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REVIEW

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Nanocarriers for intracellular delivery of proteins in biomedical applications: strategies and recent advances

Chuanda Zhu[1], Jing Mu[2*] and Ling Liang[1*]

Abstract

Protein drugs are of great importance in maintaining the normal functioning of living organisms. Indeed, they have been instrumental in combating tumors and genetic diseases for decades. Among these pharmaceutical agents, those that target intracellular components necessitate the use of therapeutic proteins to exert their effects within the targeted cells. However, the use of protein drugs is limited by their short half-life and potential adverse effects in the physiological environment. The advent of nanoparticles offers a promising avenue for prolonging the half-life of protein drugs. This is achieved by encapsulating proteins, thereby safeguarding their biological activity and ensuring precise delivery into cells. This nanomaterial-based intracellular protein drug delivery system mitigates the rapid hydrolysis and unwarranted diffusion of proteins, thereby minimizing potential side effects and circumventing the limitations inherent in traditional techniques like electroporation. This review examines established protein drug delivery systems, including those based on polymers, liposomes, and protein nanoparticles. We delve into the operational principles and transport mechanisms of nanocarriers, discussing the various considerations essential for designing cutting-edge delivery platforms. Additionally, we investigate innovative designs and applications of traditional cytosolic protein delivery systems in medical research and clinical practice, particularly in areas like tumor treatment, gene editing and fluorescence imaging. This review sheds light on the current restrictions of protein delivery systems and anticipates future research avenues, aiming to foster the continued advancement in this field.

Keywords Cytosolic protein delivery, Nanomaterial, Anti-tumor therapy, Gene editing, Fluorescence imaging

Introduction

The significance of intracellular protein delivery technology in the medical field has been widely acknowledged [1–3]. The advancement of microinjection and electro-transfection technologies has been made possible by continuous research and innovation, enabling the effective delivery of substances such as antibodies and active proteins in vitro [4, 5]. While microinjection technology enables direct injection of biological agents into the cytoplasm, its processing efficiency is relatively low, and each operation is constrained to a single cell. To enhance processing efficiency, high-throughput

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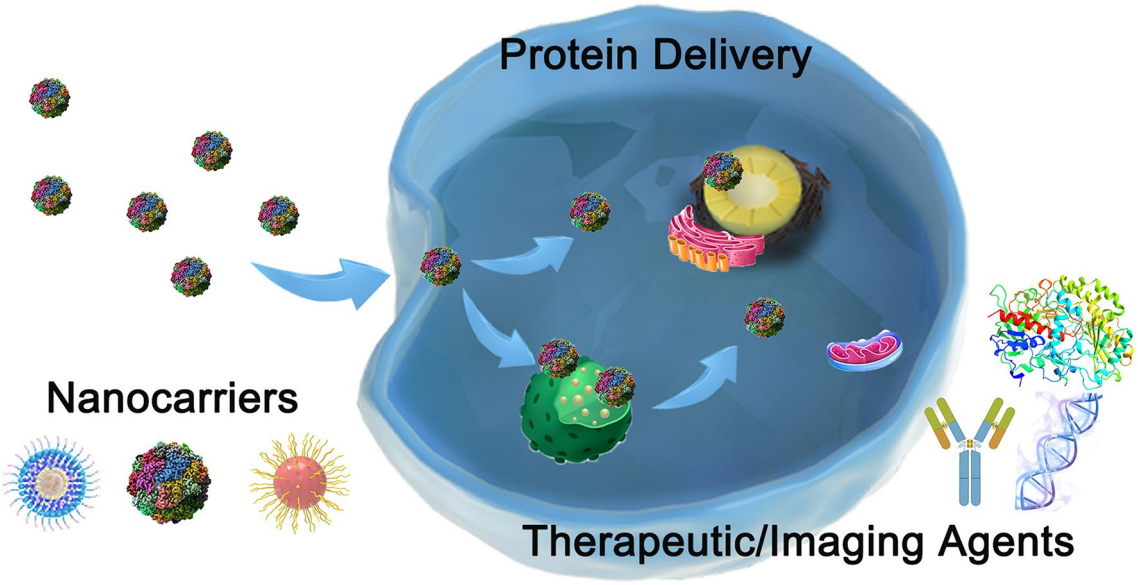
Graphical Abstract



protein delivery methodologies that can create transient pores in the cell membrane have been developed. These methods are primarily based on electroporation technology, hyperosmotic solutions, and mechanical force. However, these methods result in the destruction of the cell membrane integrity, thereby enabling the exchange of substances between the cytoplasm and the external medium. This may result in the toxic effects on the cells and interference with the normal cell functions [6]. Furthermore, these technologies necessitate the use of costly equipment and intricate operational conditions, which constrains their applicability in medical research and treatment, both in vivo and in vitro.

The advent of gene editing technology that relies on electroporation technology for protein delivery has facilitated the approval of the first gene editing therapeutic drug, Casgevy, which can be used to treat transfusion-dependent beta-thalassemia (TDT) and sickle cell disease (SCD) [7, 8]. Nevertheless, this therapeutic approach necessitates the initial acquisition of $CD34^+$ hematopoietic stem cells and progenitor cells from healthy donors, followed by the introduction of a CRISPR-Cas9 gene editing system that is specifically designed to target the BCL11A enhancer through electroporation. Notwithstanding its efficacy, the intricate treatment regimen, exorbitant treatment costs ($2.2 million), and concomitant adverse effects associated with infections and chemotherapy agents present considerable challenges [9]. Furthermore, the advancement of spatially controlled gene editing and intracellular antibody therapeutics requires sophisticated delivery platforms that enable precise protein targeting with enhanced tissue and cellular specificity [10, 11]. Therefore, innovative methods are needed to achieve sufficient interaction between protein drugs and intracellular targets without cytotoxicity [12, 13]. To address the challenges of intracellular protein delivery, various nanomaterials carriers have been developed, including polymers [14–16], lipid nanoparticles [17–19] and protein nanoparticles [8, 20]. These traditional small-molecule or nucleic acid delivery vehicles are encapsulated through physical interactions or chemical binding. Nanomaterial-based delivery systems can efficiently deliver proteins of different sizes and isoelectric points to different cells while maintaining their biological activity [21].To achieve cytoplasmic delivery, cell-penetrating peptides [22, 23] and phase-separated peptide [8, 20, 24] can be used to directly traverse the cell membrane, improving cellular entry efficiency. Biomimetic nano-carriers can protect biological macromolecules from degradation within endosomes and lysosomes, thereby improving the bioavailability of imaging agents or therapeutic agents [2, 25]. Nanomaterial-mediated cytoplasmic protein delivery has significant advantages, including protecting proteins from degradation, extending the half-life of protein drugs, responsive release, targeted delivery, and improving drug efficacy. These advantages make nanomaterials have broad application prospects in the field of drug delivery. Compared to traditional delivery methods such as electroporation, nanomaterial-mediated cytoplasmic protein delivery offers a safe, efficient, and high-uptake alternative, driving the development of intracellular target-related drugs in research.

This review provides a comprehensive and in-depth overview of the latest advancements in nano-delivery systems within the field of protein drugs. With the continuous innovation of technology, various delivery systems have emerged, designed to meet the needs of different application scenarios. Among the numerous delivery strategies, polymers, liposomes, and protein nanoparticles have become focal points of attention due to their unique advantages and broad application prospects. We have discussed in detail the specific applications of liposome-based protein delivery systems in preclinical studies such as antitumor treatment and fluorescent imaging. Additionally, we have conducted an in-depth analysis of the design principles and practical applications of protein nanoparticle and polymer nanoparticle delivery systems. Finally, we have comprehensively summarized the key factors that affect protein delivery efficiency and provided an outlook for the future development of cytoplasmic delivery systems (Fig. 1). We hope that future researchers can continue to leverage classic nanomaterials to develop new technologies for precise and personalized cytoplasmic protein delivery, continuously driving the development of novel protein drugs.

Nanoparticle-mediated protein delivery

Liposome

The combination of liposomes and protein delivery represents a novel biotechnological approach with the objective of enhancing the stability and delivery efficiency of protein drugs. As a synthetic membrane-like structure, liposomes exhibit excellent biocompatibility and controllability, enabling close binding with proteins for targeted delivery and sustained release of proteins. The ways in which liposomes enter cells mainly include direct fusion with the plasma membrane, entry through endocytosis, and lipid exchange with the plasma membrane. These methods all rely on the interaction and charge matching between liposomes and the cell membrane [26, 27]. The binding modes of liposomes and proteins can be broadly classified into two categories: encapsulation and

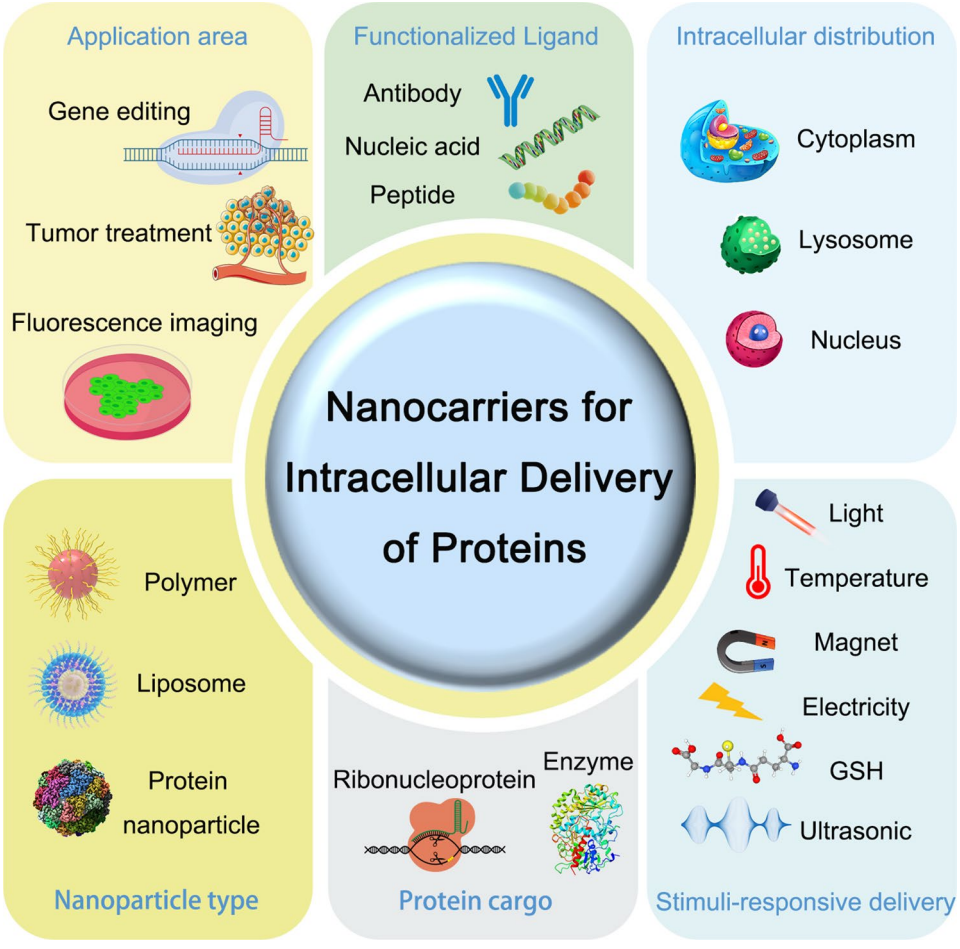


Fig. 1 Schematic illustration of various nanocarriers for cytosolic protein drug delivery in disease treatment. The diagram encompasses different types of nanocarriers, their diverse application fields, surface-modified ligands of nanocarriers, the intracellular distribution of nanocarriers, some of the protein drugs delivered by nanocarriers, and responsive delivery strategies

modification. In the context of encapsulation strategies, liposomes utilize their double-layer membrane structure to encapsulate proteins, thereby forming stable liposome-protein complexes. This strategy is particularly well-suited for the delivery of therapeutic proteins, including glucose oxidase, catalase, horseradish peroxidase, and superoxide dismutase [28–31]. Proteins such as enzymes are susceptible to various factors in the in vitro environment, leading to structural damage and loss of activity. As a protective barrier, liposomes can isolate the external environment from interfering with proteins, maintaining their stability and activity. At the same time, liposomes can also provide a suitable microenvironment that helps proteins release slowly in the body, extending their action time [32]. Liposomes can also be combined with polypeptide proteins through modification strategies. This strategy has wide applications in fields such as vaccine adjuvants [33–35]. Through specific chemical modifications, liposomes can form stable covalent or non-covalent bonds with polypeptide proteins to achieve efficient loading of proteins. As a carrier, liposomes can also target the delivery of polypeptide proteins to specific tissues and organs, improving the immune effect and safety of vaccine adjuvants [36].

Liposomes for enzyme delivery

Therapeutic enzymes have now become effective therapeutic drugs for many major diseases and play an important role in the treatment of congenital enzyme deficiency. For example, infantile neurofibromatosis, a lysosomal storage disorder characterized by the accumulation of metabolites in lysosomes due to the lack of ppt1, can lead to the formation of inclusions known as granular halophilic deposits [37, 38]. Enzyme replacement therapy (ERT) helps restore the blocked function of tissues or cells by supplementing these missing enzymes, thereby alleviating the disease. However, despite the potential efficacy of ERT, its translation into clinical applications has indeed been hindered by several factors. These obstacles may include enzyme source, stability, and immunogenicity [39].

Encapsulating enzyme-based proteins within liposomes addresses specific challenges, including enhancing their stability and mitigating immunogenicity. Santi et al. described the use of liposomal delivery of ppt1 enzyme for the treatment of infantile neurofibromatosis [37]. These liposomes containing enzymes can restore stable levels of enzyme activity in fibroblasts of CLN1 patients, promote the delivery of proteins to the central nervous system, and affect intracellular biological pathways. In addition, the delivery of catalytic enzymes, such as glucose oxidase, can help kill tumor cells (Fig. 2). In previous research, glucose oxidase was encapsulated and delivered into tumor cells by liposomes [32]. The manganese-based nanoprobes NanoMn-GOx-PTX are mainly composed of a manganese core and a phospholipid bilayer shell, which together carry glucose oxidase, paclitaxel, and fluorescent dye. The platform is capable of releasing manganese ions and payloads in a pH-dependent manner within tumor cells. Subsequently, glucose oxidase catalyzes the production of hydrogen peroxide from glucose, which is further catalyzed by manganese ions to produce reactive oxygen species. Combined with the antitumor effect of paclitaxel, it shows strong anti-tumor effects. In addition, recent research reports that Wang et al. developed a liposome-based enzyme nanoreactor, which cleverly encapsulates glucose oxidase GOx and horseradish peroxidase HRP together in the aqueous core of liposomes. Through encapsulation of liposomes, the two enzymes can work together in the same space, significantly improving the efficiency of the entire tandem reaction. GOx can effectively consume glucose in tumor cells and produce gluconic acid and hydrogen peroxide. The production of gluconic acid can reduce the pH value of the local environment and increase the concentration of hydrogen peroxide, which together promote the catalytic efficiency of HRP, resulting in the production of highly cytotoxic hydroxyl radicals ·OH, ultimately achieving the goal of killing tumor cells [17] .

Due to their unique catalytic function, enzymes can not only undergo color reactions with their substrates but also serve as tools for detecting enzyme activity [43–45]. Therefore, they are often used as model proteins to evaluate the efficiency of delivery tools. This process is usually achieved by observing fluorescence phenomena or detecting the activity of enzyme in the cytoplasm. For example, superoxide dismutase (SOD) has a special function of catalyzing the conversion process of anionic superoxide radicals in molecular oxygen and hydrogen peroxide. In the medical field, this enzyme is widely used, especially in the treatment of diseases caused by ROS, such as rheumatoid arthritis, various inflammatory diseases, and ischemia-reperfusion injury [46]. However, it is worth noting that direct administration of SOD without the assistance of a suitable delivery system faces many limitations. Its half-life in the blood is relatively short. This makes it difficult to effectively accumulate in damaged areas and is easily filtered by the kidneys [47]. In this regard, the presence of PEG on the surface of liposomes plays a crucial role [48]. It can significantly reduce the opsonization of liposomes by the mononuclear phagocytic system, thereby effectively promoting the delivery effect of SOD-loaded liposomes.

Nevertheless, despite the considerable promise of liposomes in protein delivery, the process of encapsulating proteins is not without shortcomings. A notable disadvantage is that this process may potentially impair the functionality of the proteins in question. Such damage

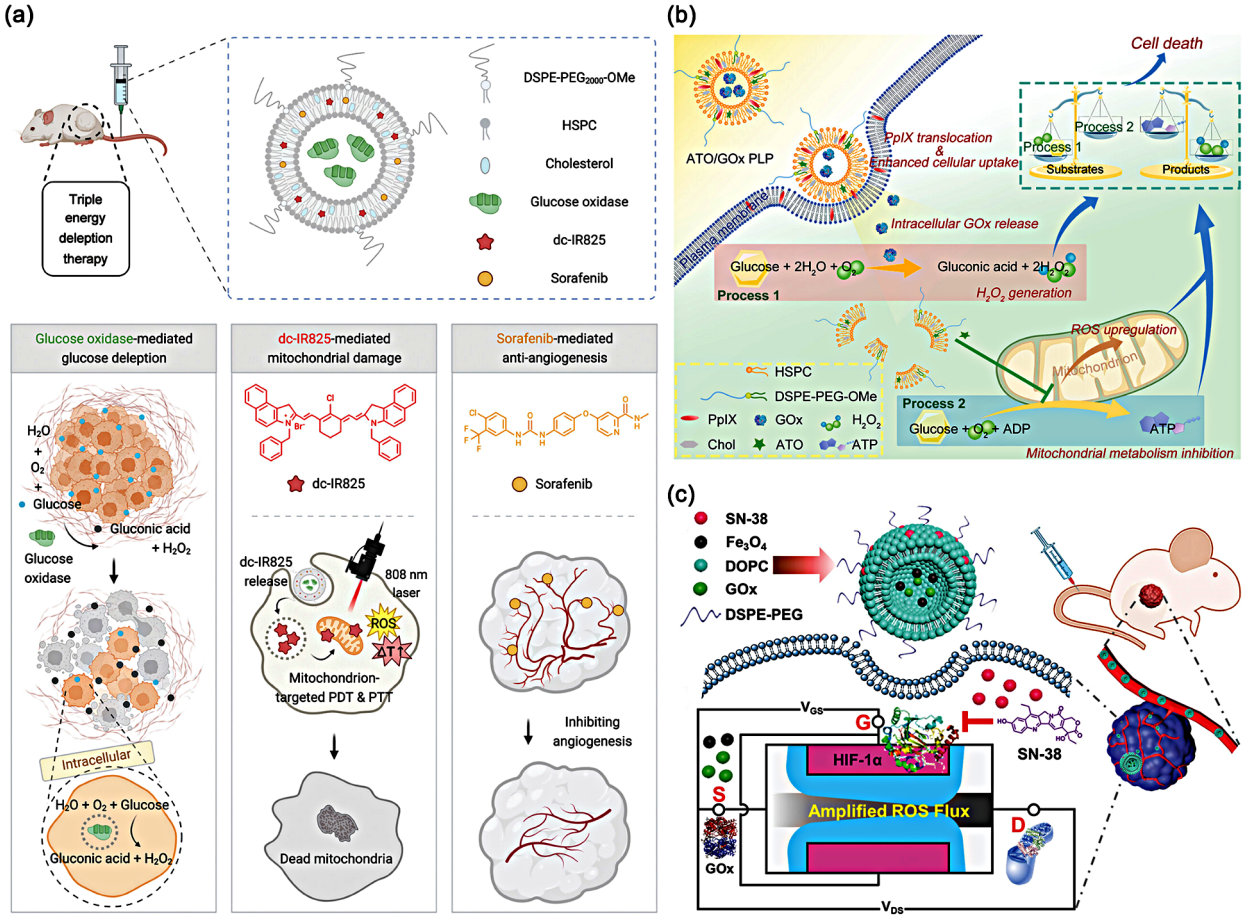


Fig. 2 Liposome-mediated delivery of GOx for antitumor therapy. (**a**) A diagrammatic representation of the GISLs' constituent parts and the anticancer mechanism exerted by GOx, dc-IR825, and sorafenib. Reproduced with permission from Ref [40]. (**b**) A schematic outlining the fabrication of the cell metabolism regulator ATO/GOx PLP and its utilization in cancer treatment through a PpIX translocation and cell respiration substrate redistribution mechanism. Reproduced with permission from Ref [41]. (**c**) A depiction of a liposomal delivery system (SN-38∩LP@Fe3O4/GOx) and a ROS field effect transistor for the enhancement of chemodynamic therapy. Reproduced with permission from Ref [42]

frequently arises from the intricate procedures involved in liposome preparation, whereby multiple steps may have a detrimental impact on the structure and functionality of proteins [49–51]. For instance, the pH value employed during liposome preparation represents a pivotal factor. The pH level exerts a direct influence on the self-assembly process of phospholipid molecules, which, in turn, affects the stability of the encapsulated proteins. Furthermore, the temperature of the solution is a significant factor that influences the activity of liposome-encapsulated proteins. It is imperative that the temperature of the solution be strictly controlled during the preparation of liposomes, as this will ensure that the phospholipid molecules can correctly self-assemble and encapsulate proteins.

Liposomes for Ribonucleoprotein (RNP) delivery

Clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) is a promising gene editing tool for treating diseases at the genetic level [52]. However, the challenge of safely and effectively delivering CRISPR/Cas9 to host cells limits its clinical application [53]. Compared to delivering genes, direct delivery of Cas9 RNP can immediately function without protein expression processes [7, 54–56]. The large size of Cas9 protein, approximately 160 kDa, prevents it from being directly delivered into cells. Liposome packaging technology can not only directly deliver Cas9 RNP into cells, but also partially protect it from degradation. The ability of liposomes to deliver intact Cas9 RNP represents a key advantage of this approach, ensuring effective and non-toxic gene editing.

Liposome-delivered gene editing protein complexes have been widely used in the study of various diseases (Fig. 3), such as gene editing in ophthalmology, otology, and dermatology [51, 54–56, 58, 59]. In the field of corneal diseases, Mirjalili Mohanna et al. tested a new LNP platform by providing pre-complexed RNPs and template DNA to cultured mouse cortical neurons, and achieved successful in vitro genome editing. Then,

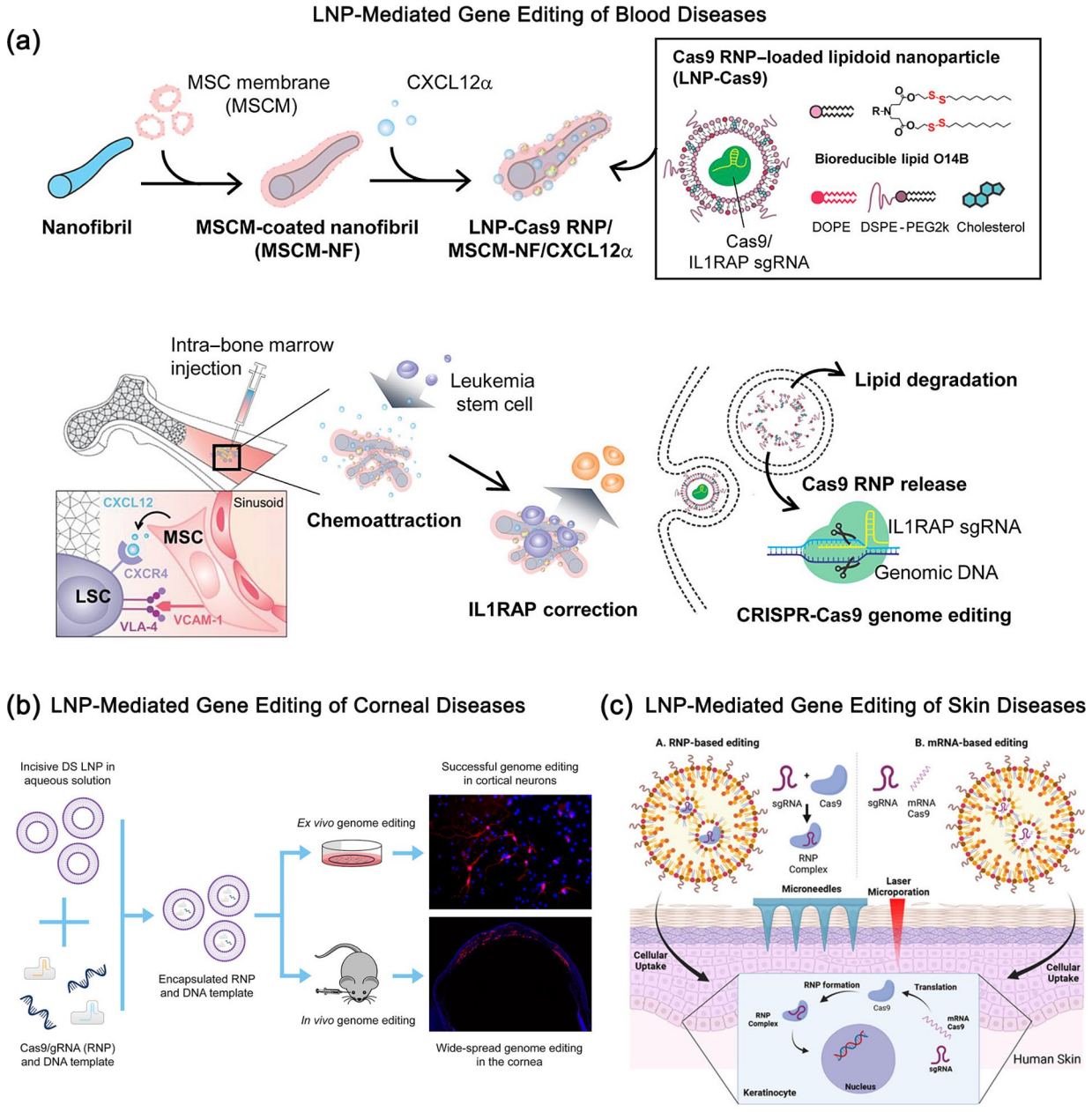


Fig. 3 Applications of Liposome-Facilitated Delivery of RNP in Diverse Diseases. (**a**) A lipid nanoparticle (LNP) encapsulated Cas9 RNP delivery system is presented. PCL nanofibrils (NFs), which mimic the bone tissue microenvironment, are coated with mesenchymal stem cell membrane (MSCM) and loaded with the CXCL12α cytokine along with LNP-encapsulated Cas9 RNP. This LNP-Cas9 RNP/MSCM-NF/CXCL12α complex can be injected into the bone marrow cavity to induce chemotaxis of leukemia blasts or leukemia stem cells (LSCs), enhancing gene editing cargo delivery efficacy. Reproduced with permission from Ref [57]. (**b**) CRISPR RNP delivery via LNPs enables widespread in vivo genome editing in the mouse cornea. Reproduced with permission from Ref [54]. (**c**) A lipid nanoparticle-mediated hit-and-run approach achieves efficient and safe in situ gene editing in human skin. Reproduced with permission from Ref [51]

the LNP-encapsulated RNP and DNA templates were directly injected into the mouse cornea to evaluate in vivo delivery. This study demonstrated extensive genome editing in the cornea using LNP-RNPs for the first time [54]. In the field of hearing disorders, Tao et al. found that the in vivo delivery of liposome-mediated CRISPR-Cas9 RNP complexes can lead to the specific editing of Obl alleles [59]. In vivo genome editing promotes the survival and restoration of function in outer hair cells, thereby restoring hearing. By lipid and AAV mediated delivery of Streptococcus pyogenes Cas9 (SpCas9) RNP complexes, researchers have improved hearing in mouse models of dominant hearing loss with hair cell origin by targeting the mutant allele of transmembrane channel-like 1 [59].

In the realm of skin diseases, Bolsoni et al. have investigated the potential of LNP to deliver gene editing tools into the living epidermis of human skin, enabling efficient in situ gene editing that could potentially cure rare monogenic skin diseases [51]. Therefore, the aforementioned studies have demonstrated the extensive applications and potential of liposome-delivered gene editing protein complexes in various disease areas.

To facilitate the clinical translation of liposome-based genome editing therapies, several novel liposome systems with responsive controlled release have been reported for the efficient delivery of CRISPR-Cas9 RNP into target cells [56, 60, 61]. Light-triggered liposome systems have been used to achieve temporal and spatial controlled release of CRISPR-Cas9 RNP [56]. By incorporating photosensitive molecules, such as Verteporfin (VP), into liposomes and exposing them to specific wavelength light, the liposomes undergo structural instability, leading to the controlled release of RNP for gene editing. For example, Aksoy et al. developed light-triggered liposomes that controllably release CRISPR-Cas9 ribonucleoprotein by incorporating the clinically used photosensitive molecule VP into the lipid bilayer and then rationally designing it. Under 690 nm wavelength light irradiation, VP reacts with available oxygen molecules and generates singlet oxygen, which rapidly oxidizes unsaturated lipid components and leads to structural instability of the liposomes and the release of ribonucleoprotein [56]. This regulatory mechanism restricts CRISPR-Cas9 activation to designated target sites, thereby achieving enhanced tissue- and cell-type specificity. Furthermore, Yan et al. reported a phosphorylated DNA-engineered liposome system capable of responding to stimuli to achieve cell-specific intracellular delivery and genome editing [61]. The liposome design mimics the viral fusion process, which can trigger membrane fusion under pH or UV stimulation to achieve cytoplasmic delivery of proteins. This strategy is highly efficient in delivering proteins of varying sizes and charges to target cells. In summary, these liposome systems offer innovative strategies for temporal and spatial control and cell-specific delivery of CRISPR-Cas9 RNP, thereby paving the way for safer and more effective genome editing therapies.

Liposomes for fluorescence imaging

Liposome-protein complexes, as efficient and precise tools, are widely applied in cell labeling and imaging techniques [43, 62]. By combining the excellent membrane fusion properties of liposomes with the specific recognition capabilities of proteins, scientists are able to accurately label and meticulously observe cells at the microscopic level. This complex not only exhibits high targeting ability, enabling precise localization to target cells or specific intracellular regions, but also produces excellent imaging results, clearly revealing the morphology, structure, and functional status of cells.

The use of liposome-protein complexes for cell labeling and imaging has enabled scientists to gain deeper insights into the biological processes occurring within cells. This encompasses a multitude of processes, including cell growth, division, metabolism, and signal transduction. Such understanding not only elucidates the enigmas of life but also furnishes a crucial theoretical foundation and practical guidance for the diagnosis and treatment of diseases. For instance, Li et al. have successfully developed a multi-faceted ultrasound molecular probe known as cell-penetrating peptide-modified 10-hydroxycamptothecin-loaded phase-transformation lipid nanoparticles, or simply iRGD-ICG-10-HCPT-PFP-NPs [63]. When combined with low-intensity focused ultrasound, liposome protein probe can be used for precise diagnosis and treatment of hepatocellular carcinoma. This probe exhibits excellent targeting ability, enabling ultrasound/photoacoustic (PA) dual-mode imaging. It can penetrate deeply into the tumor, achieving a better therapeutic effect, and thus provides new ideas and methods for the diagnosis and treatment of liver cancer. Three-dimensional optical microscopy plays a crucial role in understanding and optimizing the delivery of nanomedicines [64]. However, unfortunately, the process of tissue clearing often removes liposomes, preventing the technique from achieving three-dimensional imaging of liposomes within tissues. Fortunately, Professor Warren C. W. Chan designed a protein tag named REMNANT, which can not only attach to liposomes but also crosslink with tissues while remaining stable during the clearing process, thus enabling three-dimensional imaging of liposomes in intact tissues [25]. The REMNANT tag can also monitor the release rate of liposome contents in tissues in real time. This innovative method not only helps researchers observe the behavior of degradable materials in vivo, but also provides valuable guidance for the engineering design of imaging techniques and drug delivery vehicles.

Additionally, fluorescent proteins have been successfully utilized by researchers in combination with liposome delivery technology to target proteins, enabling clear observation of the absorption efficiency and subcellular localization of nanoparticles, providing new perspectives and tools for research in nanomedicine and drug delivery [65–67]. Yang et al. have reported the use of a high-throughput liposome screening strategy to successfully achieve intracellular delivery of OspF mediated by cationic liposomes [65]. This strategy effectively specifically inhibits the MAPK signaling pathway and tumor growth in cancer cells, as well as specifically regulates the immune response of macrophages. To further improve the encapsulation efficiency of lipid nanoparticles during intracellular delivery, researchers genetically fused

OspF with a negatively charged green fluorescent protein. This fusion promotes the self-assembly of cationic lipid nanoparticles through electrostatic interactions, and provides strong support for studying protein transport behavior at the cellular level. Overall, the application of liposome-protein complexes has not only deepened our understanding of liposome delivery mechanisms, but also opened up new prospects and potential applications for future drug delivery and disease treatment fields (Fig. 4).

Protein nanoparticles

In recent years, the cytosolic delivery of protein drugs through protein nanoparticles has emerged as a significant breakthrough in the field of biomedicine [68, 69]. This technology utilizes single or multiple protein molecules to form nanoparticles with nanoscale dimensions through self-assembly, enabling efficient cytosolic delivery. This section will focus on three innovative protein nanoparticle design methods. These methods not only demonstrate the potential of protein nanoparticles in the biomedical field, but also provide new perspectives and strategies for the development of cytosolic delivery technology. The following is a brief overview of these three design methods: The first is the design of protein nanoparticles based on phase-separated condensates. This method utilizes the interactions between different proteins and controls environmental conditions such as temperature, pH, or ionic strength to induce phase separation and subsequent formation of condensates [70, 71]. These condensates serve as drug carriers or imaging tools to perform specific functions within cells. A second innovative design approach is the use of computer-designed protein nanoparticles. The advancement of bioinformatics and computational biology has enabled the utilisation of computer simulations and algorithm optimisation in the design of protein nanoparticles with defined structures and functions. This method allows for precise control over the dimensions, configuration, and surface characteristics of nanoparticles, thereby enabling precise regulation of the drug delivery process. Moreover, computer-aided design can predict the interactions between nanoparticles and cells, thereby providing valuable insight for optimizing delivery efficiency and minimizing side effects. The third category of protein nanoparticles is based on cell-penetrating peptide nanocarriers. Cell-penetrating peptides are short peptides that possess the capacity to traverse cellular membranes. The combination of cell-penetrating peptides with proteins allows for the creation of peptide-modified nanocarriers, which can facilitate the transmembrane delivery of proteins in an efficient manner. These innovative design methods for protein nanoparticles possess unique characteristics, providing new perspectives and advanced tools for advancements in cytosolic protein delivery technology.

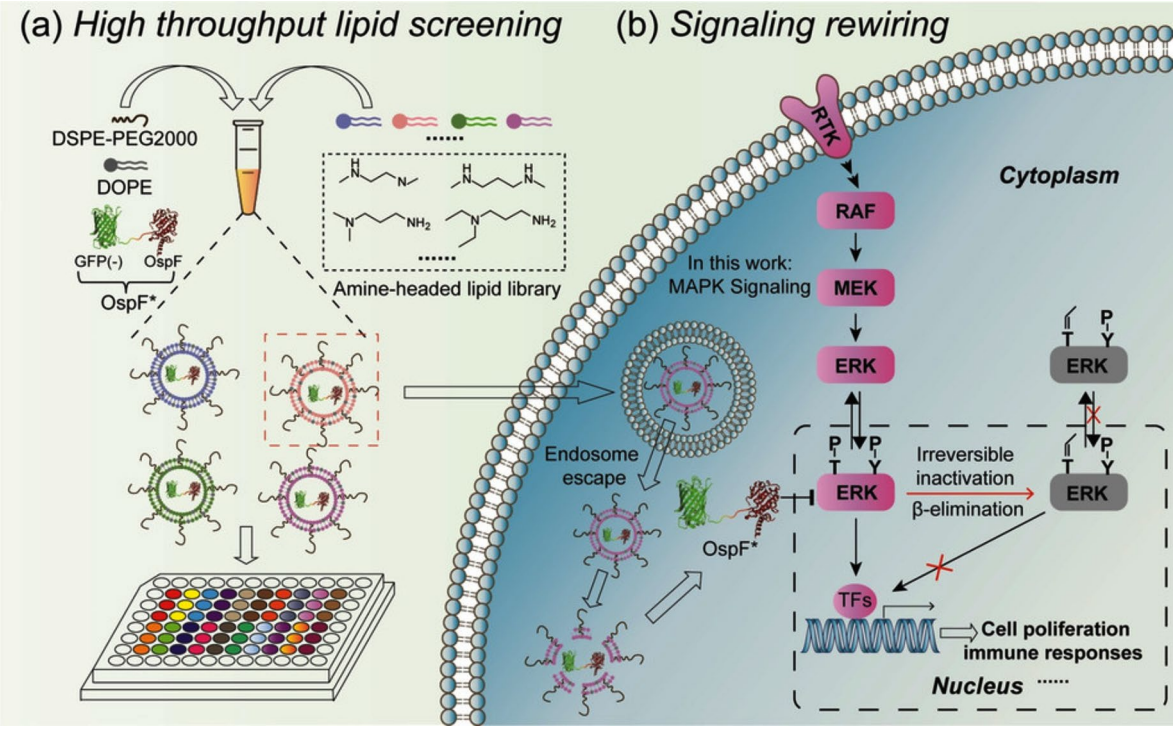


Fig. 4 Redirecting host cell signaling pathways through the utilization of bacterial effectors via cationic lipid-mediated intracellular protein delivery. Reproduced with permission from Ref [65]

Phase-separated protein self-assembly condensates

Phase-separated protein self-assembly condensates delivery is a cutting-edge biotechnology that utilizes the phase separation properties of proteins under specific conditions to form condensates through self-assembly, thereby achieving targeted delivery of proteins. Phase separation is a process where intracellular proteins or protein-RNA complexes spontaneously form distinct "phases," involving both attractive and repulsive interactions among proteins, as well as thermodynamic driving forces [72, 73]. Condensates form within cells typically through spontaneous assembly via interactions among biomacromolecules such as proteins and RNAs. These condensates are not meant to restrict molecular diffusion but rather to provide a locally high-concentration environment to facilitate specific intermolecular interactions. Within the condensates, interactions among molecules, such as electrostatic and hydrophobic interactions, cause them to tightly cluster together. However, this clustering is not completely enclosed but rather possesses a certain degree of permeability. Therefore, molecules can still diffuse to a certain extent within the condensates and between the condensates and the surrounding cytoplasm [74].

The condensates encapsulate proteins within their interior and deliver them to specific target locations through intracellular transport mechanisms [75]. The process of intracellular transport of phase-separated condensates may involve the following mechanisms: direct membrane translocation and entry into the cell through classical endocytosis. Direct membrane translocation refers to the mode of protein delivery within the cell that avoids capture by endosomes/lysosomes, directly fusing with the membrane to transport the cargo into the cytoplasm. For example, redox-responsive peptide(HBpep) coacervates and magnetically responsive peptide (DgHBP-2) coacervates [76, 77]. Phase-separated condensates can also be internalized by cells through energy-dependent endocytosis [78, 79].The classical endocytosis pathway primarily includes the macropinocytosis pathway, the clathrin-mediated endocytosis pathway, and the caveolin-mediated endocytosis pathway [80]. Some condensates are internalized through endocytosis to deliver coupled anticancer drugs to target cells. For example, Dittrich et al. developed Flutax-2 peptide nanoparticles functionalized with transferrin, which enter cells through clathrin-mediated TfR endocytosis [79]. After entering the cytoplasm, condensates can be transported by molecular motors attached to microtubules or microfilaments [81]. Furthermore, condensates in the cytoplasm may also be encapsulated within vesicles, enabling their transport inside and outside the cell through the fusion and separation processes of the vesicles with the cell membrane. For example, phase-separated YBX1 condensates recruit miRNAs and selectively sort them into exosomes [82]. Compared to other delivery vehicles, such as liposomes and polymeric nanoparticles, protein condensates form almost instantaneously, exhibiting negligible cytotoxicity from their peptide building blocks and eliminating the need for organic solvents that may reduce the biological activity of encapsulated molecules. This ensures both efficiency and safety in the delivery process.

Phase-separated protein peptides are capable of loading macromolecules, traversing cell membranes, and delivering their payloads within cells, thereby overcoming the major limitation of the difficulty in intracellular delivery of macromolecules (Fig. 5). Yu et al. reported a study on glucose-driven droplet formation in supramolecular peptide and therapeutic protein complexes. The study revealed that under specific conditions, these complexes can respond to glucose stimulation, leading to phase separation and droplet formation. This discovery not only provides a new perspective for understanding intermolecular interactions among biomolecules but also opens up new avenues for drug delivery and biological therapy [20]. In another study, Sun et al. developed short His-rich, pH-responsive beak peptide (HBpep) coacervates that bind to disulfide bonds containing self-sacrificial fragments (HBpep-SR), which can trigger the disintegration of droplets in a reducing environment, promoting the intracellular delivery of protein drugs [76]. HBpep condensates can cross cell membranes independently of endocytosis, demonstrating their potential for intracellular delivery of therapeutic agents. At low pH, histidine-rich HBpep exists as monomers, but rapidly phase separates into condensed microdroplets at neutral pH, during which process it can absorb and integrate various macromolecules from the solution. These examples fully demonstrate the versatility and practicality of phase-separated protein self-assembly condensates in the delivery of therapeutic proteins.

Currently, the technology of phase-separated protein self-assembly condensates for protein delivery has demonstrated potential application value in multiple fields, such as drug delivery, gene therapy, and cell regeneration [83, 84]. However, despite these advancements, there are some issues facing phase-separated protein self-assembly condensates, mainly centering on the stability, targeting, and systematic evaluation of the condensates. Compared to solid particles, the droplets formed by protein condensates pose certain challenges in terms of stability. Specifically, these droplets may be more susceptible to environmental factors during the delivery process, leading to their decomposition before reaching the target cells or tissues. This instability may reduce the therapeutic efficiency and even affect the overall outcome of the treatment. Future research needs to focus on precisely controlling the release kinetics of the embedded proteins and designing protein droplets with greater

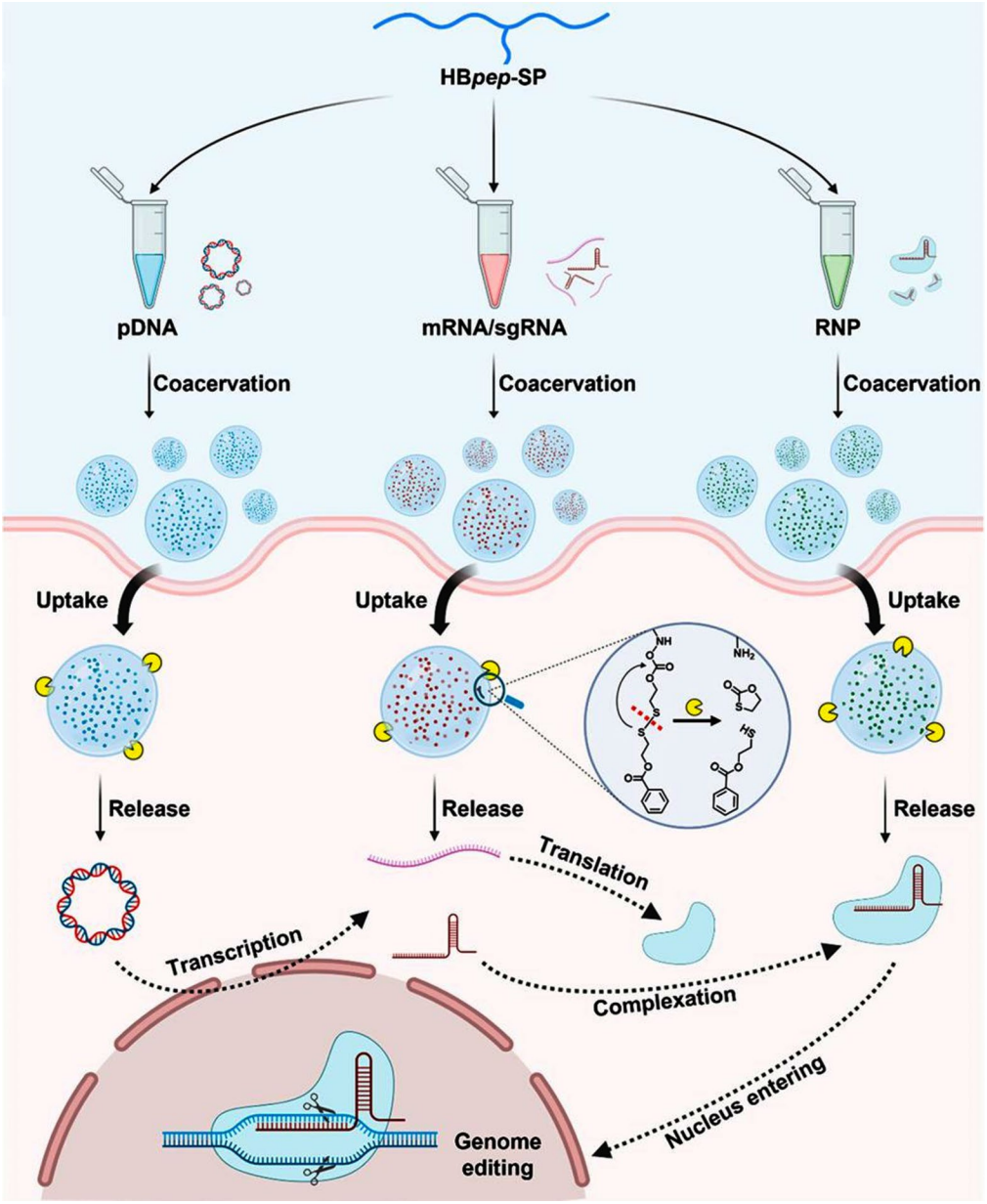


Fig. 5 Redox-Responsive Phase-Separating Peptide as a Universal Delivery Vehicle for CRISPR/Cas9 Genome Editing Machinery. Reproduced with permission from Ref [87].

cell and tumor specificity [85]. Researchers can also utilize biomarkers and other means to further optimize the targeting of protein condensates, achieving more precise therapeutic effects. Notably, there is still insufficient systematic research on protein condensates in vivo, and it is necessary to focus on evaluating their safety, effectiveness, and tissue distribution in the body [86]. With further in-depth research and continuous optimization of this technology, it is believed that it will play a more significant role in the field of biomedicine in the future.

Self-assembling protein nanoparticles

In the early 2000s, researchers began employing computational methods to design proteins with specific functions. Scientists employed sophisticated algorithms and simulation software to predict and optimize protein sequences in order to achieve the desired nanostructures. These simulations considered the interactions between protein molecules, folding, and assembly mechanisms to guarantee that the final designed nanoparticles exhibited optimal stability and functionality [88, 89]. Upon completion of the design, scientists could employ genetic engineering techniques to synthesize the proteins and induce

self-assembly into nanoparticles under suitable conditions. These nanoparticles can exhibit a variety of morphologies, including spherical, rod-like, and tubular, and can be customized according to specific requirements [90–93]. The prospective applications of computer-designed self-assembling protein nanoparticles are extensive, spanning fields such as biomedicine, drug delivery, and nanomaterials [94, 95]. For example, they can be utilized as drug carriers to facilitate the precise delivery of drugs to target tissues or cells, thereby enhancing therapeutic efficacy while reducing the incidence of adverse effects [96]. Additionally, these nanoparticles can also be employed in the construction of biosensors, tissue engineering scaffolds, and novel nanoelectronic devices [97]. These studies provide a crucial foundation for the development of novel biomedical applications (Fig. 6).

David Baker is an esteemed scientist and leader in the field of protein design, having made significant contributions to numerous areas of research, including protein folding prediction, protein-small molecule binding, self-assembling protein nanoparticles, and protein design [90–92, 94, 95, 98–100]. Recently, David Baker's team

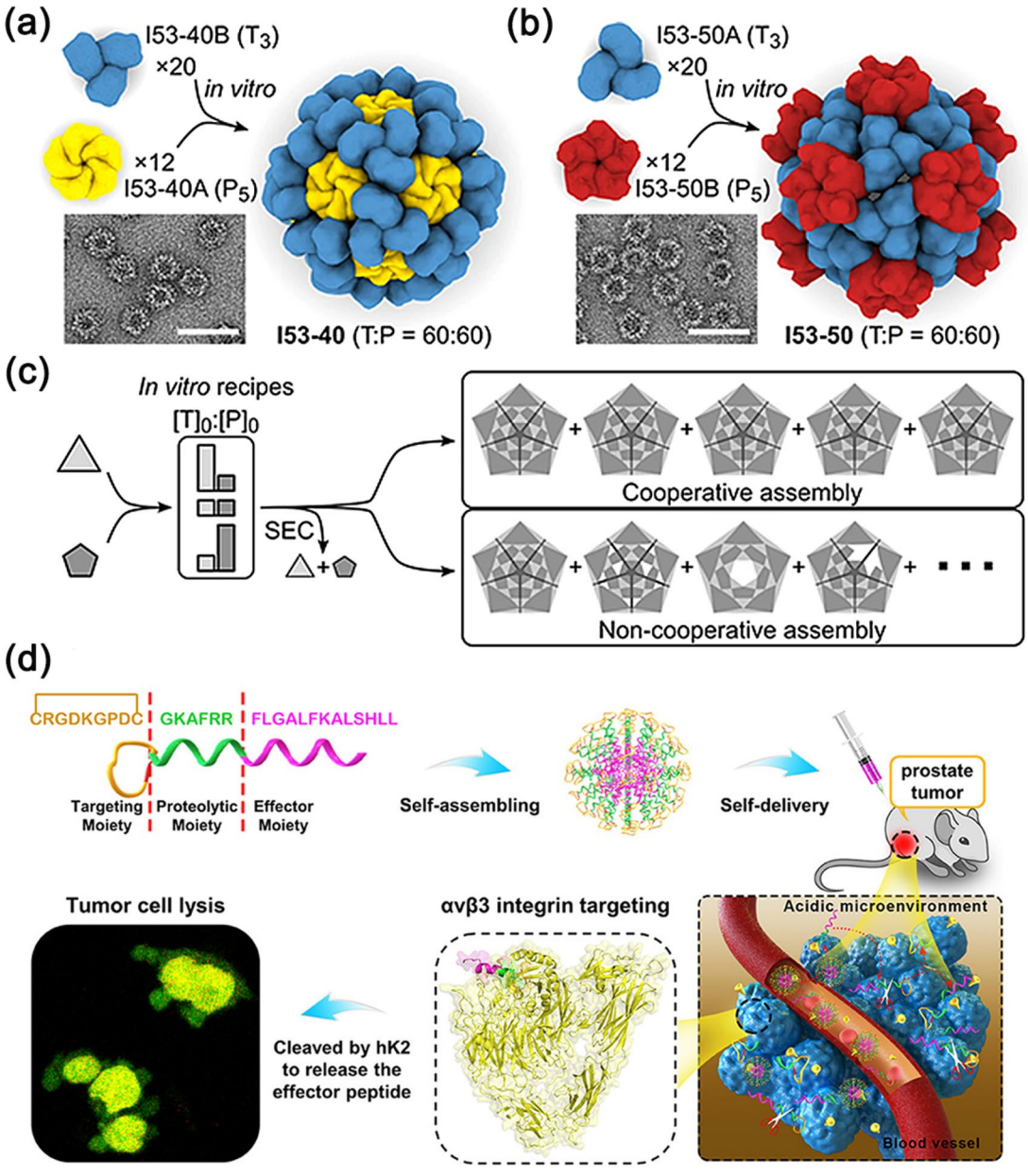


Fig. 6 Computer-designed self-assembly protein nanoparticles. (**a**-**c**) In vitro assembly analysis indicates cooperativity in most regimes. Reproduced with permission from Ref [101]. (**d**) Computer-Aided Design of Lasso-like Self-Assembling Anticancer Peptides with Multiple Functions for Targeted Self-Delivery and Cancer Treatments. Reproduced with permission from Ref [34]

has developed a reinforcement learning-based approach to protein structure design, which is known as the "top-down design method" [100]. This method is capable of designing complex protein nanomaterials with specific system properties, including: [1] Disk-shaped nanopores: Using the Monte Carlo Tree Search (MCTS) method, the researchers filled the space between two previously designed ring-shaped proteins, generating a disk-shaped structure with a central nanopore [2]. Ultra-compact icosahedra: Employing the MCTS method, the researchers created icosahedra with ultra-compact structures that do not exist in nature and are smaller and have lower porosity than known protein icosahedra. These icosahedra have the potential to display immunogenic and signaling molecules at densities that are significantly higher than those observed in naturally occurring structures. This could lead to enhanced vaccine responses and the induction of angiogenesis [3]. Icosahedra of fusion proteins: The researchers fused functional protein domains, such as the angiopoietin 1 F-domain, to the termini of icosahedra, generating bioactive nanoparticles. These particles have demonstrated potent effects in activating cell signaling pathways and vaccine applications. The top-down design method can address design challenges that are intractable with traditional methods and generate unique structures that do not exist in nature, enabling the design of complex protein nanomaterials with specific system properties. In conclusion, this study introduces novel concepts and methodologies to the field of protein design, while simultaneously paving the way for new avenues of inquiry in biomedical research and applications.

Past research methods include physical-based design methods like Rosetta, which require a large amount of computing resources and manual refinement by expert structural biologists. To simplify protein-protein interface design and make it applicable to a wide range of scientific problems, de Haas et al. used the deep learning method ProteinMPNN to design two-component tetrahedral protein nanomaterials [99]. These nanomaterials consist of two distinct trimer building blocks that are arranged on the three axes of tetrahedral symmetry, forming a structure known as "T33". The study designed protein nanomaterials capable of efficient self-assembly, which can assemble from independently purified components in vitro, crucial for biotechnological manufacturing. By using the deep learning method ProteinMPNN to design protein-protein interfaces, the researchers overcame the limitations of traditional physics-based Rosetta design methods in terms of computational efficiency and manual adjustment. Importantly, the interfaces designed by ProteinMPNN exhibit enhanced polarity, which facilitates seamless assembly of nanomaterials in vitro, crucial for efficient biotechnological manufacturing. Therefore, deep learning can enable more people to participate in protein interface design. The advanced AI technology will promote the development of the next generation of protein-based technologies faster.

Constructing protein nanomaterials often involves the docking and fusion of protein monomers or cyclic oligomers. However, due to the irregularity of protein structures, these methods often face numerous challenges and difficulties when attempting large-scale assembly based on simple geometric principles. Huddy et al. developed a simple and scalable protein nanomaterial design method to address the limitations of existing methods in terms of geometric regularity in protein assembly. This method achieves the regularity and scalability of protein nanomaterials through simple geometric rules [98]. They introduced a novel protein design method that simplifies the design of multi-component protein assemblies by using standardized protein building blocks and demonstrates how these building blocks can be used to design assemblies ranging from simple polygonal and circular oligomers to large polyhedral nanocages and unbounded linear "train track" assemblies, with adjustable sizes and geometric shapes that can be easily blueprinted. The technique of simplifying protein assembly design through the design of scalable protein building blocks provides new ideas for the design and construction of protein nanomaterials.

Computer-designed self-assembly protein nanoparticles represent a pioneering nanotechnology that integrates the tenets of computer simulation and protein engineering. This approach aims to fabricate nanostructures with defined shapes, dimensions, and functionalities. This technology enables scientists to achieve self-assembly processes at the nanoscale by precisely controlling the sequence and structure of proteins, thereby preparing nanomaterials with distinctive properties. Nevertheless, despite the considerable promise of computer-designed self-assembly protein nanoparticles, several challenges remain to be addressed, including enhancing production efficiency, optimizing stability, and ensuring biocompatibility. It is anticipated that, with the ongoing advancement of technology and further research, these challenges will be progressively addressed, thereby facilitating the exploration of new frontiers in the fields of nanomedicine and nanotechnology.

Cell penetrating peptide-modified nanocarriers

Cell-penetrating peptides (CPPs) are a class of short peptides capable of carrying macromolecules into cells. These peptides have received widespread attention due to their unique ability to penetrate cell membranes, particularly in the fields of drug delivery and biomedical applications [102]. Over the past five years, numerous CPPs have been developed and validated for their ability to effectively deliver bioactive molecules both in vitro

and in vivo. As of now, the CPPsite 2.0 database has publicly listed 1,855 distinct CPPs, which can be subdivided into three major categories based on their properties: amphipathic peptides, cationic peptides, and hydrophobic peptides [103]. CPP is often utilized as a means to modify nanocarriers, with the aim of enhancing their cellular uptake efficiency and transport capabilities [104, 105]. CPP can typically be attached to the surface of nanoparticles via electrostatic interactions or through the use of covalent coupling strategies (Fig. 7).

The delivery of CPP-modified nanocarriers across cells can be divided into two modes: direct translocation and endocytosis: [1] CPP-mediated membrane translocation: Some CPPs can be embedded in lipophilic cell membrane structures due to their unique amphipathic properties, which enables them to directly cross membrane barriers [107]; [2] CPP-mediated membrane pore formation: CPP temporarily disturbs the lipid bilayer of the cell membrane, causing minor damage to the local membrane structure. This transient membrane pore provides a passive diffusion pathway for CPP and its cargo molecules to cross the damaged cell membrane barrier [108]; [3] CPP-mediated endocytosis: CPP or its cargo-carrying complexes have also been shown to enter the interior of cells through an energy-dependent process called endocytosis [109]. This process encompasses multiple endocytic pathways, such as pinocytosis, caveolin-mediated endocytosis, and clathrin-mediated endocytosis, which are primarily responsible for cellular uptake of relatively large protein complexes [110].

In mucosal tissue areas such as the oral cavity and lungs, CPP-modified nanocarriers can significantly promote the interaction between the drug carrier and the mucus layer or epithelial tissue, thereby effectively prolonging their residence time at the target site [102, 109]. For example, Rehmani et al. introduced a novel nanocarrier modified with CPP, known as Glycosaminoglycan-binding-enhanced-transduction (GET), which functions as an efficient transepithelial delivery vector in vitro and facilitates oral insulin activity in diabetic animals [111]. By utilizing electrostatic interactions, insulin can be linked to GET to create nanocomplexes (Insulin GET-NCs). These nanocomplexes significantly boost insulin transport in vitro within differentiated intestinal epithelium models, demonstrating a more than 22-fold increase in translocation.

CPP-modified nanocarriers have also demonstrated remarkable potential in crossing the blood-brain barrier. Many anticancer drugs are hindered by the blood-brain barrier and find it difficult to penetrate into the brain [112, 113]. However, with its unique cell-penetrating ability, CPP-modified nanocarriers have opened up a new pathway for the treatment of brain diseases. For example, Barra et al. used liposomes functionalized with viral fusion peptide to evaluate the passage of a neuroprotective agent (pituitary adenylate cyclase-activating polypeptide) through a dynamic in vitro model of the blood–brain barrier [114]. Although CPP can be used to effectively deliver proteins into host cells, its instability in serum often confines it to degradation within endosomes

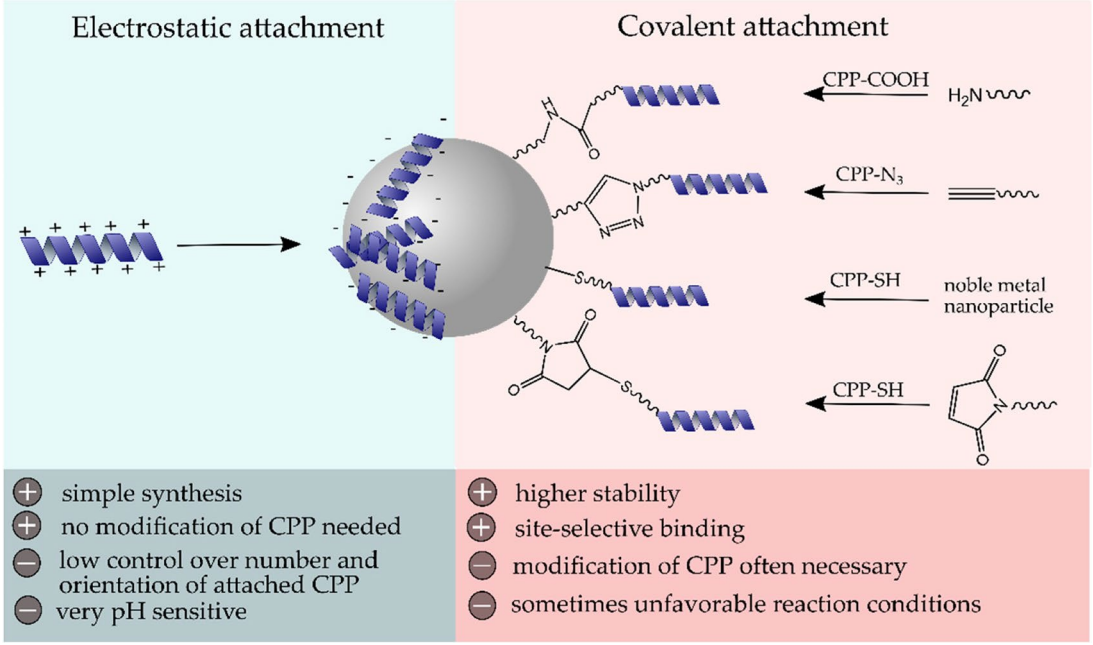


Fig. 7 This schematic illustrates the process of modifying the surface of nanoparticles using cell-penetrating peptides (CPP) through electrostatic or covalent coupling strategies, and outlines its main advantages and disadvantages. Reproduced with permission from Ref [106]

and lysosomes. Once transduced into cells, it can induce high levels of cytotoxicity, leading to suboptimal biological outcomes. To overcome this issue, the introduction of polypeptide sequences for lysosomal escape can improve the efficiency of cytosolic protein delivery [115]. Additionally, it is necessary to select appropriate CPP sequences, lengths, and modification methods, as well as to make adaptive adjustments based on specific cell types and external environmental conditions.

Polymers

Functional polymer-mediated biomacromolecular delivery occupies an important position in the field of protein delivery and has long been a focus of attention. Natural polymers such as chitosan [116], alginic acid [117], cyclodextrin [118], and chondroitin sulfate [119], as well as polymeric polymer materials such as PLGA [120]and PEI [121], can be used for protein delivery due to their good biocompatibility and biodegradability. A considerable number of excellent articles have been published introducing these materials, and they will not be elaborated in this review [122–124]. Polymer-protein complexes prepared through intermolecular interactions often exhibit instability and are prone to decomposition in buffered solutions, which is a critical challenge that needs to be overcome in this field. Compared with nucleic acids such as DNA and RNA, proteins have a relatively low net charge, which undoubtedly increases the difficulty of forming stable complexes with polymer carriers. To successfully prepare protein complexes with good stability, we urgently need to explore the effective application of cationic polymers. Grafting different functional ligands onto cationic polymers can significantly enhance the binding affinity between polymers and proteins and effectively reduce charge repulsion between cationic polymers during the formation of polymer/protein complexes. In this section, we will delve into the latest progress of polymers modified with different functional ligands in protein cytosolic delivery, aiming to provide useful references and inspirations for the future development of this field.

Fluorinated polymers

Fluorinated polymers, with their unique ionic interaction capabilities, stand out among various materials and are widely regarded by researchers as an ideal choice for binding to the anionic regions of proteins [125, 126]. These polymers can assemble synergistically through fluorophilic effects to form stable nanoformulations, a property that has extremely broad applications in the biomedical field. Such nanoformulations can not only protect proteins from external environmental influences but also effectively deliver them to target locations, providing new possibilities for the treatment of diseases. The clever introduction of fluorine chains into polymers confers dual hydrophobic and lipophobic properties to the polymers, enabling them to encapsulate proteins more precisely while maintaining their activity. According to relevant reports, the Cheng Yiyun research group was the first to reveal the "fluoroamphiphiles" phenomenon of fluorinated polymers in protein cytosolic delivery [125]. They successfully grafted different fluorinated small-molecule compounds onto polyethylenimine, establishing a rich library of fluorinated polymer materials. This discovery opened a new path for the application of fluorinated polymers in the biomedical field. To validate the effectiveness of fluoroamphiphiles, the research team further conducted extensive testing using model proteins with varying molecular weights and charge properties. The experimental results demonstrated that these fluorinated polymers can efficiently deliver unmodified proteins into cells while avoiding toxic damage to the cells. This characteristic makes fluorinated polymers highly promising for applications in the biopharmaceutical field.

Notably, the protein delivery efficiency of fluorinated polymers is closely related to the length and fluorination degree of the PFL chain. As the fluorination degree increases, the delivery efficiency also improves; however, for fluorinated polymers with longer PFLs, excessively high fluorination degrees can actually lead to a decrease in protein delivery efficiency. This finding provides important guidance for optimizing the design of fluorinated polymers. The hydrophobic properties of fluoroalkyl chains on polymers endow them with high membrane association affinity, which allows fluorinated polymers to bind more easily with cell membranes, thus achieving efficient protein delivery [127, 128]. The lipophobic property effectively prevents the amphiphilic polymers from fusing with phospholipids during endocytosis or direct membrane translocation, ensuring the precision and efficiency of the delivery process. Compared to non-fluorinated analogues combined with aliphatic lipids, fluoropolymers exhibit significant performance advantages, including high tissue permeability and effective cytoplasmic delivery capabilities [129, 130]. They can rapidly penetrate deep into tissues and accurately deliver drugs to target locations, thereby enhancing treatment effectiveness [131, 132] (Fig. 8). However, positively charged fluorinated polymer/protein complexes are often rapidly cleared by the reticuloendothelial system during systemic delivery. To address this issue, researchers cleverly utilize anionic polymers to shield the positive charges on the surface of the complexes [126]. This innovative application strategy not only enhances the stability of the complexes in the bloodstream but also improves their biocompatibility, opening new avenues for the further development of fluorinated polymers in the field of biomedicine.

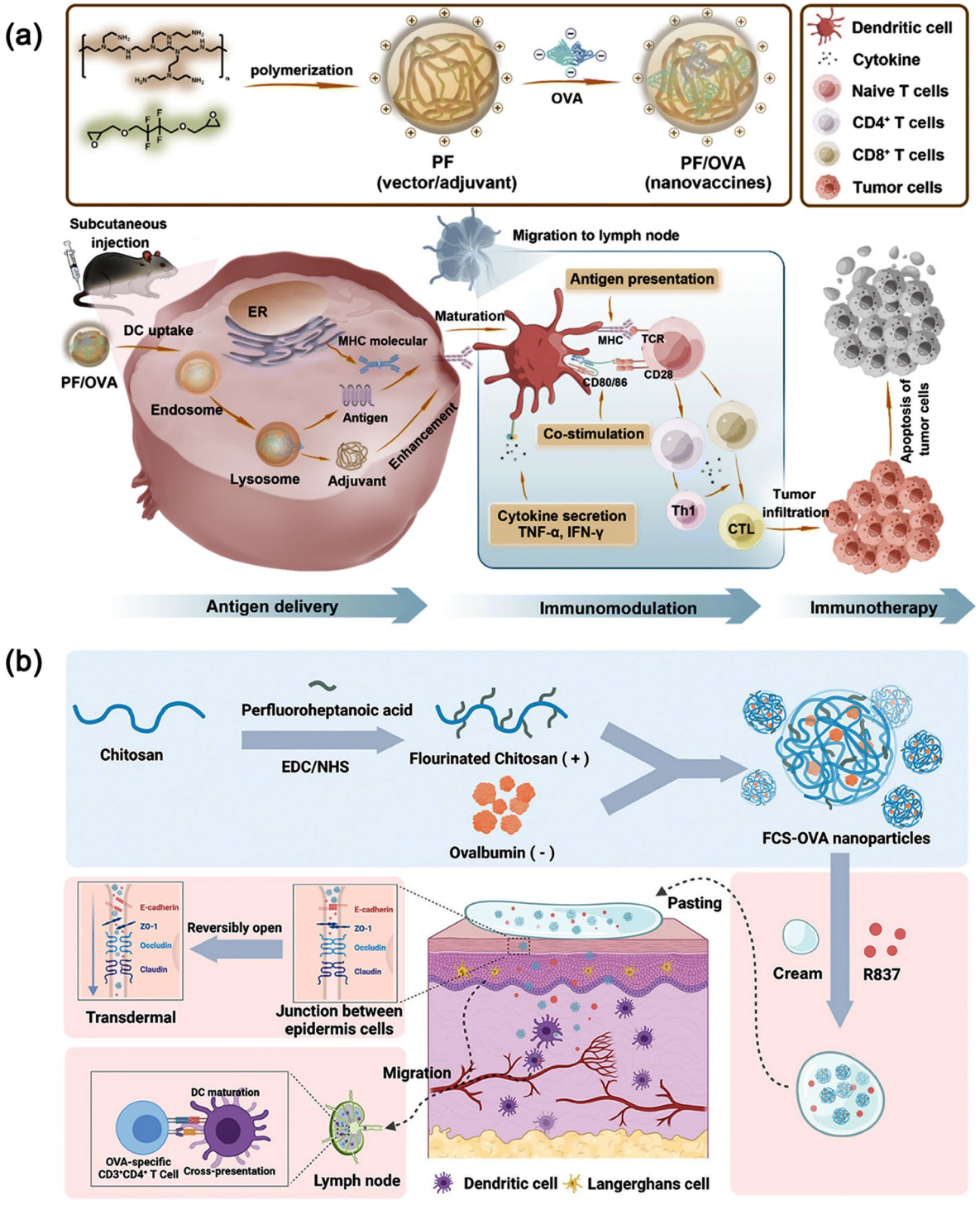


Fig. 8 Using Fluorinated Polymers as Delivery Vectors for Cancer Immunotherapy. (**a**) Illustrative diagram of the production of fluoropolymer PF, creation of PF/OVA nanovaccines, and the PF/OVA-facilitated immunotherapy procedure. Reproduced with permission from Ref [133]. ; (**b**) A non-invasive and needle-free cancer vaccine application in cream patch form, utilizing fluorinated chitosan as the base material. Reproduced with permission from Ref [134].

Boronated polymers

Boronated polymers have successfully garnered widespread attention in the biomedical field due to their unique N-B coordination mechanism [135]. The application of boronated polymers in drug delivery exhibits excellent performance and potential. Firstly, boronated polymers can precisely identify tumor cells due to their high affinity for sialic acid residues on cell

membranes [136]. This recognition ability allows boronated polymers to selectively bind to tumor cells, thus avoiding damage to normal cells [137]. Secondly, through multiple mechanisms such as nitrogen-boronic acid coordination, guanidinium-π interaction, and ionic interaction, boronated polymers achieve deep binding with cationic and anionic regions on the protein surface [138]. This deep binding not only enhances the interaction between drugs and cells but also improves the stability and bioavailability of drugs [139]. In addition, boronated polymers also demonstrate low cytotoxicity and excellent serum stability [135]. This means that during drug delivery, boronated polymers will not cause damage to normal cells while maintaining stability in complex biological environments. Therefore, boronation has become a universal and reliable strategy for polymer functionalization, providing strong support for drug delivery and tumor treatment (Fig. 9).

For example, phenylboronic acid (PBA), as a typical electron-deficient group of boronated polymers, plays a crucial role in the application of these polymers. PBA can form stable nitrogen-boronic acid coordination with primary amines and imidazoles rich in lone pair electrons, thereby achieving efficient binding with proteins [140]. Furthermore, the guanidinium part of arginine on proteins can also tightly bind to PBA through guanidinium-π interaction, further enhancing the affinity between boronated polymers and proteins. When PBA is grafted onto cationic polymers with a high density, these polymers can achieve deep binding with cationic and anionic regions on the protein surface through multiple interaction mechanisms. This deep binding not only improves the interaction between drugs and cells but also enhances the targeting ability and therapeutic effect of drugs. However, for polymers with relatively low PBA grafting rates, improving their affinity with proteins becomes a challenge. To address this issue, researchers cleverly introduced natural polyphenols. Natural polyphenols are rich in phenolic hydroxyl groups and aromatic rings, enabling them to form strong hydrogen bonding and hydrophobic interactions with biological molecules such as proteins and nucleic acids. This interaction can compensate for the limitations of insufficient PBA grafting rates, thus improving the efficiency of boronic acid-modified polymers. Boronate-modified polymers can achieve specific binding with catechin groups on the protein surface through catechin-boronic ester bonds. This dynamic covalent bond remains stable under physiological conditions but can be cleaved in acidic microenvironments. This feature allows for precise

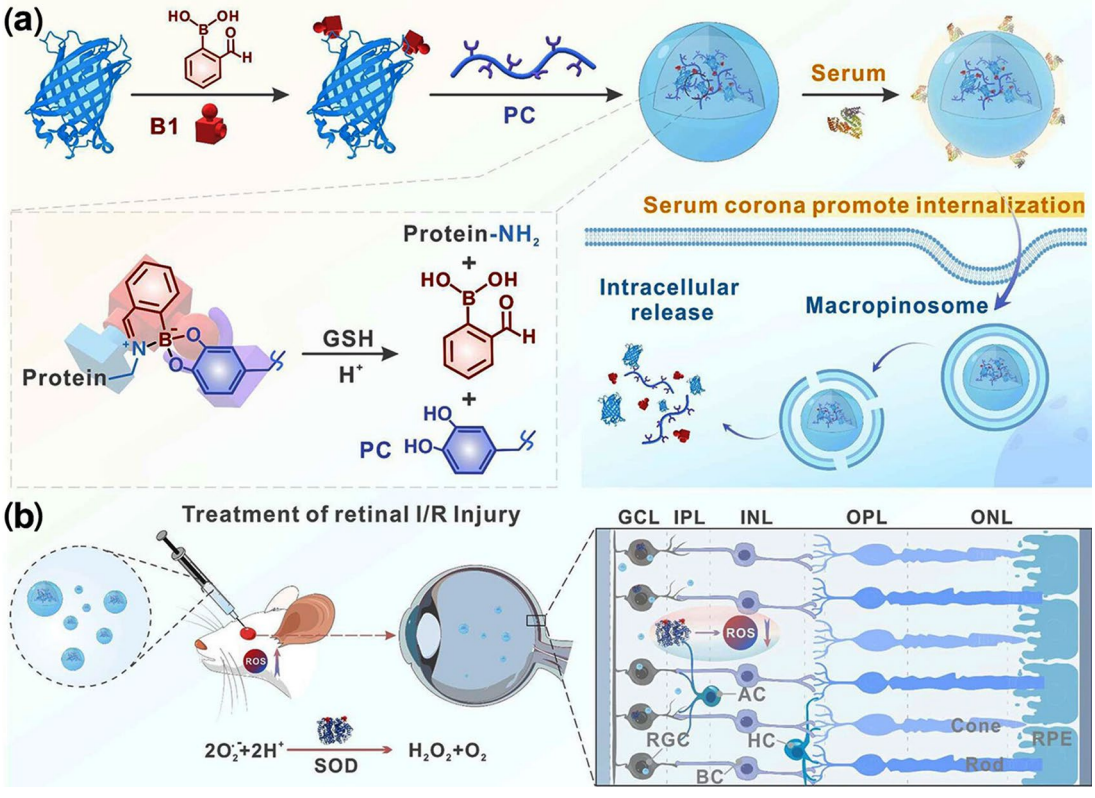


Fig. 9 Reversible Nanocomplex Formation for Intracellular Protein Delivery with Enhanced Serum Stability. (**a**) Utilizing a Heterobifunctional Adaptor B1 to Assemble Cargo Proteins and Polycatechols (PC) into Serum-Stable Nanoparticles. (**b**) Treating Retinal Ischemia/Reperfusion (I/R) Injury through Intravitreal Injection of SOD Nanoparticles Prepared via the Reversible Assembly Delivery System. Reproduced with permission from Ref [135].

control of the boronate-modified polymers during drug delivery, ensuring drug release at the target location, thereby enhancing therapeutic effects and reducing side effects. Studies have shown that the addition of natural polyphenols increases the efficiency of boronate-modified dendrimer polymers by at least five times [135]. This achievement opens new avenues for drug delivery and tumor treatment, potentially providing a more efficient and safe drug delivery system for future medical treatments.

Guanidinium-functionalized polymers

Guanidinium-functionalized polymers have demonstrated remarkable potential in biomedical applications. Due to their unique chemical properties, these polymers significantly enhance their binding ability with proteins and effectively promote interactions between cell membranes, leading to increased intracellular uptake [141]. This characteristic makes guanidinium-functionalized polymers promising candidates for protein delivery, drug delivery, and cell imaging. Firstly, guanidinium, as a strongly basic group with a pKa value of 13.6, exhibits superior alkalinity among different amino acids. Compared to arginine (with an R-group pKa of 12.5) and lysine (with an R-group pKa of 10.5), guanidinium displays a more prominent basicity. Consequently, guanidinium can form robust salt bridges and hydrogen bonding interactions with carboxyl groups of glutamic acid or aspartic acid in proteins [142]. This interaction not only enhances the stability of polymer-protein binding but also facilitates the intracellular localization and distribution of the polymers.

Guanidinium-rich peptides, polymers, and nanoparticles have garnered significant attention due to their remarkable membrane translocation activity. These materials can rapidly bind to cell membranes and enter cells through endocytosis, enabling efficient protein or drug delivery. Wang et al. modified poly (β-amino ester) (PAE) with phenylguanidine groups to enhance its applicability in cytosolic protein delivery (Fig. 10). The guanidine-rich PAE exhibited strong protein binding ability and high protein internalization efficiency, successfully delivering CRISPR-Cas9 RNP to HeLa cells expressing GFP and achieving over 80% GFP expression knockout [143]. The phenylbiguanide conjugated polymers have exhibited higher activity in protein binding and polymer internalization. Compared to monoguanidine polymers, the phenylbiguanide conjugated polymers have demonstrated higher efficiency in protein delivery. This polymer not only has a stronger ability to bind to proteins but also can more effectively promote cell membrane interactions and internal uptake. This discovery provides a new strategy for developing more efficient and safer protein delivery systems. Notably, despite the higher charge density of biguanidines compared to monoguanidines, surprisingly, the resulting polymers exhibit lower toxicity. The specific reasons for this finding still need further investigation, but it may provide important clues for the development of novel low-toxicity and high-efficiency protein delivery vectors.

Heterocyclic polymers

This regulatory capability is particularly crucial for the delivery of cytosolic proteins, as it ensures their stability and activity during the delivery process. Heterocyclic

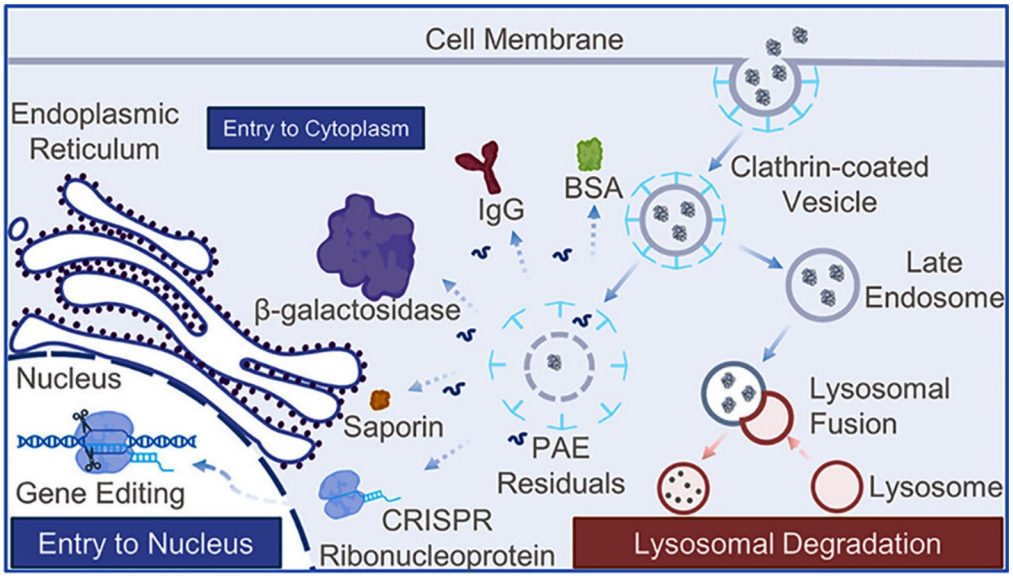


Fig. 10 Schematic illustration of Guanidyl-Rich Poly (β Amino Ester) for Universal Functional Cytosolic Protein Delivery and Gene Editing. Reproduced with permission from Ref [143]

organic compounds occupy a pivotal position in the development of biological macromolecule delivery systems. The heteroatoms such as N, O, and S in these compounds, with their notable high electronegativity, interact with the heterocyclic rings through inductive effects, thereby fine-tuning the charge distribution of the heterocycles. This unique property endows heterocyclic polymers with inherent advantages in optimizing the delivery efficiency of biological macromolecules. For instance, functionalized polyethylenimines and polyethylene glycols can precisely regulate charge and cargo binding capabilities through the modulation of coordination and hydrogen bonding [16, 18, 59, 144–146].

In the biomedical field, polyethylene glycol (PEG) has extensive applications in biological conjugation, drug delivery, surface functionalization, and tissue engineering [20, 32, 48, 148]. For example, PEGylation covalently couples PEG with proteins, significantly improving the pharmacokinetic properties of peptides and proteins. This improvement includes enhanced solubility, prolonged stability, and reduced immunogenicity, greatly enhancing their application effectiveness within biological systems [149]. Notably, pyridine thiourea-modified polyethylenimines exhibit exceptional performance in cytosolic protein delivery [145]. They can tightly bind to cargo proteins like GFP through a combination of ionic and hydrophobic interactions, ensuring that the proteins do not detach or lose activity during delivery. Moreover, heterocyclic polymers can be combined with other biocompatible materials such as liposomes and nanoparticles to form delivery systems with specific functions. These systems not only enable targeted delivery of proteins but also enhance their stability and bioavailability in vivo. It is worth noting that despite the tremendous potential of heterocyclic polymers in biological macromolecule delivery, they still face some challenges in practical applications. For instance, precise control over the synthesis and modification processes of heterocyclic polymers is necessary to ensure good biocompatibility and stability [129, 149]. For example, Malhotra et al. achieved long-term stability of nanoparticles using a biocompatible phosphonate-based polymer coating [147]. Furthermore, optimizing the design and preparation processes of delivery systems is crucial to enhance their delivery efficiency and specificity. Heterocyclic polymers play a significant role in the development of biological

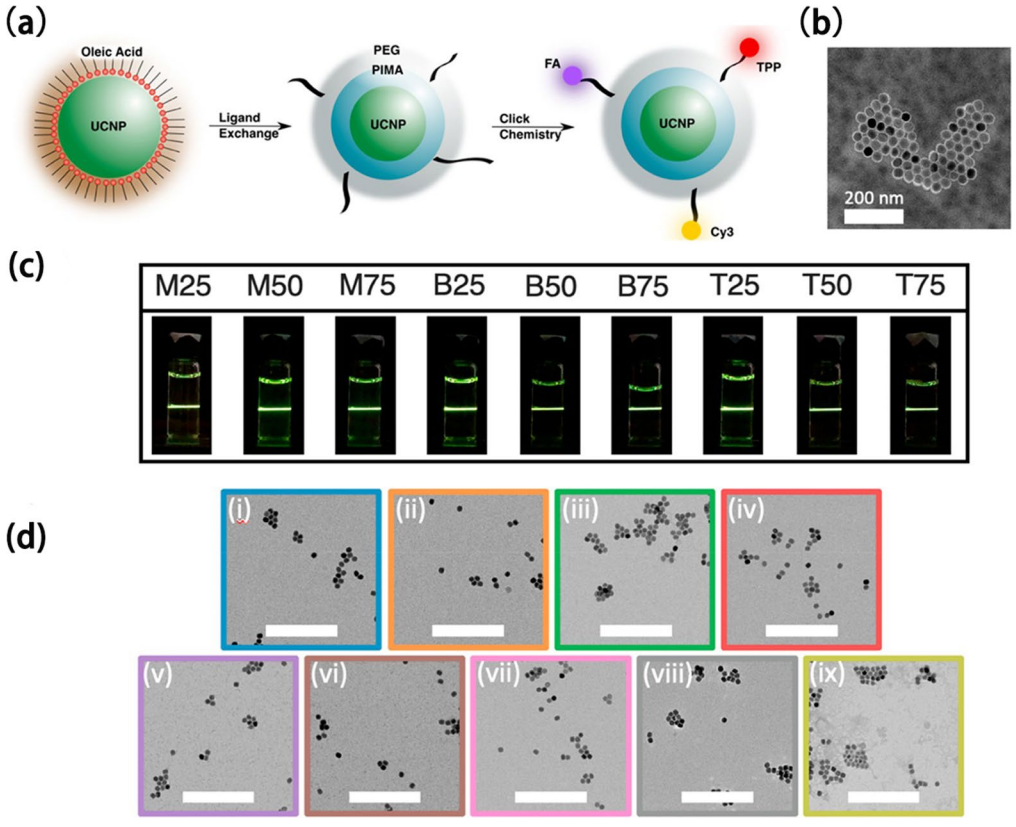


Fig. 11 Polymer-Coated Upconversion Nanoparticles (UCNPs) with Ligand Exchange and Functionalization for Protein Delivery. (**a**) Fabrication of functionalized polymer-coated upconversion nanoparticles (UCNPs) via ligand exchange with oleic acid coating. (**b**) TEM Characterization of B75 polymer-coated UCNPs with negative staining. (**c**) Photoluminescence imaging of UCNPs under 980 nm Excitation (800 mW Laser). (**d**) TEM imaging of the UCNP-polymer composite systems. Reproduced with permission from Ref [147]

macromolecule delivery systems (Fig. 11). Through further investigation of their properties and application mechanisms, combined with advanced preparation techniques and evaluation methods, it is expected that more efficient and safe biological macromolecule delivery systems will be developed in the future, making significant contributions to the advancement of the biomedical field.

Challenges

The rapid advancement of biotechnology and nanotechnology has led to an increased utilization of nanocarriers in the domain of protein delivery. Nevertheless, despite considerable advancement, nanocarriers continue to encounter a multitude of challenges in protein delivery, including issues pertaining to targeting capability, the efficiency of cell-penetrating peptides, lysosomal escape efficiency, limited reversible release capabilities, and the maintenance of protein activity. To address these challenges, researchers must continuously optimize the design of nanocarriers and explore new delivery strategies and technologies in order to achieve more efficient and precise protein delivery.

Targeting ability

Firstly, targeting ability is one of the significant challenges for nanocarriers in protein delivery. Protein delivery requires precise targeting to specific organ, tissue, and cell types to ensure the accurate delivery of drugs to the target cells [1, 150]. However, due to the complexity of the biological environment, the distribution and targeting of nanocarriers in vivo are often affected by various factors such as blood circulation, intercellular spaces, and cell membranes. Therefore, achieving precise targeting of nanocarriers is a crucial issue that researchers need to address. Here, RGD protein, a representative peptide with targeting ability to the cell attachment receptor integrin αvβ3 [63, 151, 152]. Compared to the linear RGD peptide, the cyclic RGD (cRGD) peptide possesses a more stable structure and exhibits stronger binding affinity and selectivity towards integrin receptors [153]. Since this integrin is overexpressed in various tumor cells, cRGD has become a highly sought-after target in tumor diagnosis and treatment [154]. Through clever modifications, liposomes can utilize the targeting ability of cRGD to actively seek and lock onto tumor cells, enabling precise delivery [155]. However, liposomes also face some challenges in their application. Due to their unique properties, liposomes are easily recognized and cleared by the mononuclear phagocyte system, which limits their application in cargo delivery to some extent [156]. Nevertheless, researchers are continuously exploring and optimizing the design of liposomes to overcome this challenge and enable them to play a greater role in tumor diagnosis and treatment. Li et al. have integrated liposomes with exosomes to construct a biomimetic hybrid nanoparticle called miR497/TP-HENP, successfully encapsulating the chemotherapeutic drug TP and miR497 [151]. By leveraging the homologous targeting effect of tumor cell-derived exosomes and cRGD targeting, the delivery system effectively targets tumor sites, significantly enhancing its targeting and retention capabilities at cancer locations. In the research of recombinant proteins, RGD protein also plays a crucial role. For instance, RGD4C, a cyclic RGD polypeptide containing two disulfide bonds, exhibits strong binding capacity to tumor neovascular endothelial cells [157–159].

Efficiency of cell-penetrating peptides

Cell-penetrating peptides (CPP) play an important role in protein delivery systems, as they have the ability to directly transport large molecules such as proteins, DNA, and RNA into cells, which is of great significance in drug development, disease treatment, and other fields [160, 161]. However, in practical applications, the delivery efficiency of CPP has become a key issue that needs to be urgently addressed. The efficiency of CPP delivery is constrained by a variety of complex factors [162]. Firstly, the sequence, length, and chemical modification of CPPs can significantly affect their ability to penetrate cell membranes [163]. Secondly, cell type is also an important factor affecting the efficiency of CPP delivery. It is worth noting that different types of cells have different membrane composition and structure, and therefore respond differently to CPP [164].

Lysosomal escape efficiency

When nanoparticles are relatively large (>100 nm), they often find it difficult to directly penetrate the cell membrane. In such cases, endocytosis becomes the primary pathway for nanoparticles to enter cells [165, 166]. Through endocytosis, the cell membrane forms a vesicle that encapsulates the nanoparticle, and then this vesicle detaches from the cell membrane and enters the interior of the cell. After entering the cell via endocytosis, nanoparticles are typically enclosed within lysosomes. Lysosomes are acidic environments containing various hydrolytic enzymes that can degrade foreign substances that enter them, including nanoparticles and their cargo (such as enzymes and antibodies). To maintain the integrity and biological activity of nanoparticles and their cargo, the nanoparticles need to be designed with the ability to escape from lysosomes. This allows them to successfully release from lysosomes into the cytoplasm, where they can bind to intracellular targets and thereby exert the desired biological effects. Additionally, when the surface of nanoparticles is modified with specific ligands, such as transferrin, antibodies, and RGD proteins, these modifications enable them to bind to receptors on the

cell membrane. This binding can also trigger endocytosis, resulting in the phagocytosis of the nanoparticles by the cell. It is worth noting that different types of cells have different preferences for endocytosis; for example, macrophages have a greater tendency to engulf foreign substances through endocytosis.

The pH level of the cellular microenvironment has a significant impact on lysosomal escape efficiency, and the acidity of the endo/lysosomal compartment has been used as a stimulus to activate the endosomal escape of nanocarriers. For example, cationic polymer nanoparticles can achieve lysosomal escape through the proton sponge effect [146]. After absorbing protons from the lysosome, the pH value inside the lysosome increases, leading to a decrease in the stability of the lysosome membrane, which subsequently triggers lysosome rupture and releases its target protein into the cytoplasm. Given the differential expression of enzymes in the endo/lysosomal environment of specific cells, we can also utilize the high content of proteases in cancer cells to provide a unique triggering mechanism for the activation of nanocarriers. For example, the activity of lysosomal cysteine proteases such as cathepsin B (CTSB) in the endo/lysosomes of cancer cells is much higher than in normal cells [167, 168]. Chen et al. utilized nanocarriers that sense endo/lysosomal enzymatic activity for selective intracellular delivery of antibodies in cancer cells. They selected anti-nucleopore complex antibodies (anti-NPC) as the model protein and constructed an endosome-soluble polymer based on PEG-pAsp(DET). Through a CTSB-sensitive val-cit linker, this polymer was conjugated with anti-nasopharyngeal carcinoma antibodies to form an antibody-polymer conjugate nanocarrier [16]. It is particularly noteworthy that the 1,2-diaminoethane moiety in the PEG-pAsp(DET) polymer has been experimentally proven to possess a strong membrane destabilizing effect in the endo/lysosomal environment, which is crucial for the endosomal escape process of nanocarriers [169, 170].

Limited reversible release capabilities

A key challenge that needs to be addressed urgently in protein delivery with nanocarriers is the difficulty in releasing proteins due to excessive binding forces [58, 171]. Although researchers have designed and synthesized various functional polymers, such as guanidine-rich polymers and boronate-rich polymers, in the hope of achieving effective cytoplasmic protein delivery, these polymers often result in delayed intracellular protein release due to their excessive binding forces, thereby reducing their biological activity [172, 173]. This largely limits their widespread application in the field of protein delivery. Ideal nanocarriers should possess precise and controllable release mechanisms to ensure effective protein release at specific times and locations, thereby achieving efficient cellular function regulation.

Maintenance of protein activity

The loss of protein activity during the preparation of nanocarriers remains a challenging problem that urgently needs to be addressed [174, 175]. Nanocarriers often undergo a series of complex physical and chemical treatments during the encapsulation of protein drugs. These treatment conditions, including high temperature, high pressure, the use of specific chemical reagents, and extreme pH values, may pose a threat to the stability of proteins such as enzymes [176, 177]. In addition, multi-step synthesis routes not only increase the complexity of operations, making the entire preparation process more cumbersome, but also potentially affect enzyme activity at each step [178]. When polymer monomers are used to covalently or non-covalently modify the amino acid side chains of proteins, such as fluorination, heterocyclic modification, and the use of organic solvents, they may alter the structure and charge properties of the proteins. For example, these modifications may block or expose reactive binding sites of proteins, thereby inhibiting or activating their functional activity [122, 179]. To overcome this challenge, researchers need to continuously innovate and explore new encapsulation methods and techniques to achieve efficient delivery of protein drugs while maintaining enzyme activity. When these modification strategies are being designed and applied, their impact on protein structure and function must be carefully weighed, which may involve comprehensive consideration and innovation in material selection, optimization of synthesis conditions, and encapsulation strategies.

Conclusions and perspectives

In recent years, there have been notable advancements in the field of nanocarrier research for intracellular protein delivery. This review examines the latest strategies for cytosolic protein delivery through the use of nanocarriers based on liposomes, protein self-assembly, and polymers. By meticulously controlling the dimensions, configuration, and surface characteristics of nanocarriers, researchers have made noteworthy advancements. Such advances include improvements in the stability and biological activity of proteins, as well as the ability to target and deliver them to specific cells with greater precision and efficiency. For example, the control of nanocarrier size ensures their stable transmission in intracellular and extracellular environments [165]. Additionally, the optimization of nanocarrier shape facilitates their traversal within cells, helping to avoid obstacles and quickly reach target areas. The efficiency of protein delivery by nanocarriers is closely related to the electronegativity of their

functional ligands, which may contain fluorine, boronate, or guanidine-rich polymers [139, 143, 180]. The efficacy of polymer materials in protein delivery can be enhanced by the targeted modification of ligands with diverse electronegativity groups. Furthermore, the advent of stimulus-responsive nanocarriers has infused the field of protein delivery with a new sense of dynamism. Such nanocarriers are capable of undergoing structural alterations or the release of proteins in response to particular environmental stimuli, including temperature, pH, or light [61]. This enables the precise timing and location of protein release, thereby further enhancing delivery efficiency.

In the field of protein delivery, the impact of protein design technology is profound and multifaceted. Firstly, protein design technology offers new possibilities for new drug development. Presently, a considerable number of proteins are regarded as undruggable targets due to the absence of small molecule binding sites, exemplified by KRAS and MYC [181]. The application of protein design technology enables researchers to obtain proteins with specific structures and functions, thereby facilitating the development of protein drugs with enhanced binding capabilities [182];Furthermore, protein design technology can be employed in domains such as synthetic biology and enzyme preparations, where it can enhance protein activity and provide novel delivery vectors [183]. Conventional protein delivery systems frequently necessitate intricate preparation procedures and costly materials. Nevertheless, the application of protein design technology can not only streamline the preparation of delivery systems but also reduce delivery costs, thereby extending the benefits of advanced protein delivery technology to a greater number of patients. This is of great significance for the advancement of drug development and clinical application. Furthermore, protein design technology provides substantial support for precision medicine. To illustrate, through the rational design of binder proteins or antibodies that specifically bind to receptors on the surface of cancer cells, researchers can achieve precise targeted delivery for tumor treatment and efficient gene editing applications [184]. As technology advances and its applications expand, protein design technology is poised to become an increasingly pivotal force in the domain of protein delivery.

The clinical development of nanocarriers loaded with proteins is still in its infancy. To further advance this field, future research should focus on the following specific directions: [1] Developing novel delivery vectors: Efforts should be directed towards researching and developing new delivery vectors with higher encapsulation efficiency, superior biocompatibility, and stronger targeting ability. In this process, we can draw inspiration from natural transport proteins and injection systems to create biotechnology platforms with efficient and precise delivery capabilities. For instance, delivery technologies based on bacterial extracellular contractile injection systems and virus-particle-based delivery systems; [2] Optimizing protein modification techniques: By applying protein modification techniques such as chemical modification and genetic engineering, we can enhance the stability and activity of proteins while improving their ability to cross biological barriers. This will help proteins better exert their therapeutic effects and provide more robust support for disease treatment; [3] Enhancing the controllability of delivery systems: To achieve temporal and spatial control of delivery systems, we can introduce advanced technologies such as light-stimulated release mechanisms and chemically induced release mechanisms, providing patients with safer and more effective treatment options; [4] Promoting interdisciplinary collaboration: Strengthening interdisciplinary collaboration among materials science, chemistry, biology, and medicine is crucial for driving innovation and development in protein delivery technology.

In conclusion, nanoparticle-mediated protein delivery systems demonstrate considerable potential for application and offer promising prospects for the delivery of therapeutic protein drugs. With the deepening of scientific research and advancements in technology, we have reason to believe that this field will bring revolutionary changes to the treatment of human diseases.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

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Competing interests
The authors declare no competing interests.

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