Title

Recent developments in intracellular protein delivery

Authors

Zhang, Yumiao
Røise, Joachim Justad
Lee, Kunwoo
et al.

Publication Date

2018-08-01

DOI

10.1016/j.copbio.2018.02.009

Peer reviewed

HHS Public Access

Author manuscript

Curr Opin Biotechnol. Author manuscript; available in PMC 2019 August 01.

Published in final edited form as:

Curr Opin Biotechnol. 2018 August ; 52: 25–31. doi:10.1016/j.copbio.2018.02.009.

Recent Developments in Intracellular Protein Delivery

Yumiao Zhang[1], **Joachim Justad Røise**[2], **Kunwoo Lee**[3], **Jie Li**[1], and **Niren Murthy**[1,*]

[1]Department of Bioengineering, University of California, Berkeley, Berkeley, CA, USA, 94720

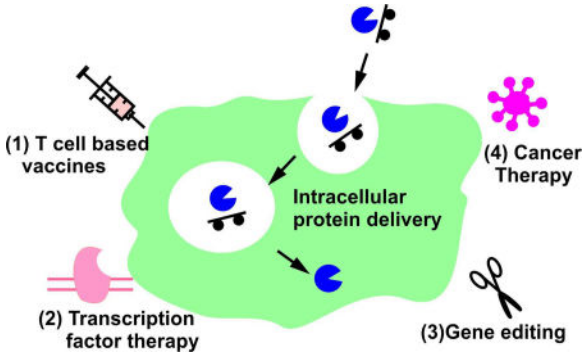
[2]Department of Chemistry, University of California, Berkeley, Berkeley, CA, USA, 94720

[3]GenEdit, Berkeley, CA, USA, 94720

Abstract

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, (1) Vaccine development, (2) Transcription factor therapies, (3) Gene editing and (4) Cancer therapeutics. These exciting new advances raise the prospect of developing cures for several un-treatable diseases.

Graphical abstract



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

*Correspondence should be addressed to nmurthy@berkeley.edu.

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.

There is therefore great interest in developing strategies that can enable proteins to enter the cytoplasm of cells. However, despite 40 years of research the development of therapeutics that work intracellularly remains a major challenge. Delivering proteins intracellularly is challenging because it requires developing multifunctional delivery vehicles that can perform the functions of triggering endocytosis and endosomal disruption and this frequently requires assembling macromolecular complexes. This is inherently challenging with protein therapeutics because of their large size, their sensitivity to chemical modifications and the difficulties in characterizing and purifying macromolecular complexes. Nevertheless, several innovative strategies have been developed to deliver proteins intracellularly, and these delivery strategies provide a roadmap for developing intracellular protein therapeutics. In this review article we will discuss recent progress in 4 areas of intracellular protein therapeutics, which are focused on the following four classes of therapeutics, (1) Vaccine development, (2) Transcription factor therapies, (3) Gene editing and (4) Cancer therapeutics (Figure 1).

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 delivery vehicles are avoided. In addition, APCs and dendritic cells are phagocytic cells and robustly internalize particles between 100 nm–2 microns 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 [14–17]. 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 $t_{1/2}$ 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 pG5E4T-Fluc. Adamantane 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 chemical 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 DARTs 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. S. Kim et al. demonstrated that Cas 9 protein with gRNA delivery using electroporation efficiently induced gene editing [31]. S. 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]. JA Zuris *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]. Bio-reducible 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 DNA, 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 Duchene 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 therapeutics 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 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 un-treatable diseases. In the future clinical studies with these new delivery vehicles will hopefully be performed so that their translation potential can be determined.

Acknowledgments

This work was supported by the National Institutes of Health (R21AI119115, R01AI117064; RO1EB2000801A1; RO1EB023776).

References and recommended reading

Papers of particular interest, published within the period review, have been highlighted as:

• of special interest

•• of outstanding interest

1. Rincon-Restrepo M, Mayer A, Hauert S, Bonner DK, Phelps EA, Hubbell JA, Swartz MA, Hirosue S. Vaccine nanocarriers: Coupling intracellular pathways and cellular biodistribution to control CD4 vs CD8 T cell responses. Biomaterials. 2017; 132:48–58. [PubMed: 28407494]
2. Alving CR, Wassef NM. Cytotoxic T lymphocytes induced by liposomal antigens: mechanisms of immunological presentation. AIDS Res Hum Retroviruses. 1994; 10(Suppl 2):S91–94. [PubMed: 7532427]
3. Higgins DE, Shastri N, Portnoy DA. Delivery of protein to the cytosol of macrophages using Escherichia coli K-12. Mol Microbiol. 1999; 31:1631–1641. [PubMed: 10209738]
4. Wilson JT, Postma A, Keller S, Convertine AJ, Moad G, Rizzardo E, Meagher L, Chiefari J, Stayton PS. Enhancement of MHC-I antigen presentation via architectural control of pH-responsive, endosomolytic polymer nanoparticles. AAPS J. 2015; 17:358–369. [PubMed: 25501498]
5. Martin S, Niedermann G, Leipner C, Eichmann K, Weltzien HU. Intracellular processing of hapten-modified protein for MHC class I presentation: cytoplasmic delivery by pH-sensitive liposomes. Immunol Lett. 1993; 37:97–102. [PubMed: 8258464]
6. Zhou F, Huang L. Liposome-mediated cytoplasmic delivery of proteins: an effective means of accessing the MHC class I-restricted antigen presentation pathway. ImmunoMethods. 1994; 4:229–235. [PubMed: 7820454]
7. Mandal M, Lee K-D. Listeriolysin O-liposome-mediated cytosolic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency, activity, and tumor protection. Biochim Biophys Acta. 2002; 1563:7–17. [PubMed: 12007619]
8. Reis e Sousa C, Germain RN. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. J Exp Med. 1995; 182:841–851. [PubMed: 7650490]
9. Wilson JT, Keller S, Manganiello MJ, Cheng C, Lee C-C, Opara C, Convertine A, Stayton PS. pH-Responsive Nanoparticle Vaccines for Dual-Delivery of Antigens and Immunostimulatory Oligonucleotides. ACS Nano. 2013; 7:3912–3925. [PubMed: 23590591]

10. Oh YK, Harding CV, Swanson JA. The efficiency of antigen delivery from macrophage phagosomes into cytoplasm for MHC class I-restricted antigen presentation. Vaccine. 1997; 15:511–518. [PubMed: 9160518]

11. Waeckerle-Men Y, Groettrup M. PLGA microspheres for improved antigen delivery to dendritic cells as cellular vaccines. Adv Drug Deliv Rev. 2005; 57:475–482. [PubMed: 15560953]

12. Sharp FA, Ruane D, Claass B, Creagh E, Harris J, Malyala P, Singh M, O'Hagan DT, Pétrilli V, Tschopp J, et al. Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP3 inflammasome. Proc Natl Acad Sci U S A. 2009; 106:870–875. [PubMed: 19139407]

13•. Newman KD, Samuel J, Kwon G. Ovalbumin peptide encapsulated in Poly(d,l lactic-coglycolic acid) microspheres is capable of inducing a T helper type 1 immune response. J Controlled Release. 1998; 54:49–59. One of the first papers demonstrating that PLGA can itself act as an adjuvant.

14. Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides. J Controlled Release. 2003; 89:365–374.

15•. Murthy N, Campbell J, Fausto N, Hoffman AS, Stayton PS. Bioinspired pH-Responsive Polymers for the Intracellular Delivery of Biomolecular Drugs. Bioconjug Chem. 2003; 14:412–419. This paper demonstrated that amino-benzaldehyde acetals have potential for drug delivery because of their unique pH sensitivity. [PubMed: 12643752]

16•. Murthy N, Thng YX, Schuck S, Xu MC, Fréchet JMJ. A Novel Strategy for Encapsulation and Release of Proteins: Hydrogels and Microgels with Acid-Labile Acetal Cross-Linkers. J Am Chem Soc. 2002; 124:12398–12399. This paper demonstrated that methoxy-benzaldehyde acetals have potential for drug delivery because of their unique pH sensitivity. [PubMed: 12381166]

17. Murthy N, Xu M, Schuck S, Kunisawa J, Shastri N, Fréchet JMJ. A macromolecular delivery vehicle for protein-based vaccines: Acid-degradable protein-loaded microgels. Proc Natl Acad Sci. 2003; 100:4995–5000. [PubMed: 12704236]

18. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126:663–676. [PubMed: 16904174]

19. Huang P, He Z, Ji S, Sun H, Xiang D, Liu C, Hu Y, Wang X, Hui L. Induction of functional hepatocyte-like cells from mouse fibroblasts by defined factors. Nature. 2011; 475:386–389. [PubMed: 21562492]

20. Bailus BJ, Pyles B, McAlister MM, O'geen H, Lockwood SH, Adams AN, Nguyen JTT, Yu A, Berman RF, Segal DJ. Protein delivery of an artificial transcription factor restores widespread Ube3a expression in an Angelman syndrome mouse brain. Mol Ther. 2016; 24:548–555. [PubMed: 26727042]

21•. Liu Y, Wang H, Kamei K, Yan M, Chen K-J, Yuan Q, Shi L, Lu Y, Tseng H-R. Delivery of Intact Transcription Factor by Using Self-Assembled Supramolecular Nanoparticles. Angew Chem Int Ed Engl. 2011; 50:3058–3062. This paper demonstrated that DNA can be used as a scaffold to deliver transcription factors. [PubMed: 21370360]

22. Lee K, Rafi M, Wang X, Aran K, Feng X, Lo Sterzo C, Tang R, Lingampalli N, Kim HJ, Murthy N. In vivo delivery of transcription factors with multifunctional oligonucleotides. Nat Mater. 2015; 14:701–706. [PubMed: 25915034]

23. Schwarze SR, Dowdy SF. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol Sci. 2000; 21:45–48. [PubMed: 10664605]

24. Zhao X, Dong Y, Zhao Z, Guo J, Liu J, Huang P, Dong D, Fan H, Guo Q, Yang X, et al. Intracellular delivery of artificial transcription factors fused to the protein transduction domain of HIV-1 Tat. Protein Expr Purif. 2013; 90:27–33. [PubMed: 23648869]

25. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science. 2012; 337:816–821. [PubMed: 22745249]

26. Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. Multiplex Genome Engineering Using CRISPR/Cas Systems. Science. 2013; 339:819–823. [PubMed: 23287718]

27. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-Guided Human Genome Engineering via Cas9. Science. 2013; 339:823–826. [PubMed: 23287722]

28. Cho SW, Kim S, Kim JM, Kim J-S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat Biotechnol. 2013; 31:230–232. [PubMed: 23360966]

29. Yin H, Song C-Q, Dorkin JR, Zhu LJ, Li Y, Wu Q, Park A, Yang J, Suresh S, Bizhanova A, et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. Nat Biotechnol. 2016; 34:328–333. [PubMed: 26829318]

30. Wang L, Li F, Dang L, Liang C, Wang C, He B, Liu J, Li D, Wu X, Xu X, et al. In Vivo Delivery Systems for Therapeutic Genome Editing. Int J Mol Sci. 2016; 17

31. Kim S, Kim D, Cho SW, Kim J, Kim J-S. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins. Genome Res. 2014; 24:1012–1019. [PubMed: 24696461]

32. Ramakrishna S, Kwaku Dad A-B, Beloor J, Gopalappa R, Lee S-K, Kim H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. Genome Res. 2014; 24:1020–1027. [PubMed: 24696462]

33••. Zuris JA, Thompson DB, Shu Y, Guilinger JP, Bessen JL, Hu JH, Maeder ML, Joung JK, Chen Z-Y, Liu DR. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo. Nat Biotechnol. 2015; 33:73–80. This paper demonstrated for the first time that Cas9 RNP could edit genes in vivo. [PubMed: 25357182]

34. Wang M, Zuris JA, Meng F, Rees H, Sun S, Deng P, Han Y, Gao X, Pouli D, Wu Q, et al. Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles. Proc Natl Acad Sci. 2016; 113:2868–2873. [PubMed: 26929348]

35. Sun W, Ji W, Hall JM, Hu Q, Wang C, Beisel CL, Gu Z. Self-Assembled DNA Nanoclews for the Efficient Delivery of CRISPR–Cas9 for Genome Editing. Angew Chem Int Ed. 2015; 54:12029–12033.

36••. Lee K, Conboy M, Park HM, Jiang F, Kim HJ, Dewitt MA, Mackley VA, Chang K, Rao A, Skinner C, et al. Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng. 2017; This paper demonstrated for the first time that gene mutations can be corrected in vivo by delivering Cas9 RNP and donor DNA. doi: 10.1038/s41551-017-0137-2

37. Bolhassani A, Jafarzade BS, Mardani G. In vitro and in vivo delivery of therapeutic proteins using cell penetrating peptides. Peptides. 2017; 87:50–63. [PubMed: 27887988]

38. Shin MC, Zhang J, Min KA, Lee K, Byun Y, David AE, He H, Yang VC. Cell-penetrating peptides: achievements and challenges in application for cancer treatment. J Biomed Mater Res A. 2014; 102:575–587. [PubMed: 23852939]

39. Akishiba M, Takeuchi T, Kawaguchi Y, Sakamoto K, Yu H-H, Nakase I, Takatani-Nakase T, Madani F, Gräslund A, Futaki S. Cytosolic antibody delivery by lipid-sensitive endosomolytic peptide. Nat Chem. 2017; 9 nchem.2779.

40. Procko E, Berguig GY, Shen BW, Song Y, Frayo S, Convertine AJ, Margineantu D, Booth G, Correia BE, Cheng Y, et al. A computationally designed inhibitor of an Epstein-Barr viral Bcl-2 protein induces apoptosis in infected cells. Cell. 2014; 157:1644–1656. [PubMed: 24949974]

41. Li J, Zhang L, Liu Y, Wen J, Wu D, Xu D, Segura T, Jin J, Lu Y, Wang H. An intracellular protein delivery platform based on glutathione-responsive protein nanocapsules. Chem Commun. 2016; 52:13608–13611.

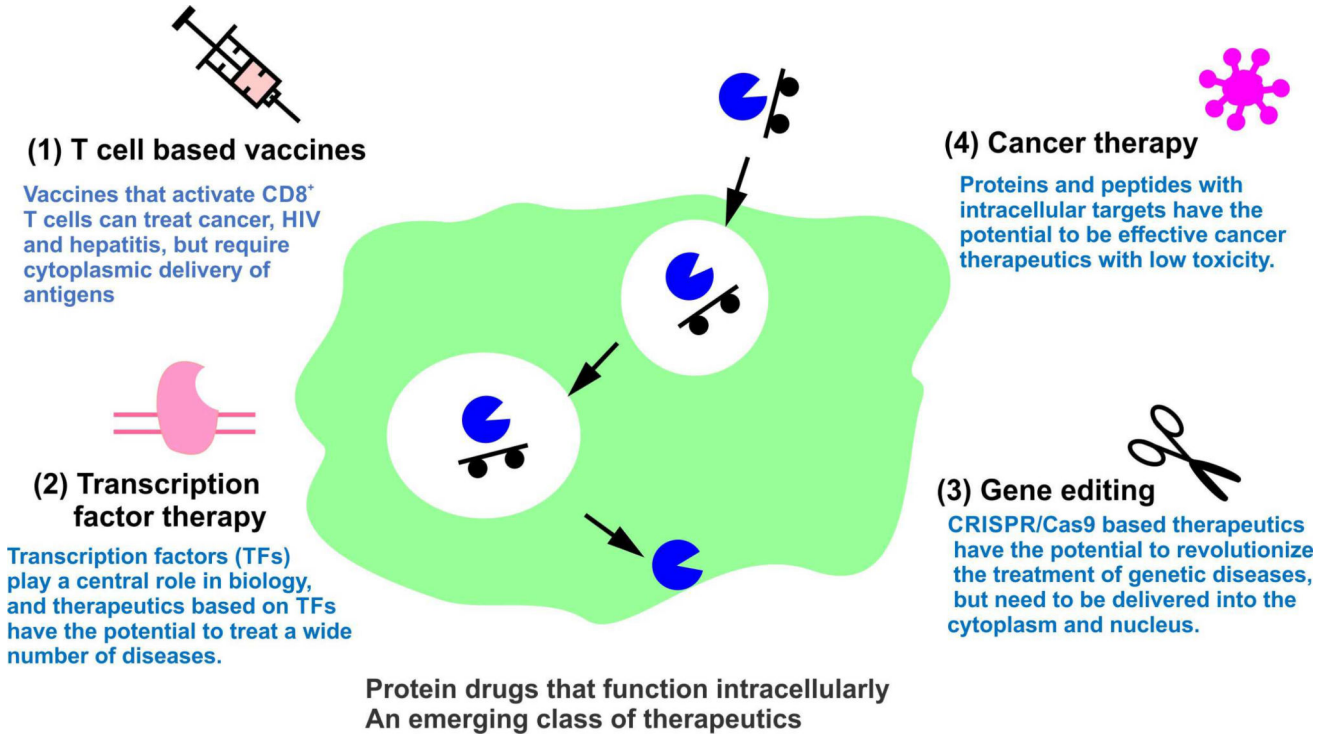


Figure 1. Protein therapeutics that function inside of cells: An emerging class of therapeutics

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.

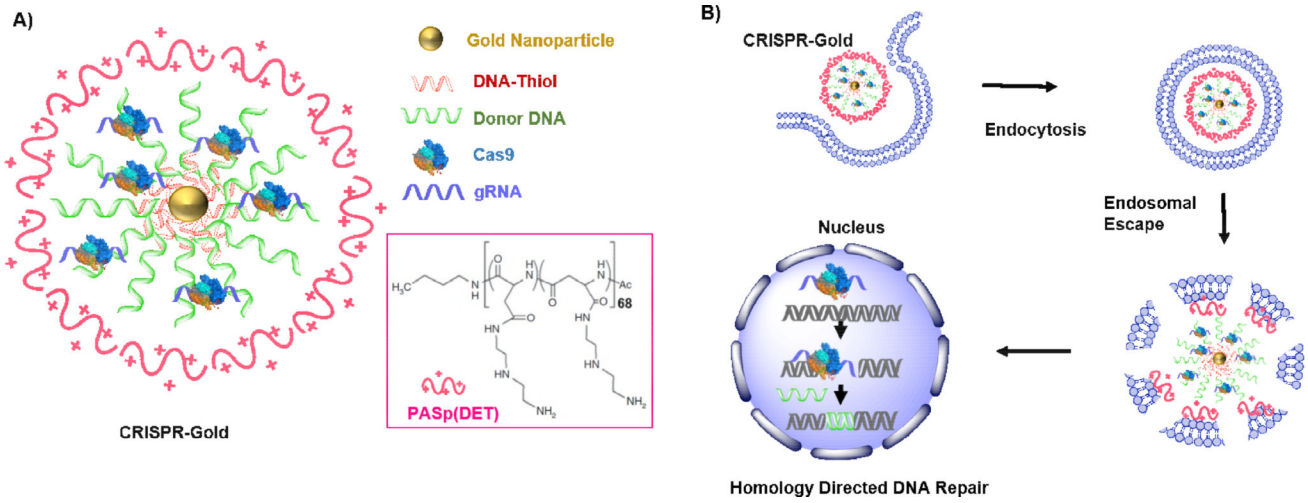


Figure 2. CRISPR–Gold can deliver Cas9 RNP and donor DNA in vivo and induce HDR

A) CRISPR–Gold is composed of 15 nm GNPs conjugated to thiol modified oligonucleotides (DNA-Thiol), which are hybridized with single-stranded donor oligonucleotides and subsequently complexed with Cas9 RNP and the endosomal disruptive polymer PAsp(DET), where ‘DET’ is diethylenetriamine. **B)** CRISPR–Gold is internalized by cells in vitro and in vivo via endocytosis. This triggers endosomal disruption and releases Cas9 RNP and donor DNA into the cytoplasm. Nuclear delivery results in HDR.

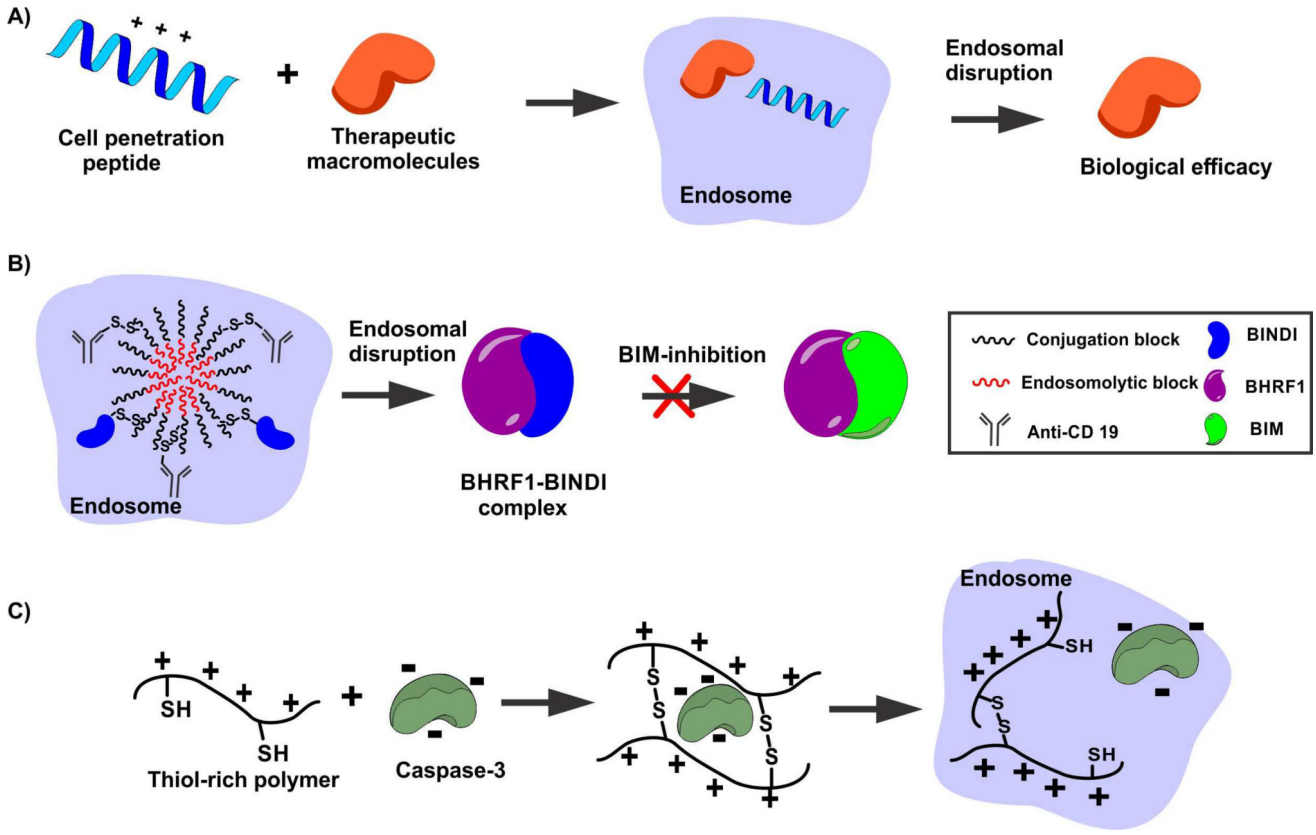


Figure 3. Strategies for delivering protein based cancer therapeutics intracellularly

(a) Cell-penetrating peptides (CPPs) co-administered with macromolecules can disrupt the endosomal membrane and enable the cytosolic delivery of macromolecular therapeutics. (b) Endosomolytic micelles conjugated with antibodies target cancer cells and deliver BIND1 intracellularly (an inhibitor of BHFR1), and treat lymphatic cancers caused from EBV infections. (c) Encapsulation of caspase-3 in a positively charged disulfide cross-linked polymer micelle increases its serum stability, cellular uptake and intracellular delivery.