival in a mouse model of EBV-positive lymphoma (Figure 3b).

A recent paper by Li et al. reported a protein delivery system based on positively charged polymers that self-assemble around negatively charged proteins through cross-polymer disulfide bonds [41]. The crosslinked micelles increased the serum stability of encapsulated proteins (in this case caspase-3, a pro-apoptotic cancer therapeutic), and released them after intracellular delivery through glutathione mediated disulfide exchange (Figure 3c).
In summary, in the past 10–20 years, several new strategies for delivering proteins intracellularly have been developed. These exciting new advances raise the prospect of developing cures for several untreatable diseases. In the future clinical studies with these new delivery vehicles will hopefully be performed so that their translation potential can be determined.

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References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest


One of the first papers demonstrating that PLGA can itself act as an adjuvant.


This paper demonstrated that amino-benzaldehyde acetals have potential for drug delivery because of their unique pH sensitivity.


This paper demonstrated that methoxy-benzaldehyde acetals have potential for drug delivery because of their unique pH sensitivity.


This paper demonstrated that DNA can be used as a scaffold to deliver transcription factors.


This paper demonstrated for the first time that Cas9 RNP could edit genes in vivo.


This paper demonstrated for the first time that gene mutations can be corrected in vivo by delivering Cas9 RNP and donor DNA.


