Review

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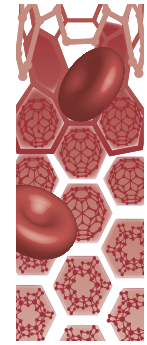
Nanomedicine



Intracellular delivery of proteins by nanocarriers

Intracellular delivery of proteins is potentially a game-changing approach for therapeutics. However, for most applications, the protein needs to access the cytosol to be effective. A wide variety of strategies have been developed for protein delivery, however access of delivered protein to the cytosol without acute cytotoxicity remains a critical issue. In this review we discuss recent trends in protein delivery using nanocarriers, focusing on the ability of these strategies to deliver protein into the cytosol.

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Keywords: cytosolic delivery • nanoparticle-stabilized nanocapsules • nuclear targeting • protein • punctate fluorescence

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Proteins play a crucial role in maintaining all cellular functions [1]. Therefore, malfunctioning or poor expression of proteins in cell is the origin of most genetic and many acquired diseases [2]. Replacing dysfunctional macromolecules via *in vitro* or *in vivo* delivery of proteins can be the most secure and unambiguous method for treating diseases [3]. Protein therapy directly addresses the disease, without the random and sometimes permanent integration of genetic material observed with gene therapy [4].

There are two major challenges in delivering proteins into the cells [5]. The first barrier is the uptake of protein by the cell. Proteins can be modified or conjugated to use the endocytic pathway as a route of cellular entry, providing high delivery efficiency [6]. A much harder challenge, however, is getting the protein into the cytosol. In most cases, delivered proteins are trapped in endosomes, and are degraded by cathepsins in the endosomal/lysosomal pathway [7]. As a result, the amount of protein reaching the cytosol is low, with concomitantly low efficacy of the delivered protein [8]. Although, progress has been made in increasing the efficiency of endosomal escape of delivered proteins [9], these methods are still generally of rather low efficiency [10].

One of the most common approaches for protein delivery is to fuse the proteins with protein transduction domains (PTDs) or cell-penetrating peptides (CPPs) [11]. PTDs and CPPs typically use endocytic pathway for cellular entry [11]. However, in majority of the cases, PTD/CPP fused proteins are trapped in the endosomes, hence unable to escape to the cytosol [12]. A variety of membrane-destabilizing agents have been used to disrupt the endosomes and release the proteins from these vesicles [13–15]. But to date, these systems generally feature low delivery efficiency [16] or high toxicity [17,18].

In the recent years, nanocarrier-based protein delivery approaches have emerged. Nanocarriers have certain key features that make them attractive alternatives for intracellular protein delivery [19,20]. First, the physical and chemical properties of these vehicles can be tuned via chemical modifications. As such, key surface properties, such as, charge,

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size and displayed ligands on the surface of the carrier can be tailored for cellular uptake, endosomal escape and target specificity [21]. Furthermore, nanocarriers can be loaded with proteins via covalent or noncovalent modifications that can protect the target proteins from denaturation via proteolysis [22,23].

In this review, we will discuss recent methodologies developed for intracellular protein delivery using nanocarriers. Limitations and advantages of these approaches along with future opportunities and challenges will also be reported.

Liposomes

Liposomal carriers have been widely used to deliver therapeutic proteins, antibodies, enzymes and cytokines [24–26]. The lipid bilayers of liposomes inhibit access of extracellular and endosomal proteases, maintaining protein stability. Using the cationic lipid-mediated carriers referred to as RNAiMAx, Liu and colleagues [27] have recently demonstrated a strategy of delivering proteins, such as, GFP, Cre recombinase, TALE and clustered, CRISPR–Cas9 protein *in vitro* and *in vivo* as described in Figure 1. The authors have fused engineered, supernegatively charged GFP (-30 GFP) to the above mentioned target cationic proteins and delivered them into mammalian cells after complexing with cationic liposomes. All of the above proteins were delivered rapidly and efficiently into mammalian cells. In fact, the genetic engineering of -30GFP variants or the interaction between Cas9 and highly anionic sgRNA mediates the electrostatic self-assembly of the protein with cationic lipids, further

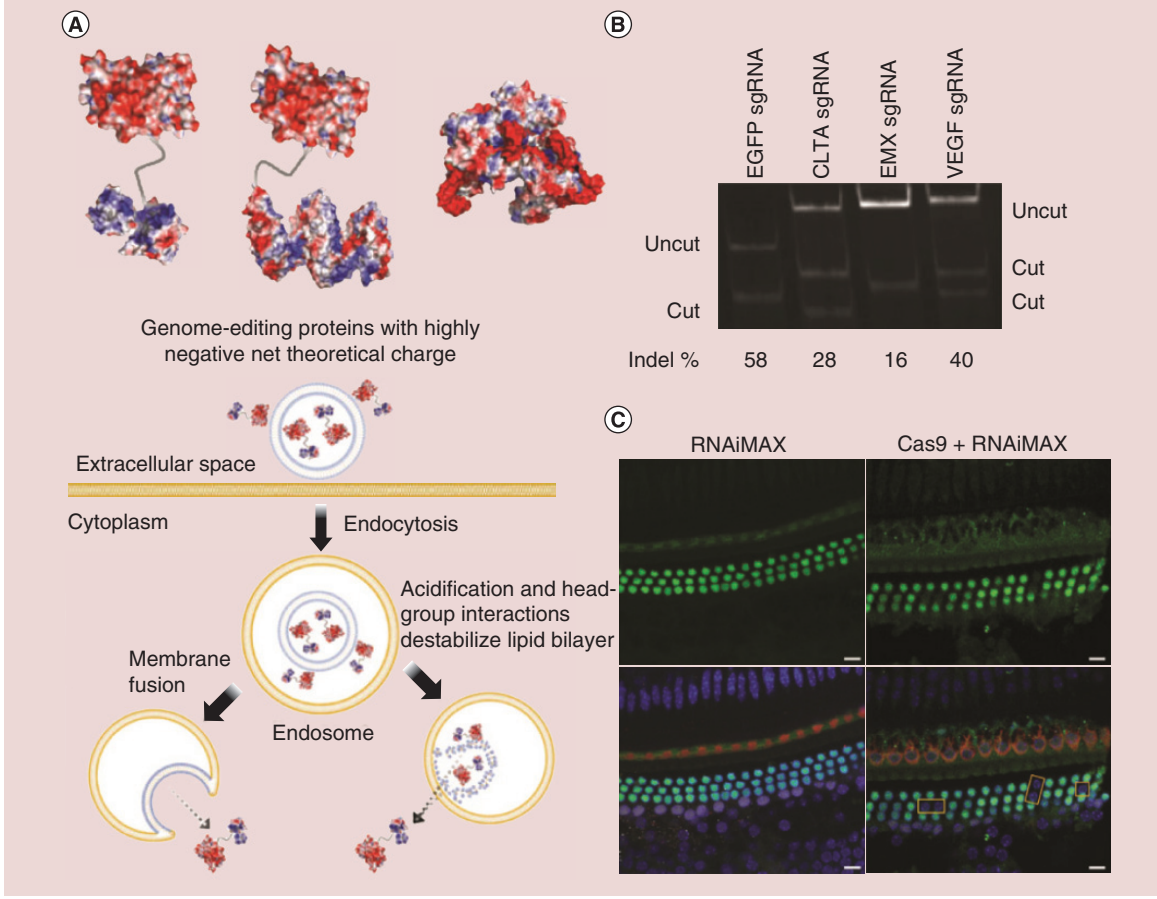

Figure 1. Intracellular delivery of TALE and Cas9 proteins using cationic lipids. **(A)** Recombinases, TALE proteins and Cas9 endonucleases fuse with supernegatively charged proteins, (-30) GFP, first, and complex with cationic lipids, resulting highly anionic proteins or protein–nucleic acid complexes and mediating their delivery into mammalian cells. **(B)** T7EI assay of simultaneous genome modification at *eGFP* and three endogenous genes in U2OS cells 48 h after a single treatment of 100 nM Cas9 protein, 25 nM of each of the four sgRNAs shown (100 nM total sgRNA) and 0.8 ml RNAiMAX. **(C)** Cas9-mediated gene disruption results in the loss of GFP expression when visualized 10 days later. The upper panels show GFP signal only, whereas lower panels include additional immunehistological markers. Yellow boxes in the lower panels highlight hair cells that have lost GFP expression. All scale bars (white), 10 μm.
GFP: Green fluorescent protein.
Adapted with permission from [27].

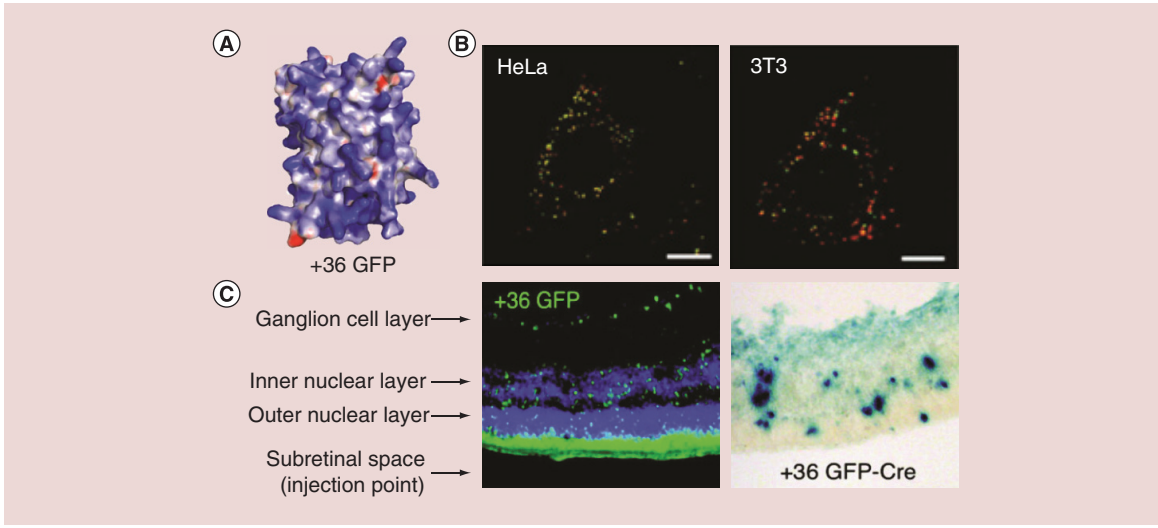

Figure 2. (A) Calculated electrostatic surface potentials of +36 GFP, colored from -25 kT/e (dark red) to +25 kT/e (dark blue). **(B)** Confocal fluorescence microscopy of live cells incubated with 100 nM +36 GFP–mCherry for 4 h at 37°C. Red color represents mCherry signal; green color represents +36 GFP signal. The scale bar is 15 μm. **(C)** Fluorescence microscopy of a retinal section of a CD1 adult mouse injected with 0.5 μl of 100 μM +36 GFP. The retina was harvested and analyzed 6 h after injection. GFP fluorescence is shown in green and DAPI nuclear stain is shown in blue. Lower right: retinal sections of neonatal RC::PFwe mouse pups harboring a nuclear LacZ reporter of Cre activity. Three days after injection of 0.5 μl of 40 μM +36 GFP–Cre, retinas were harvested, fixed and stained with X-gal. Dots on the graph represent the total number of recombined cells counted in each retina.
DAPI: 4′,6-Diamidino-2-phenylindole; GFP: Green fluorescent protein.
Adapted with permission from [36,37].

improving the nanoparticle-based protein delivery. Most importantly, the delivery of (-30)d GFP (non-fluorescent variant) fused Cas9–sgRNA complexes via RNAiMAx in U2OS-eGFP reporter cells showed loss of eGFP expression in 48% of cells along with a cleavage efficiency of 58% at the eGFP locus. Further, the delivery of Cas9–sgRNA was tested *in vivo*. RNAiMAx-mediated delivery of Cas9–sgRNA in the mouse cochlea showed loss in eGFP fluorescence by 13% in outer hair cells. Nevertheless, protein delivery using this method includes intramuscular injection into the inner ear of the mice, leading to functional protein delivery only near the injection site. This method looks promising. However, the efficiency of delivery is demonstrated only in the confined space of mouse inner ear. Hence, this delivery method is unlikely to work for systemic delivery, limiting its clinical potential. More recently, in another study [28], RNAiMAx was replaced by bioreducible lipid nanoparticles for CRISPR–Cas9 delivery and genome editing both *in vitro* and *in vivo*. The integration of bioreducible disulfide bonds into the hydrophobic tail of the lipid facilitates endosomal escape and cargo release in response to the reductive intracellular environment. In another liposome study, Xu and colleagues [29] have created a combinatorial design of cationic lipid-like materials, referred to as lipidoids, coupled with a reversible chemical protein engineering approach to deliver proteins into cells. Using lipidoid nanoparticles, EC16–1, the authors have demonstrated delivery of two representative cytotoxic proteins, RNase A and saporin, along with the cis-aconitic anhydride modified versions (RNase A-Aco and saporin-Aco) into cancer cells. Modification of protein with acid-labile and chemically reversible cis-aconitic anhydride increases the electrostatic binding of the protein with cationic lipidoids. When FITC-RNase A-Aco was used for tracking cellular uptake, punctate fluorescence was observed, demonstrating endosomal localization of the protein. Moreover, conjugation of the protein with cis-aconitic anhydride had only minor effect on its bioactivity.

Fusogenic liposomes

Fusogenic liposomes (FLs) are a separate group of liposomes that is worth mentioning. These FLs can fuse directly with the cell membrane and deliver cargo into the cell cytosol. Using FLs, recently, Csiszár and colleagues [30] have reported efficient cytosolic delivery of different size of proteins, such as, eGFP and R-phycoerythrin. Most importantly, here the charge of the proteins played a major role in delivery. Effective complex formation between positively charged FLs and negatively charged proteins is the key factor that drives the protein internalization in cell. With further study, FLs can emerge as a powerful vehicle for protein delivery [31] in future.

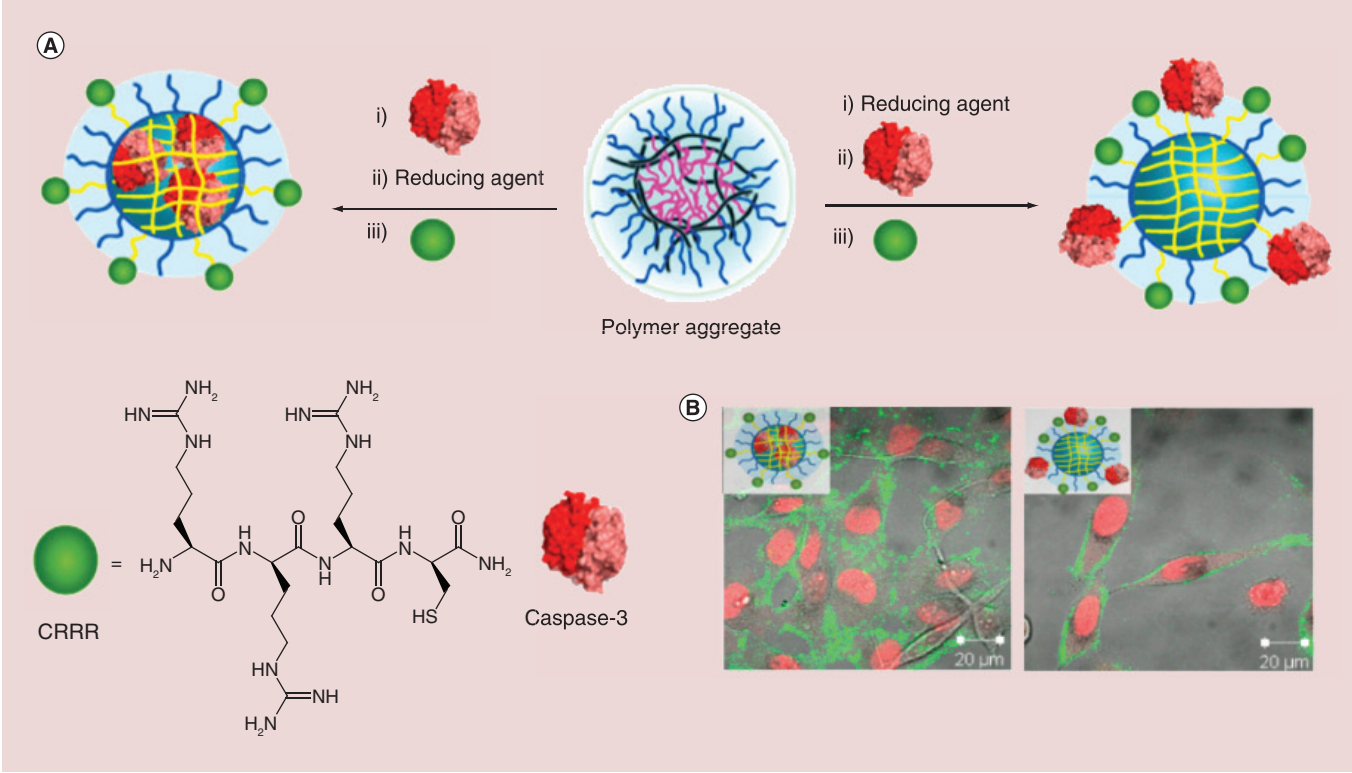

Figure 3. Preparation and delivery of nanogel-caspase-3 conjugates. (A) Preparation of nanogel–caspase-3 conjugates: covalent conjugation of caspase-3 in the interior or on the surface of polymeric redox-sensitive nanogels through disulfide linkages. **(B)** Cellular internalization: NG-FITC–Casp–InRRR and NG-FITC–Casp–OutRRR at 0.5 mg/ml on HeLa cells. The images are overlap of FITC channel (green; caspase-3), DRAQ5 channel (red; nucleus) and differential interference contrast. This experiment was performed with triplicate visualization on 1 day. One representative field is shown for each condition.
FITC: Fluorescein isothiocyanate.
Adapted with permission from [45].

Virus-like particles

Virus-like particles (VLPs) are self-assembling protein cages that are derived from a modified version of the viral genome. They contain viral capsid proteins that are devoid of genetic material and viral enzymes. VLPs are commonly used in vaccine development, including commercially used vaccines for human papilloma and hepatitis B viruses. Reiser and colleagues [32] have demonstrated efficient encapsulation of proteins like GFP into polyoma VLPs. However, delivery of proteins showed GFP mostly trapped in intracellular vesicular structures. Chatterjee and colleagues [33], on the other hand, have demonstrated the delivery of functional proteins into cells using VLPs derived from avian retrovirus. These VLPs were composed of Gag fusion proteins which were the anchoring proteins of VLPs and were fused with the target protein. This system described did not contain viral enzymes protease, reverse transcriptase and integrase, hence was not able to replicate in human cells. Moreover, the authors have demonstrated effective cytosolic delivery of proteins, such as, GFP, Cre recombinase, Caspase 8, etc. along with inducing appropriate signaling in cells by displaying protein ligands like TRAIL and INF-γ on the surface of the VLPs. Recently, Savithri and colleagues [34] have generated Sesbania mosaic virus loop B by self-assembling *Staphylococcus aureus* protein A to deliver multiple antibodies. The authors have demonstrated that this strategy can be used for targeting cellular as well as surface-exposed antigens. Additionally, there is no report of any toxic effect in cells.

Supercharged proteins

Supercharged proteins are a class of engineered proteins that can be fused with the target proteins for superior membrane penetration properties [35], providing an alternative to CPP 'tags' [11]. Liu and colleagues [36] have recently demonstrated a potent delivery vehicle for proteins using supercharged GFP (+36 GFP). These proteins are highly positive (theoretical net charge of +36 on the surface) in charge (Figure 2A) and can be uptaken by a variety of mammalian cells via nonspecific electrostatic interaction with sulfated proteoglycans present on the surface of the cells. Further, the authors fused different proteins, such as, mCherry, Cre

recombinase and ubiquitin to +36 GFP [37] and demonstrated that the proteins rapidly (within few minutes) entered different mammalian cells (Figure 2B). The efficiency of delivery was approximately 100-fold greater in mCherry and approximately tenfold greater in Cre recombinase than that of corresponding fusions with PTDs and CPPs. Additionally, ubiquitin-fused +36 GFP was observed to be partially deubiquitinated, suggesting cytosolic release of the protein. However, the cells were treated with chloroquine (an inhibitor of lysosomal protein degradation) to increase the cytosolar access of the protein, since the fusion protein was mostly trapped in endosomes. Moreover, for *in vivo* study, Cre fused with +36 GFP was injected intramuscularly and functional delivery was only observed at the injection site since most of the fusion protein precipitated (Figure 2C).

DNA nanoclews

DNA nanoclews (NCs) are a new class of nanocarriers that has been recently used to effectively deliver proteins, antibodies, enzymes and cytokines into cells. These are yarn-like DNA nanoparticles that are synthesized by rolling circle amplification with palindromic sequences for self-assembly of NPs. Recently, Gu and colleagues [38] have used NCs to deliver CRISPR–Cas9 into mammalian cells. Specific DNA NCs were designed complimentary to the CRISPR–sgRNA it will carry. This allows the CRISPR–Cas9 complex–sgRNA bound to a Cas9 protein – to loosely attach itself to the NCs. A coating of cationic polymer, polyethylenimine was applied after loading DNA NCs with Cas9–sgRNA complex in order to facilitate endosomal escape. These complexes once delivered were uptaken by the cell through lipid rafts and micropinocytosis. The activity of the delivered Cas9 was evaluated by indel formation through targeted DNA cleavage and repair by nonhomologous end joining pathway. The authors have reported an indel (insertion-deletion) of 28% for the cells treated with DNA NCs loaded with Cas9–sgRNA complex. Intratumoral injection of DNA NCs loaded with Cas9–sgRNA complex in U2OS-eGFP tumor-bearing mice showed no eGFP expression locally, suggesting local delivery of protein and its functional activity at the injection site only. In another study, the same group has reported tumor microenvironment responsive cell membrane targeted delivery of cytokine, TRAIL, using NCs [39]. Here, the authors have used phospholipase A2 degradable liposome as a shell and NCs decorated with TRAIL as the core. After phospholipase A2 activation, TRAIL was targeted to cancer cell receptors amplifying apoptotic signal with reduced TRAIL internalization.

Polymer

Polymers are macromolecules that can be biocompatible [40], designed for controlled release [41] and provide multivalences to interact to complementary moieties on proteins. Polymeric nanoparticles [42] and nanogels [43] have been reported to achieve intracellular delivery of drugs and biomolecules. A representative example is reported by Tang and colleagues [44]. In this work, authors presented a degradable polymeric nanocapsule for the delivery of recombinant maltose-binding protein fused apoptin. Here the recombinant apoptin was reversibly encapsulated in a positively charged water soluble polymer shell, and was released in its native form in response to reducing conditions. Confocal images reported the presence of rhodamine-labeled protein in early and late endosomes. The authors also claimed nuclear trafficking of the protein due to the colocalization of rhodamine-labeled apoptin with DAPI. However, no diffusion of rhodamine fluorescence was observed inside the nuclei, suggesting further investigation to be done to prove nuclear translocation of the protein. More recently, Thayumanavan and colleagues [45] reported a self-assembly strategy

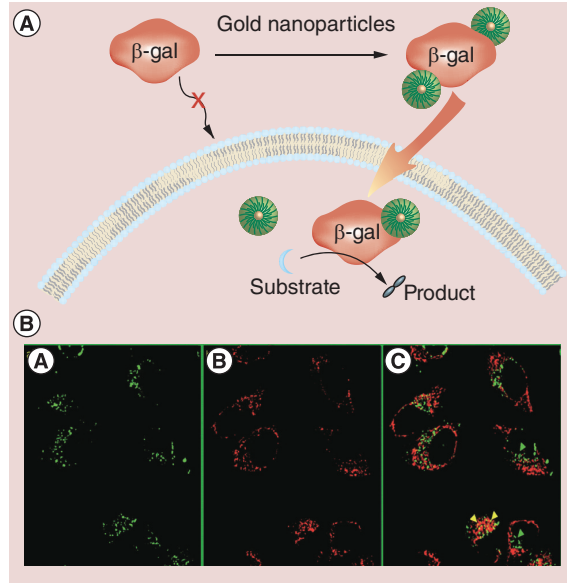

Figure 4. Delivery of large anionic protein β-gal (473 kDa) with gold nanoparticles into cells. (A) Intracellular delivery of functional protein using gold nanoparticles. **(B)** Colocalization study using confocal microscopy after protein transfection (NP_Pep/FITC-gal: 100 nM/50 nM) of HeLa cells in 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).
Adapted with permission from [59].

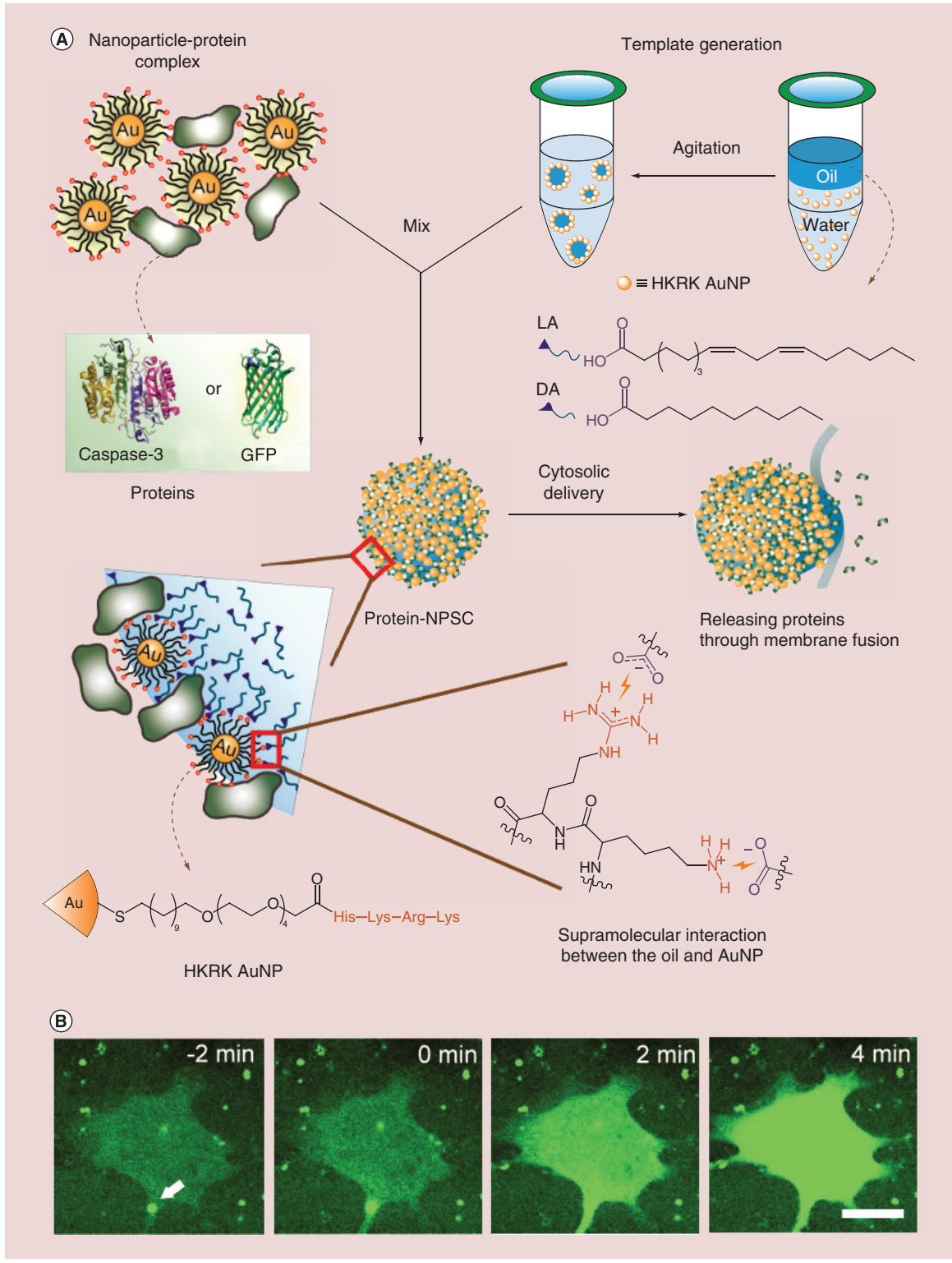

Figure 5. Intracellular protein delivery by nanoparticle-stabilized nanocapsules. (A) Schematic showing the preparation of the protein NPSC complex containing caspase-3 or GFP and proposed delivery mechanism. **(B)** Live cell imaging of rapid GFP release into the cytosol of HeLa cell by NPSCs. Scale bar: 20 μm. AuNP: Gold nanoparticle; GFP: Green fluorescent protein; NPSC: Nanoparticle-stabilized nanocapsule. Adapted with permission from [60].

to conjugate active enzymes, such as, caspase-3, to polymeric nanogels. In this system, the enzyme was conjugated on the surface or encapsulated inside the polymeric redox-sensitive nanogel through disulfide linkages as shown in Figure 3A. These nanogels were then conjugated with the CPP, cysteine-containing tri-arginine peptide (CRRR). Authors reported that in both cases the activity of caspase-3 was turned off when conjugated with the nanogel, and it was reverted back in the reducing environment of the cytosol. Additionally, the authors have reported a receptor-mediated endocytic uptake of the CRRR-modified nanogels in the cells. This report has been supported by confocal images (Figure 3B) that showed punctate fluorescence of the proteins, suggesting an endosomal entrapment of the same [46]. However, confocal images did not show endosomal escape of the protein in the cell cytosol, which is one of the major criteria for the efficiency of the protein delivery.

Carbon nanotubes

Water soluble and biocompatible functionalized carbon nanotubes have been widely used in various delivery applications. Dai and colleagues [47] reported the internalization of fluorescently labeled single-walled carbon nanotubes (SWCNT) and SWCNT–biotin–streptavidin conjugates into human promyelocytic leukemia cells (HL60) and human T cells (Jurkat). Fluorescently labeled SWCNT themselves exhibited slight toxicity to HL60 cells while SWNT–biotin–streptavidin conjugates showed a dose-dependent cytotoxicity due to the delivery of streptavidin into cells. The nanotubes were internalized via an endocytic pathway, as demonstrated by the colocalization of the green fluorescence of SWCNT conjugates with red-stained endosomes. The endocytosis pathway for the internalization of SWCNT conjugates was mainly clathrin dependent rather than caveolae- or lipid rafts mediated. However, cytosolic release of protein was not observed. Recently CNTs have been used frequently for protein delivery purpose [48,49]. In particular, Li *et al.* [50] have exhibited a CNT-mediated system for delivery and activation of proteins inside cells by near-infrared (NIR) light irradiation. Here, eGFP was conjugated to SWCNTs via a streptavidin–desthiobiotin linkage, where the protein activity was blocked. NIR irradiation cleaved eGFP–SWCNT linkage releasing eGFP inside the cells. Interestingly, eGFP was taken up by cells most likely through endosomes, while, after NIR irradiation, the protein localized into the nuclei due

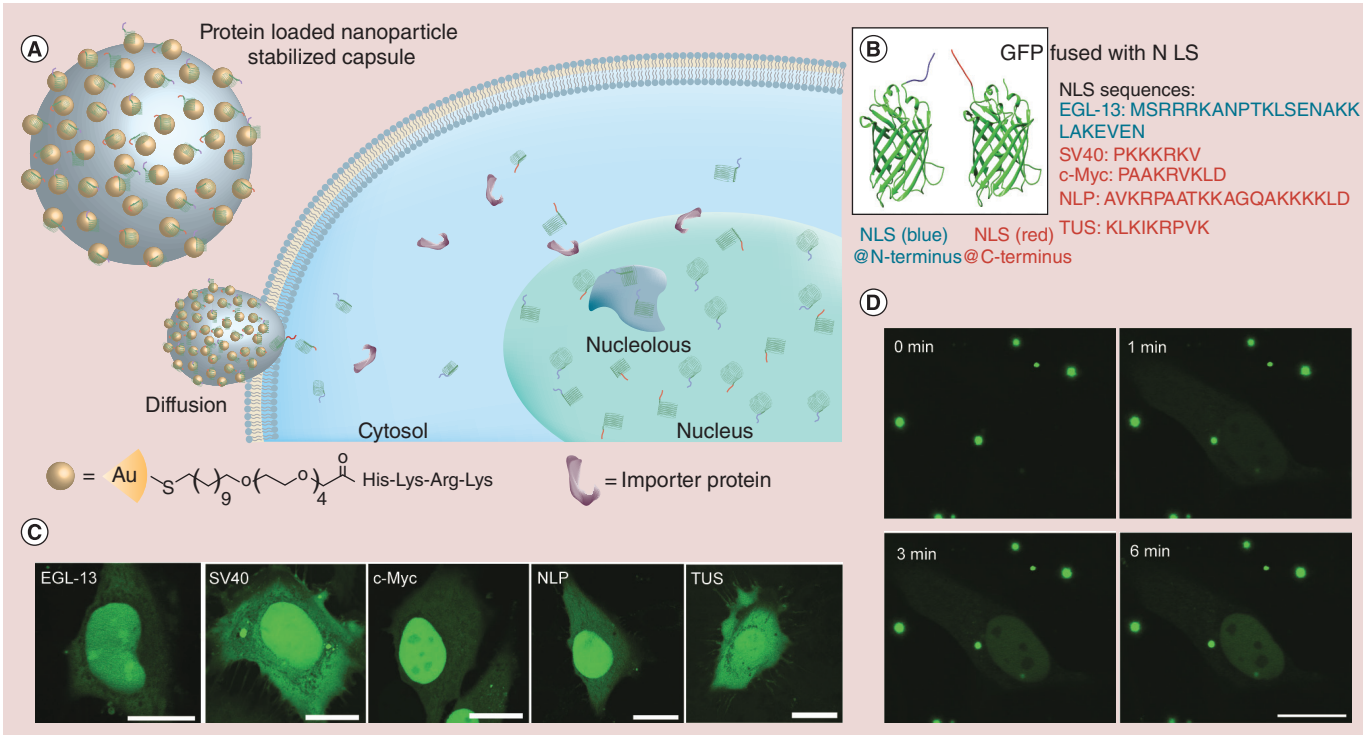

Figure 6. Delivery of eGFP fused with nuclear localization signals to cells using nanoparticle-stabilized nanocapsules. (A) Schematic representing the cytosolic delivery and nuclear accumulation of proteins with NLSs. **(B)** Structure of enhanced (e)GFP fused with NLSs. **(C)** LSCM images showing different cellular distribution patterns of eGFP fused with NLSs. Bars: 20 μm. **(D)** Time-lapse LSCM images unveil the kinetics of nuclear import of $NLS^{c\text{-}Myc}$–eGFP.
GFP: Green fluorescent protein; LSCM: Laser scanning confocal microscope; NLS: Nuclear localization signal.
Adapted with permission from [62].

to the nuclear localization signal attached to the protein. Apart from endocytic pathway of internalization, Pantarotto *et al.* [51] and Lu *et al.* [52] reported another uptake mechanism of SWCNT via insertion and diffusion of nanotubes directly into the cytosol through the lipid bilayer of cell membrane. Even though the present mechanism allowed direct cytosolic delivery of protein cargos, it was associated with thrombus formation by activation of store-operated calcium entry in human blood platelets as reported by Simak and colleagues [53] and highlighted by the US FDA [54].

Silica nanoparticles

Mesoporous silica nanoparticles (MSNs) are promising nanocarriers for the intracellular delivery of membrane-impermeable proteins [55]. The large pore volumes of MSNs allow ample loading of the protein. Additionally, these particles protect the protein molecules from proteases and denaturing chemicals by encapsulating proteins in their protective shell. Lin and colleagues [56] demonstrated intracellular delivery of cytochrome c using MSNs. Confocal fluorescence imaging of FITC–cytochrome c–MSNs exhibited punctate fluorescence, indicating internalized proteins being mostly trapped in endosomes. Recently, De Cola and colleagues [57] designed breakable hybrid organosilica nanocapsules where the protein cargo was encapsulated within a breakable hybrid shell comprising of disulfide bridges embedded in a silica network. These organosilica nanocapsules were used for the intracellular delivery of eGFP and highly cytotoxic protein, human TRAIL Apo2 ligand and Onconase into C6 glioma cells. After internalization, the active proteins were released breaking the shell of the capsule. However, the delivery of eGFP–nanocapsule conjugate evidenced eGFP fluorescence mostly colocalized with lysosomes, indicating only a small portion of eGFP being released into the cytosol.

Gold nanoparticles

Gold nanoparticles (NPs) have been widely used for delivery applications. The core size of the NPs can be tuned to commensurate with the protein size [58]. Moreover, the surface of the NPs can also be tailored with appropriate ligands to provide versatility to NP–protein interaction. Rotello and colleagues [59] have reported delivery of β-gal using NPs as carrier in different cell lines (Figure 4). NPs with core size of 2.5 nm were used for this study. These NPs were functionalized with cationic ligands terminated with a cationic

Table 1. Summary of the characteristics of different nanocarriers described.

Nanocarrier	Mechanism of uptake	Advantages	Limitations	Therapeutic potential
Liposomes	Endocytosis	Protein stability	Endosomal entrapment	Topical delivery
Fusogenic liposomes	Membrane fusion	Efficient cytosolic delivery	Only for charged proteins	Used for cancer immunotherapy
Virus-like particles	Endocytosis	No toxicity	Tedious manufacturing procedure	Tested *in vitro*
Supercharged proteins	Endocytosis	Efficient uptake due to high charge of the protein	Endosomal entrapment	Topical delivery
DNA nanoclews	Endocytosis	Protein stability	Limited delivery and functional activity *in vivo*	Topical delivery
Polymer	Endocytosis	Biocompatible, controlled release	Endosomal entrapment	Tested *in vitro*
Carbon nanotubes	Endocytosis/ membrane fusion	Low toxicity/cytosolic delivery	Endosomal entrapment/thrombus formation	Tested *in vitro*
Silica nanoparticles	Endocytosis	Efficient loading, protein stability	Endosomal entrapment, low release in cytosol	Tested *in vitro*
Gold nanoparticles	Endocytosis	Versatility, retained enzymatic activity	Endosomal entrapment	Tested *in vitro*
Nanoparticle-stabilized nanocapsules	Membrane fusion	Rapid cytosolic delivery and intracellular targeting	Efficiency *in vivo* to be demonstrated	Yet to be tested *in vivo* Delivered in cancer cells

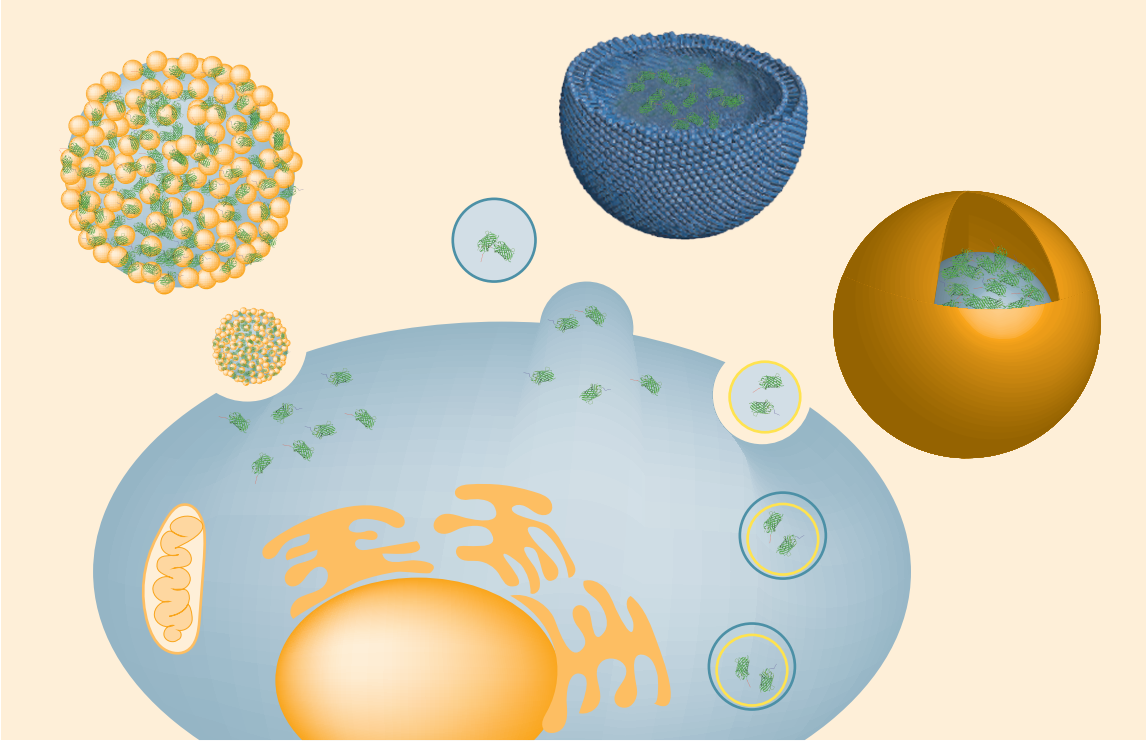

Figure 7. Schematic representation of intracellular delivery of proteins via nanocarriers.

peptide (His-Lys-Arg-Lys, HKRK). NP–HKRK when complexed with FITC–β-gal in a molar ratio of 2:1, β-gal successfully penetrated the plasma membrane and got delivered into the mammalian cells. Confocal images of the delivery showed punctate fluorescence, however, the fluorescence of the protein did not colocalize with the endosomal tracking agent. This suggests that the protein was released from endosomes, but was otherwise sequestered. Significantly, the enzymatic activity of the protein was retained in the cells.

Nanoparticle-stabilized nanocapsules

As mentioned in the beginning, direct transport of the protein through the cell membrane and rapid cytosolic release of the protein are the keys for efficient protein delivery. In the recent years, Rotello and colleagues [60,61] have developed an efficient delivery system, nanoparticle-stabilized nanocapsules (NPSCs) that can effectively and rapidly deliver the payloads into the cytosol (Figure 5). These NPSCs consist of an oil core (a mixture of linoleic acid and decanoic acid) that directly fuses with the cell membrane via hydrophobic interaction while delivering proteins. The NPSCs are stabilized by hydrogen bonding and electrostatic interaction between guanidinium moieties of the NPs and carboxylate groups on the oil core. Anionic proteins also provide lateral stabilization to the NPSCs via electrostatic interaction with the cationic NPs. Using these NPSCs, the Rotello group has demonstrated efficient intracellular delivery of GFP. In contrast to the delivery systems mentioned above, the NPSC-based delivery method was capable of overcoming the major obstacle of endosomal entrapment and was able to release the payloads uniformly in the cytosol. As a result, delivered proteins can be easily trafficked to the desired subcellular compartment where they are required for their activity. In addition, the authors have demonstrated the efficiency of this system in delivering functional therapeutic protein capase-3 in target cells to induce apoptosis.

Furthermore, the versatility of the NPSC-mediated delivery system was demonstrated by intracellular targeting of the protein to nucleus (Figure 6A). In this work, the authors [62] chose five different nuclear localization sequences: NLS^{EGL-13}, $NLS^{c\text{-}Myc}$, NLS^{NLP}, NLS^{SV40} and NLS^{TUS} and fused them with eGFP for monitoring nuclear protein trafficking and comparing targeting efficiencies of these NLSs (Figure 6B). NPSCs containing NLS-fused GFPs when delivered into mammalian cells distributed throughout the cell with obvious accumulation in the nucleus (Figure 6C). Quantitative comparison of the nuclear accumulation demonstrated 160% increase in nuclear intensity of $NLS^{c\text{-}Myc}$–eGFP compared with that in the cytosol. Further, the authors studied the import dynamics of $NLS^{c\text{-}Myc}$–eGFP in the nucleus. Results showed that the protein substantially accumulated in the nucleus within 60 s of cytosolic delivery and reached an

equilibrium in 6 min (Figure 6D). Therefore, NPSCs proved to be efficient *in vitro* delivery vehicle for cytosolic delivery as well as intracellular targeting. However, their application *in vivo* is yet to be demonstrated.

Future perspective

Protein delivery into cells is a potentially powerful strategy for the development of new therapeutics since it can replace poorly expressed or dysfunctional proteins, minimizing off-target effects. However, efficient delivery of protein is essential to achieve this goal. First and foremost, we need to focus more on what happens once proteins are inside the cell. Improved methods for endosomal escape as well as mechanisms that avoid endocytosis altogether provide the promise of considerable improvement beyond current vehicles. Thinking further ahead, efficient access to the cytosol will enable intracellular targeting, providing another strategy that will further increase the efficacy of protein-based therapy.

Financial & competing interests disclosure

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Executive summary

- Intracellular protein delivery is an important strategy for protein therapy replacing missing, dysfunctional or poorly expressed proteins in cells.
- Two major requirements for protein delivery:
  - Efficient uptake of protein by the cell;
  - Rapid cytosolic delivery of the protein without being trapped in the endosomes.
- Proteins fused with protein transduction domains/cell-penetrating peptides is the most common approach for protein delivery. Nevertheless, this approach can have efficiency and toxicity concerns.
- Nanocarrier-based protein delivery approaches are attractive due to the tunable chemical properties of the carrier.
- Nanocarriers, such as, liposomes, polymer, supercharged proteins, nanoparticles, carbon nanotubes, etc. have been widely used for intracellular protein delivery. Most of these delivery systems have demonstrated intracellular delivery of proteins. However, it seems that, in general, these nanocarriers have not completely solved the problem of the endosomal scape.
- Nanoparticle-stabilized nanocapsules, on the other hand, have exhibited efficient and rapid release of protein in the cytosol via membrane fusion mechanism, avoiding any vesicular entrapment. Additionally, nanoparticle-stabilized capsules have efficiently delivered proteins to subcellular organelles, such as, nucleus and peroxisomes.
- Protein delivery has emerged as a powerful strategy for development of new therapeutics. However, more effort should be given in developing strategy for efficient cytosolic access of the protein in order to increase the efficacy of protein-based drugs.

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• **Comprehensive review on intracellular protein delivery. This is a tutorial review that talks about methodologies developed for intracellular delivery of proteins using nanocarriers.**

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