Chemistry & Biodiversity

WILEY-VCH

CHEMISTRY & BIODIVERSITY

REVIEW OPEN ACCESS

Advances in Cytosolic Delivery of Proteins: Approaches, Challenges, and Emerging Technologies

Wenyan Zhang[1] | Huiling Liu[2] | Bingdong Zhu[3] | Wen Li[2] | Xue Han[2] | Jiaojiao Fu[1] | Renjie Luo[1] | Haiyan Wang[1] | Jinxia Wang[1]

[1]First Clinical Medical College, Gansu University of Chinese Medicine, Lanzhou, Gansu, China | [2]Gansu Provincial Hospital, Lanzhou, Gansu, China | [3]Lanzhou University, Lanzhou, Gansu, China

Correspondence: Huiling Liu (liuhuiling75@163.com)

Received: 14 July 2024 | **Revised:** 6 February 2025 | **Accepted:** 6 February 2025

Funding: This study was supported by the National Natural Science Foundation of China (82260557), Lanzhou Science and Technology Plan Project (2023-ZD-21), and Gansu Provincial People's Hospital (22GSSYD-39).

Keywords: nanocarriers | protein chemical modification | protein delivery

ABSTRACT

Although therapeutic proteins have achieved recognized clinical success, they are inherently membrane impermeable, which limits them to acting only on extracellular or membrane-associated targets. Developing an efficient protein delivery method will provide a unique opportunity for intracellular target-related therapeutic proteins. In this review article, we summarize the different pathways by which cells take up proteins. These pathways fall into two main categories: One in which proteins are transported directly across the cell membrane and the other through endocytosis. At the same time, important features to ensure successful delivery through these pathways are highlighted. We then provide a comprehensive overview of the latest developments in the transduction of covalent protein modifications, such as coupling cell-penetrating motifs and supercharging, as well as the use of nanocarriers to mediate protein transport, such as liposomes, polymers, and inorganic nanoparticles. Finally, we emphasize the existing challenges of cytoplasmic protein delivery and provide an outlook for future progress.

1 | Introduction

Proteins regulate nearly every biological activity, including signal transduction, cellular metabolism, gene expression, cell division, cell proliferation, and programmed cell death [1]. Importantly, proteins hold exceptional promise as a therapeutic approach for tackling numerous hard-to-treat diseases, thanks to their unmatched precision and adaptability [2]. The synthesis and development of biopharmaceutical proteins, such as recombinant therapeutic proteins, enzymes, synthetic vaccines, monoclonal antibodies, and antibody-drug couplings, have facilitated protein therapeutics in the 40 years since recombinant human insulin (INS) Humulin was first introduced [3]. Unfortunately, the highly hydrophobic membrane impermeability, caused by the protein's hydrophilicity, charge, and rapid degradation, greatly limits the application and clinical translation of the protein's therapeutic ability [4, 5]. Currently, clinically authorized protein treatments like monoclonal antibodies, cytokines, and growth factors mainly target extracellular receptors or secreted proteins [6]. More than 70% of biosynthesized proteins localize and execute their functions within the cell. Overcoming proteins' membrane impermeability would significantly increase the number of therapeutic targets and would directly benefit a variety of therapeutic areas, such as oncology, infectious diseases, and genetic disorders.

In order to introduce the protein into the cell, one strategy is to transfect the target cell with a plasmid encoding the

target protein. In this case, the nucleic acid delivery problem can replace the protein delivery problem. However, plasmid DNA needs to be localized to the nucleus for transcription and translation, which is difficult to achieve in permanent cells [7]. Compared with plasmid DNA, the delivery of mRNA only needs to be delivered to the cytoplasm and translated into the target protein, which is feasible in permanent cells [8]. Nevertheless, it is important to note that nucleic acid delivery methods lack control over protein dosage and administration intervals. Direct intracellular delivery of proteins not only bypasses this issue but also enables comprehensive physicochemical and molecular characterization. Proteins, unlike nucleic acids, exhibit greater stability and do not require ultra-cold transportation and storage. They can be mass-produced efficiently by leveraging commonly utilized and readily accessible platforms, thereby minimizing production expenses. Various methods for direct protein delivery have been established. For instance, high-intensity energy or force produced by electric, magnetic, or light fields is used in physical procedures (e.g., microinjection and electroporation) to create transitory gaps in cell membranes for protein transport [9]. However, because physical approaches are ineffective, scalable, and potentially harmful to cells, they cannot be applied to in vivo protein delivery. Viral vectors are very effective because they come with an internal mechanism for getting past cellular barriers. However, because of their toxicity and immunogenicity, they should only be used sparingly. Protein entry into cells can be facilitated by chemical changes, such as coupling cell-penetrating peptides (CPPs), enhancing the hydrophobic characteristics of target proteins, and applying protein supercharging. Furthermore, membrane-permeable non-viral nanocarriers that transport functional proteins into the cell interior are an enticing platform for delivering intact proteins into the cell's interior without any further modification and without altering their natural function [1]. Typically, these nanocarriers form complexes with cargo proteins by noncovalent binding. By balancing ionic, hydrophobic, and hydrogen bonding (HB) interactions between macromolecules, complexes can be stabilized, interacted with the cell membrane, and released proteins into the cytoplasm [10]. Over the past decades, several nanocarriers, such as liposomes [11, 12], polymers [13, 14], and inorganic nanoparticles (NPs) [15, 16], have been explored for the delivery of biomolecules across cell membranes. Although intracellularly delivered proteins have great potential for treating a range of diseases, delivery vectors face several challenges in efficiently transporting proteins. These challenges include ensuring sustained retention in the circulation, selective accumulation in pathological tissues, effective cellular uptake, and the release of therapeutic cargoes from endosomes and facilitation of intracellular transport. Existing delivery technologies have substantial challenges in effectively overcoming these biological barriers to obtain satisfactory therapeutic results. Furthermore, the cytotoxic impacts and delivery platform instability commonly pose limitations to their practical application within biological systems, amplifying the critical demand for a resilient, adaptable, and biocompatible strategy for intracellular protein transportation.

In this work, we comprehensively overview various mechanisms of cellular protein intake and emphasize crucial attributes for effective transportation via these pathways. Next, we provide a comprehensive overview of the latest developments in protein covalent modification and the use of nanocarriers, including liposomes, polymers, and inorganic NPs, for protein delivery. Specifically, we discuss the difficulties encountered by delivery carriers in reaching their intended targets and the various strategies that have been developed to conquer these obstacles. Although these technologies have facilitated the practical application of protein therapies, the utilization of proteins for therapeutic objectives is still hindered by the requirement for precise, effective, and secure delivery methods. Finally, we emphasize the key obstacles that protein delivery encounters and present a perspective on potential advancements.

2 | Pathways for Intracellular Protein Delivery

Two main pathways are used by proteins to enter the cell: either directly across the cell membrane or by encapsulation in endosomes and transport through endocytosis (Figure 1).

2.1 | Proteins Cross the Cell Membrane Directly

There are multiple methods for delivering proteins directly across the cell membrane intracellularly, such as membrane perforation (physical) [17], membrane fusion (biological) [18], or membrane translocation (chemical) [19]. Mammalian cells possess an inherent capacity to withstand a certain degree of membrane harm, facilitating protein ingress via transient membrane breaches [20]. Physical techniques, such as microinjection, sonoporation, and electroporation, can induce temporary openings in the cell membrane to facilitate direct protein delivery. However, these methods are not only ineffective but also unsuitable for in vivo applications. Chemicals or pore-forming toxins, such as streptolysin O (SLO) and listeriolysin O (LLO), have the ability to create pores in cell membranes. However, this process often results in cell death. Therefore, this review does not emphasize membrane damage-associated protein delivery.

Proteins can be transported into the cell not only via transient pores in the membrane but also by fusing with it utilizing synthetic carriers composed of lipid bilayers. These carriers transport proteins into the cell, bypassing the need for endosomal capture. Exosomes are natural vesicles that can fuse with cell membranes and release their cargo by utilizing their unique lipid components [21]. Moreover, scientists prefer incorporating cellular components (e.g., cell membranes) into nanostructures to create biomimetic NPs (Figure 2). The membrane coating maintains the source cell membrane's natural biological characteristics, which not only directly fuses with the cell membrane to promote cellular uptake but also serves to prolong the somatic circulation. Prolonged in vivo circulation is critical for drug delivery as it allows nanocarriers to have a higher chance of accumulating at the target site. In conclusion, these biomimetic strategies enable conventional NPs to acquire the properties and functionalities of their biological counterparts. Additionally, they retain the efficient loading and delivery capabilities of NPs, making them promising systems for protein delivery [22].

In addition to the preceding techniques, proteins can utilize direct membrane translocation as an alternative route for entry. Although most proteins are impermeable, certain peptides and proteins can enter via this route. These peptides, predominantly

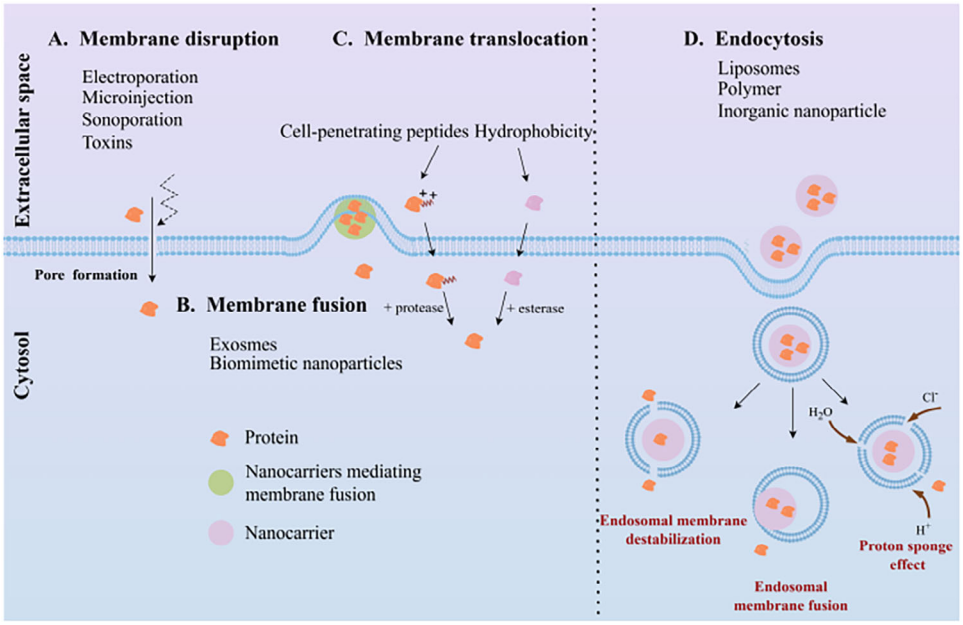

FIGURE 1 | Schematic illustrating the various pathways for external protein internalization, including (A) membrane disruption, (B) membrane fusion, (C) membrane translocation, and (D) endocytosis. *Source:* By Figdraw.

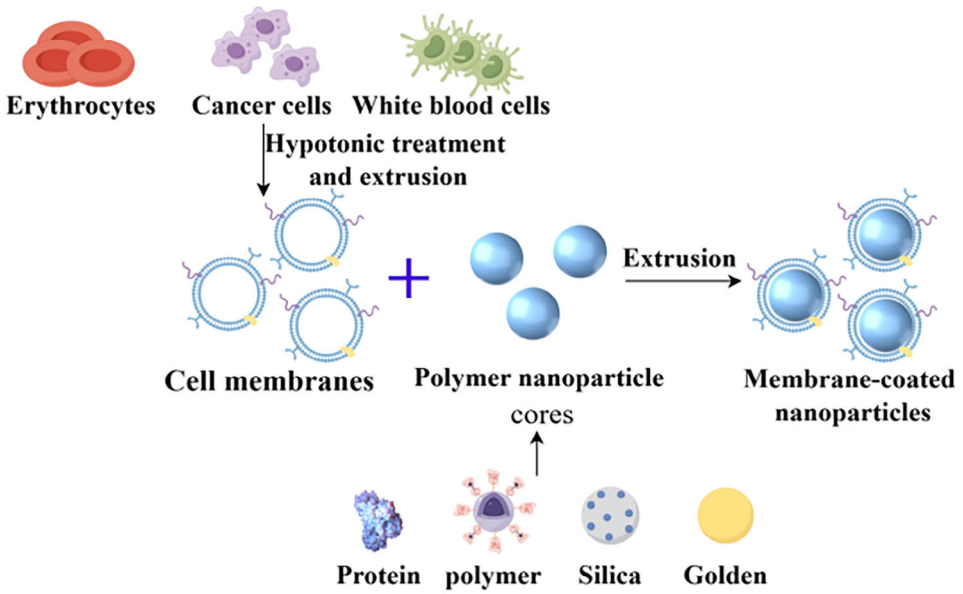

FIGURE 2 | Schematic diagram of the preparation process of cell membrane-camouflaged nanoparticles. Cell membrane vesicles, derived from the cell membrane, are expelled surrounding a polymer nanoparticle core to create cell membrane-encapsulated nanoparticles. *Source:* By Figdraw.

derived from pathogenic viruses and bacteria, have evolved to invade and infect human host cells. One of the most intensively researched strategies involves coupling payload proteins with such peptides for effective cellular entry and cytoplasmic protein delivery. Chemical membrane translocation, involving polymer functionality transfer through bond interaction with membranes, proves advantageous for in vivo macromolecular conveyance compared to cellular manipulation techniques.

The most prominent example of direct membrane translocation domains or proteins is the CPP domain. It has the ability to enhance the effectiveness of intracellular delivery by modifying proteins or carriers. CPPs are typically α-helical and highly cationic peptides. They are usually composed of less than 30 amino acid residues and exhibit strong interactions with the negatively charged cell membrane surface. In comprehensive investigations of CPP interactions with artificial bilayer structures, it has been observed that well-interacting CPPs are "interfacially active" and can bind noncovalently to model membranes [23, 24]. Several arginine-rich CPPs are currently utilized for protein cytoplasmic delivery, yielding desired experimental results [19]. The guanidino group, present in arginine-rich CPPs, is paramount due to its cationic charge, which facilitates binding to cell membranes via electrostatic forces. The membrane's anion forms a stronger,

bidentate HB with the peptide's guanidinium group, enhancing adhesion compared to the interaction with lysine's amino group [25]. Therefore, arginine-rich peptides that form guanidinium cation-like charge pairs can aggregate in the lipid bilayer, stabilizing the transient pore for CPPs to translocate into the cell [26, 27]. However, CPPs do not enter cells exclusively through direct translocation; it is also possible that they enter through energy-dependent pathways, such as endocytosis. Because the plasma membrane is constantly cycling with vesicular compartments, the mechanism of CPPs' cellular entry poses an intricate and ongoing research topic. Still, it is important to remember that covalently coupling CPPs to proteins can be effective in helping proteins cross the cell membrane.

2.2 | Proteins Cross the Cell Membrane Indirectly

Endocytosis is another major method of intracellular protein delivery. The process of endocytosis primarily involves the invagination/ruffling of the cell membrane, which then forms endocytic vesicles. Endocytosis can be divided into two main types: phagocytosis and pinocytosis [28]. Phagocytosis involves the uptake of large particles (≥0.5 μm) and is carried out solely by specialized cells. Several studies have reported the phagocytosis of NPs, including silver [29], gold [30], and polymeric NPs [31]. Pinocytosis is related to liquid uptake, including caveolin-mediated endocytosis [32], clathrin-mediated endocytosis [33], clathrin/caveolae-independent endocytosis, and macropinocytosis. Endocytosis is a cellular vesicular transport system. After endocytosis occurs, early endosomal vesicles rapidly mature into late endosomes, the pH value is reduced from 6.5 to 4.5, and finally fuses with the lysosome. The combined process ultimately results in the thorough breakdown of engulfed protein substances by lysosomal enzymes and acidic milieu. Membrane receptors identify certain proteins, facilitating their entry through energy-dependent endocytosis. However, the majority of proteins require sufficient carriers to cross the membrane. Typically, protein delivery employs structures like liposomes, polymers, micelles, inorganic NPs, and massive protein assemblies to enhance endocytic uptake. These nanocarriers offer numerous benefits in protein delivery, especially through the endocytosis pathway. Nanocarriers that encapsulate proteins offer protection against neutralization by serum and extracellular macromolecules, as well as minimize immunogenic responses, in contrast to unencapsulated proteins [34]. Nanomaterials shield proteins from endosomal protease-rich acidic conditions, prevent degradation, and enhance functional protein delivery. Additionally, NPs are purposefully crafted to leverage material traits or surface-bound ligands for targeted tissue interaction. Some can be programmed to react to cellular compartments like endosomes or cytosol, thereby promoting endosomal release or enabling protein delivery into the cytoplasm and restoring their native state. Clathrin-mediated endocytosis is the most studied endocytic mechanism and is considered one of the most important mechanisms for the uptake of NPs. The detailed process of clathrin-mediated endocytosis has been reported in several articles [35, 36]. In addition, many factors affect the internalization of NPs, including the physical properties of NPs, such as size, shape, composition, stiffness, and surface chemistry. At present, the process of how the physical properties of NPs affect the uptake of NPs by cells has been reviewed [37].

3 | Transduction by Protein Covalent Modification

Recently, the transformative potential of protein chemical modification has gained significant attention for augmenting both structural adaptability and functional diversity [38]. More interestingly, specific covalent modifications on the surface of proteins can directly facilitate intracellular delivery or enhance affinity with nanocarriers in cargo-transport systems.

3.1 | Coupling CPPs

CPPs, featuring low molecular weight, exhibit an exceptional capacity to traverse cellular barriers and convey an array of therapeutical biomolecules, ranging from small molecules and nucleic acids to larger compounds like proteins and NPs [39]. This characteristic enhances cellular uptake significantly and holds immense promise for drug delivery applications. CPPs bind to proteins through different strategies, including production by genetic engineering, covalent introduction by chemical means, and noncovalent complexation or simple co-incubation of proteins and peptides [40–42]. Expressing the protein as a protein–peptide fusion facilitates easy integration of CPPs [43]. Nonetheless, due to their high charge and inherent disorder, most CPPs can disrupt the stability and functionality of targeted proteins. An example is the protein precipitation caused by incorporating a highly charged nonaarginine peptide (R9) into the sequence of the nucleic acid endonuclease Cas9 [44]. Covalently attaching CPPs to target proteins is an interesting modification strategy. Still, attention needs to be paid to protein functional activity and coupling methods such as peptide affixation with site selectivity.

The first known CPP was a cationic peptide isolated from the HIV-1 transactivator of transcription proteins called TAT, which has been coupled to many proteins for intracellular delivery [45]. TAT peptide cellular entry relies heavily on basic amino acids like arginine and lysine, which bind to the negative phospholipid heads on the plasma membrane. For example, TAT specifically penetrates mouse hypoxic tumor cells when fused to an oxygen-dependent degradation (ODD)-β-galactosidase (β-Gal) [46]. When TAT couples other membrane-impermeable proteins, such as ribonuclease A (RNase A) and horseradish peroxidase (HRP), it can facilitate the transport of these proteins into the cytoplasm for their biological roles [47, 48]. Following the identification of TAT, numerous synthetic and natural CPPs were found, such as annexin-derived peptides [49], rabies virus glycoprotein [50], and fibroblast growth factor four-derived peptides [51]. Through cationic lysine-rich areas, these peptides and their imitators usually enhance cellular absorption [45]. These peptides' internalization process is influenced by various elements, including the cargo attached, membrane composition, concentration, and peptide sequence and structure [52]. Although numerous clinical trials are in progress [53, 54], several technological challenges remain to be resolved. The positively charged character of CPP renders it highly susceptible to forming stable complexes with negatively charged large molecules like serum albumin, thereby significantly impeding cellular uptake. This problem can be overcome by enhancing the oligomerization of CPP, which ultimately increases the local concentration inter-

acting with the plasma membrane [55]. Moreover, numerous technological obstacles persist, including severe dose-related toxicity, insufficient cell specificity, and covalent or unbreakable connections between CPPs and cargoes that result in endosomal entrapment, famously known as the "endosomal escape problem" [56].

In the past few years, protein-CPP fusions and conjugates have made important progress in solving these problems, which can better promote increased cellular uptake, reduce cytotoxicity, cause endosomal membrane damage, and promote escape from the endocytosis pathway. These advancements encompass various strategies like minimal cationic CPPs, integration of disulfide bonds, cyclic structures, reduction of arginine content, and incorporation of stimuli-responsive elements for regulated release. For example, multimeric CPP delivery has surpassed first-generation linear ones, yet most remain in the micromolar requirement [57]. In addition, existing multimeric CPPs reportedly enhance delivery, though their synthesis is relatively more intricate. Cyclic CPPs have shown superior ability in membrane perforation, and augmenting arginine content enhances membrane translocation [58]. Reports indicate that cyclic TAT (cTAT) accumulates at higher intracellular concentrations, entering cells, on average, 15 min sooner than its linear counterpart [59]. Long polypeptides' cationic transduction domains have been shown to increase protein transfection effectiveness in comparison to the traditional short, linear CPPs. Yin et al. screened bacterial cultures for long, lysine-rich polypeptides that yielded transduction sequences of up to 360 amino acids with a maximum theoretical charge of up to +60. Upon binding to cargo proteins, K4 exhibits exceptional efficiency in transporting proteins with a wide range of weights, from approximately 10 to 160 kDa, which is superior to TAT [60].

Typically, CPPs exhibit poor performance and are susceptible to endosomal trapping [61]. Cyclization of specific arginine-rich CPPs improves their endosomal escape, as discovered [62]. However, these CPPs are typically short peptides of up to seven amino acids, as larger peptides are not effectively absorbed by cells [63]. Certain folded mini-proteins possessing arginine sequences can be discharged from initial endosomes [64]. This modality comprises a helical structure featuring five arginines strategically positioned at its three distinct vertices [65]. Some CPPs are specifically designed to achieve endosomal escape, known as endosomal escape peptides (EEPs), which facilitate the release of macromolecules from endosomes through pore formation and fusion or cleavage activity [66]. For example, the amphiphilic peptide GALA, characterized by 30 amino acids and responsiveness to pH, was originally designed to explore viral fusion mechanisms. In addition to encouraging endocytosis, GALA is frequently employed to improve the endosomal escape of proteins and siRNAs, both in vivo and in vitro. Chen et al. constructed a novel nanoconjugate of antibody-drug coupling (NDC) by introducing GALA. In their study, it was discovered that GALA peptides form helical structures when situated near negatively charged lipids, which could significantly enhance cytosolization and provide a general strategy for endocytosis-dependent cellular delivery efficiency [67]. Kuehne et al. coupled GALA to anti-transferrin antibodies and demonstrated that these couplings were capable of releasing up to 10 kDa of fluorescent dextrose anhydride from liposomes. Furthermore, their study also revealed that increased dextran molecular weight necessitates a correspondingly elevated peptide-to-lipid or peptide-to-antibody ratio (PAR) [68]. Nonetheless, augmenting the PAR value can result in unfavorable pharmacokinetics due to changes in the antibody's biophysical characteristics, like its isoelectric point. Considering the distinct compositions of cellular and endosomal membranes, along with the notable pH disparity (endosomal at approximately pH 5 and extracellular at around pH 7), it is feasible to design pH-responsive membrane cleavage peptides and polymers. Most are currently designed to preferentially interfere with endosomal membranes through protonation of carboxylates under acidic conditions in endosomes [69, 70]. For example, Sakamoto's team synthesized the new cleavage peptide L17E by using a cationic amphiphilic peptide, M—lycotoxin, as a template and introducing a single negatively charged glutamic acid residue onto the potentially hydrophobic surface of the peptide, which efficiently attenuates the cytotoxicity of the original peptide and modulates the cleavage activity of the peptide. This peptide is positively charged, and its electrostatic interaction with the negatively charged endosomal membrane can interfere with the endosomal membrane, thus possessing membrane cleavage activity. L17E has been shown to promote cellular uptake by inducing macropinocytosis, as well as to cause significant release of antibodies (immunoglobulin G) from intracellular bodies [41]. Du et al. modified biodegradable silica NPs (BSNPs) with different nuclear-localized signaling peptides (NLS) and encapsulated cargo proteins. These particles were then able to enter cells by endocytosis with the aid of L17E. L17E-induced endosomal disruption facilitated NPs release into the cytoplasm, thereby achieving targeted nuclear delivery of natural proteins/antibodies [71]. However, the endosomal membrane cleavage activity of L17E still fell short of expectations. In order to obtain a more potent pH-sensitive endosomal membrane cleavage activity, Sakamoto's team subsequently synthesized the cleavage peptide HAad based on the cleavage peptide L17E by replacing the glutamate on the peptide with $_{L}$-2-aminoadipic acid (Aad, p*K*a 4.21) and by replacing the histidine on the peptide with methyl-containing alanine. Compared to glutamate, HAad is more hydrophobic and has a higher p*K*a of δ-COOH (4.21), making it more conducive to disrupting the endosomal membrane and facilitating endosomal escape. At the endosomal pH, histidine is positively charged or protonated, which decreases the peptide's hydrophobicity. The hydrophobic contacts and membrane disruption ability of the peptide can be greatly enhanced by substituting alanine with a methyl group on the side chain, which is uncharged and promotes the formation of an α-helical structure, for histidine. The findings indicate that HAad can disrupt endosomal membranes in addition to facilitating the entry of loaded macromolecules into the cell [42]. The team then employed a protein nanocage as a model framework, which was formed by the self-assembly of a β-cyclic peptide with 24 amino acids in water. Nitrilotriacetic acid (NTA) was attached to the nanocage's interior (N-terminal) in the presence of Ni^{2+}, allowing it to effectively encapsulate the protein, and the cationic amphiphilic peptide HAad was attached to the nanocage's surface (C-terminal). The findings demonstrated that HAad had successfully induced cargo protein endocytosis uptake and endosomal escape and that 75% of the cells' cytoplasm contained the target proteins [72].

In addition to coupling directly to proteins for delivery, CPPs can also bind proteins via reversible noncovalent binding, which

facilitates enhanced endosomal escape [73]. Depending on their chemical structures, CPPs are usually classified as amphiphilic, hydrophobic, and cationic, with most of them belonging to the cationic type. Cationic CPPs and negatively charged proteins can be noncovalently complexed via weak hydrophobic and electrostatic interactions. Although they can be prepared as CPP-protein complexes, this technique usually leads to structural ambiguity. In addition, this complexation is limited to the delivery of negatively charged proteins. On the basis of this, Morris et al. constructed a novel peptide carrier Pep-1, containing 21 amino acids, which is composed of three structural domains: a hydrophobic tryptophan-rich segment for effective membrane targeting and protein interactions, a hydrophilic SV-40-derived lysine-rich area for enhanced intracellular transport and peptide solubility, and a flexible linker area separating the above contrasting sequences. The amphiphilic Pep-1 peptide exhibits remarkable efficacy in transporting an extensive array of peptides and proteins to diverse cell lines, maintaining their full biological activity upon complex formation with the payload proteins, achieved by a straightforward blending of the constituent components [74]. Positively charged CPPs can be attached to protein-loaded NPs in addition to the above-described direct coupling of proteins. This promotes cellular absorption through electrostatic interactions with negatively charged areas on the cell surface, which is crucial for delivery systems. It is significant to note that positively charged NPs may inhibit the uptake of NPs by cells and encourage the development of protein crowns [75]. Anionic coatings can be created on the surface of NPs to overcome this restriction. For example, Zsák et al. modified the carboxyl terminus of the hydrophilic CPP poly-L-lysine (PLL) with lipophilicity by amidation reaction with oleylamine (OA), which was then grafted onto fatty acid chains to enable it to be anchored on the outside of the nanoemulsions (NEs). Through electrostatic contact, sodium tripolyphosphate was encapsulated on the surface of NEs to produce an anionic coating. Once alkaline phosphatase (ALP)-enriched target tissues were reached, ALP facilitated the quick dephosphorylation of sodium tripolyphosphate and exposed the *N*-amino group on the surface of NEs. This led to a notable change in zeta potential, which increased the success of target cargo uptake by three times, from −22.4 to +8.5 mV [12]. The benefits and limitations of nanocarrier delivery of proteins are described in detail below.

3.2 | Supercharging and Increasing Hydrophobicity

CPPs' highly cationic character enables electrostatic interaction with anionic cell membranes, typically taken up through endocytosis. This property of CPPs provides ideas for protein delivery, that is, increasing the positive charge of proteins through various genetic and covalent modifications can enhance cellular delivery efficiency. In addition, the hydrophobic nature of the plasma membrane's inner layer, attributed to fatty acid chains in phospholipids, restricts penetration to lipophilic compounds below 500 kDa. Although amino acids can move freely, proteins' hydrophilic character and larger size significantly impede their passive diffusion [76]. Direct membrane translocation can be facilitated by increasing the target protein's hydrophobic groups, which allow it to pass across extremely hydrophobic cell membranes.

Anionic supercharging, unlike cationic, impairs direct transport but is widely employed for intracellular cargo delivery. Typically, negatively charged nucleic acids (DNA, mRNA, siRNA) adhere to positively charged delivery systems in the form of NPs via electrostatic attraction. Charge-neutralizing nucleic acid complexes with nanostructures have a strong attraction to negatively charged cell membranes and stimulate cytophagy. However, unlike nucleic acids such as DNA and RNA, protein molecules possess a restricted number of net charges. This is due in part to the hindrance of intrinsic non-systematic charges, that is, positive charge of amine groups and negative charge of carboxyl groups [77]. As a result, it is challenging to form stable complexes with polymer carriers using electrostatic complexation strategies similar to those used with nucleic acids. Thus, this issue might be resolved by anionic supercharging of proteins. Rather than directly increasing delivery, anionic supercharging increases the electrostatic interactions between proteins and cationically charged NPs, is a useful technique for facilitating the entry of carrier-encapsulated protein cargoes into cells via endocytosis or non-endocytosis delivery mechanisms. The method targets imparting proteins with a net negative charge akin to that of nucleic acids. Liu et al., for instance, fused a gene-editing enzyme with negatively charged green fluorescent protein (GFP) [34] or other naturally derived anionic proteins [78], illustrating cationic lipid-assisted gene-editing enzyme delivery. Protein amino groups were combined with acid-unstable carboxyl groups to create integral anionic polyelectrolytes, which are better able to achieve electrostatic complexation with cationic delivery carriers [79]. Still, the bioactivity of the proteins has to be ensured. Cui et al. carboxylated the proteins with amine-reacted *cis*-aconitic anhydride, which was efficiently encapsulated into a multilayer nanostructured carrier composed of a multi-block copolymerized cationic ring Arg–Gly–Asp (RGD)-polyethylene glycol (PEG)-GPLGVRG-polylysine (thiol). Carboxylated proteins, when subjected to acidic endosomal pH, restore the original amine group through a charge reversal decarboxylation process and effectively increase the charge density, disrupting the anionic endosomal membrane and facilitating the endosomal escape of the nanocomposites [80]. Wang et al. conjugated free lysine residues on cytotoxic proteins, such as RNase A and saponins, to *cis*-aconitic anhydride, thereby altering the positively charged lysine moiety into a negatively charged carboxylic acid moiety. Chemically changed cargo proteins have a stronger binding to cationic lipid NPs (LNPs) and can be applied in vivo to limit tumor growth [81]. It is crucial to recognize that charge modification by indiscriminate binding to surface residues may have a deleterious effect on protein function. Negative charge introduction techniques that are site-specific and controlled are better at preventing protein inactivation while enabling electrostatic interactions with charged lipids.

The creation of stimulus-responsive proteins that allow for precise and spatiotemporal control of protein activity within the cell can also benefit from direct chemical modification of proteins. For example, Li et al. used propargyloxycarbonyl to specifically replace the key amino acids on the target protein to inactivate the protein. After using the palladium catalyst, a deprotection reaction can be triggered to remove the structure, thereby quickly restoring protein activity in living cells [82]. Zhao et al. chemically coupled *ortho*-nitrobenzyl to luciferase to generate photoresponsive proteins, which can rapidly activate intracellular

protein functions under UV light irradiation [83]. The restricted tissue penetration of UV radiation and the intrinsic toxicity of heavy metal ions limit the therapeutic applicability of these technologies, despite their ability to precisely and efficiently control protein function. This type of chemical modification of proteins can be combined with simultaneous enhancement of the interaction with NPs. Wang et al. underwent a strategic modification of RNase A by incorporating 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (NBC), enabling the successful conjugation of an aryl boronic ester to the protein's lysine residue via a stable carbamate linker. RNase A binding to the lysine of NBC reverses its positive surface charge, decreases its isoelectric point (p*I*), and enhances electrostatic binding to cationic LNPs. High concentrations of reactive oxygen species (ROS) in tumor cells efficiently broke the NBC binding when RNase A-NBC was liberated from the NPs. They reactivated the anti-tumor activity of RNase A [84].

Traditionally, protein biocoupling techniques lacked precise control over modification site and orientation, resulting in heterogeneous products with varied activity. The development of site-specific protein coupling techniques made it possible to display delivery fractions more adaptable, improving protein activity, circulation characteristics, and targeting specificity.

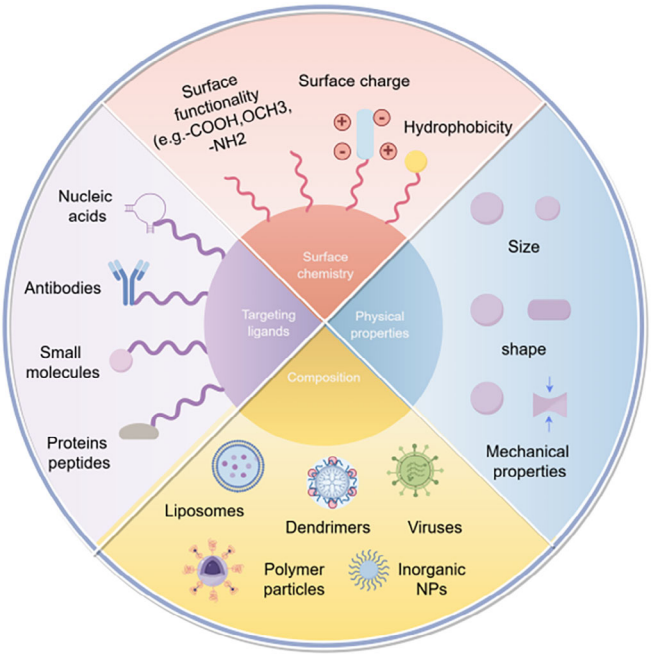

FIGURE 3 | Schematic diagram of the main physical and chemical properties of nanocarriers. *Source:* By Figdraw.

4 | Transduction With Nanocarrier Systems

Nanoparticles (NPs) are effective delivery systems for drugs, vaccines, peptides, and proteins. In recent years, NPs have gained popularity due to their distinct characteristics such as size-dependent mobility, enhanced drug solubility, extended circulation duration in living organisms, stimulus-responsive payload delivery, combating drug resistance, the capacity to actively and passively target specific biological tissues, and the ability to achieve controlled release of drugs [85]. The cellular uptake, internalization pathways, and intracellular fate of NPs directly impact the efficiency of nanotherapeutic approaches for disease management. By coupling targeted ligands (e.g., small molecules, nucleic acids, peptides, and proteins), chemical modifications, and changing physical properties, more efficient NPs can be created (Figure 3). Protein-based technology translation into clinical practice has been facilitated through the use of NP delivery systems. However, the requirement for targeted, effective, and secure delivery methods continues to be a barrier to the therapeutic use of proteins, and their long-term toxicity and complexity continue to be major problems in clinical translation [86]. In this section, we discuss in great depth the developments and obstacles encountered in the delivery of proteins by NPs composed of lipids, polymers, and inorganic materials.

4.1 | Liposome

Liposomes, initially formulated by Alec D. Bangham in the 1960s at the Babraham Institute, Cambridge University, consist of biocompatible and biodegradable phospholipid bilayers, showcasing their unique properties for various applications [87]. Compared to viral delivery systems, lipid delivery systems significantly lower the risk of infection, carcinogenicity, and immunogenicity [88]. Lipids and lipid derivatives are mainly composed of a head group, a linker group, and a hydrophobic tail. During the manufacturing process, the hydrophilic head is oriented toward the aqueous medium. In contrast, the hydrophobic tail forms the inner region of the membrane, resulting in the formation of a bilayer [89].

Liposomes are categorized into three distinct types: cationic lipids, lipids with ionizable properties, and amphiphilic lipids, each differing in their head group attributes (e.g., number of amines, type of amine, number of substituents, and hydrophobicity). Figure 4 displays some common liposome structures. Cationic liposomes have a positive charge on the surface, which can interact with the negatively charged cell surface to promote intracellular protein delivery. However, this property of cationic liposomes is usually associated with considerable cytotoxicity, such as ROS production, energy metabolism disruption, and cell death [90, 91]. Ionizable liposomes have ionizable groups, and a number of ionizable amino head groups have been produced as essential ingredients in the production of ionizable liposomes. Ionizable liposomes are neutral under physiological conditions (pH 7.4), with an overall surface charge close to neutral, when protonated under acidic conditions (pH < 6.0), thus exhibiting a positive charge. After systemic injection, they are less toxic and have longer circulation durations than cation delivery systems, which enables them to reach a wide range of tissues. Following this line of reasoning, Hirai's group created a dioleoylglycerophosphate–diethylenediamine affix (DOP–DEDA), a new pH-responsive charge-reversible LNP. In the neutral extracellular environment, it remains negatively charged to prevent toxic interactions with cells. Upon entry into the acidic confines of the endosome, the lipid acquires a positive charge, subsequently engaging with the membrane and initiating a disruption. The results showed that the delivery system successfully carries out endosomal uptake and endosomal escape while effectively wrapping short interfering RNAs (>95%) and cargo

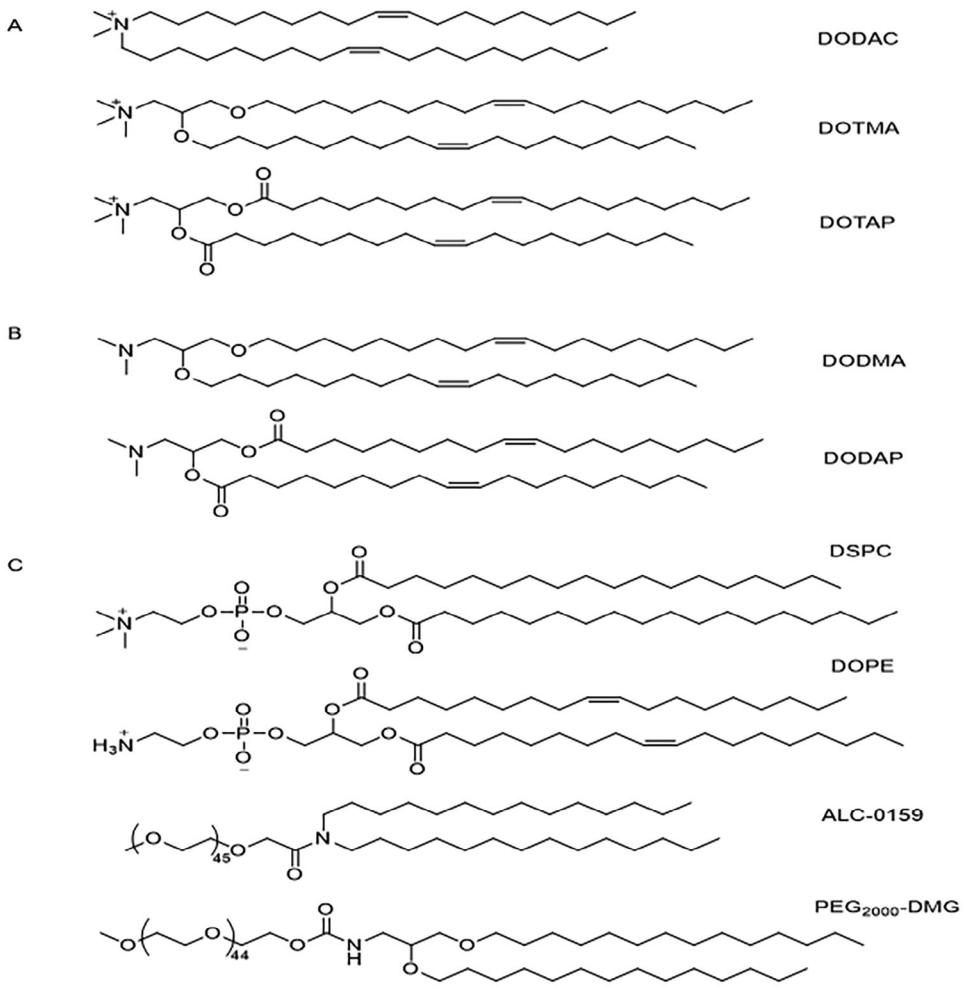

FIGURE 4 | Chemical structures of lipids and lipid derivatives: (A) cationic lipids, (B) ionizable lipids, and (C) other types of lipid. DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; PEG, polyethylene glycol.

proteins (~80%) [92, 93]. Lipids carrying both positive and negative charges on their heads are known as amphipathic liposomes. In medicine delivery, gene delivery, and other sectors, they exhibit superior biocompatibility and minimal toxicity because of their charge-balancing feature, which also has a more mild effect on organisms.

The ideal set of linkers in a lipid (e.g., ester, amide, and C–N bond) should be biodegradable and have excellent cyclic stability. Lipophilicity, transition temperature, and cargo transfer efficacy of a lipid can be affected by the hydrophobic tail's structure, which includes heteroatoms, degradable bonds, and tail quantity and length. Although liposomes are simple to make, they are not very effective at encapsulating proteins. LNPs, which are highly prevalent in non-viral delivery platforms, boast superior cargo encapsulation efficiency compared to liposomes. As illustrated in Figure 5, LNPs primarily consist of ionizable lipids, cholesterol, PEG-anchored lipids, and supporting phospholipids. By filling the gaps between the phospholipids, cholesterol stabilizes the lipid bilayer membrane, improves the stability of the LNPs, and prevents the leakage of therapeutic drugs. In addition, PEG can also modify the liposomes' surface. This enhanced liposome design prolongs in vivo circulation by strategically restricting electrostatic and hydrophobic contacts with plasma proteins, thus

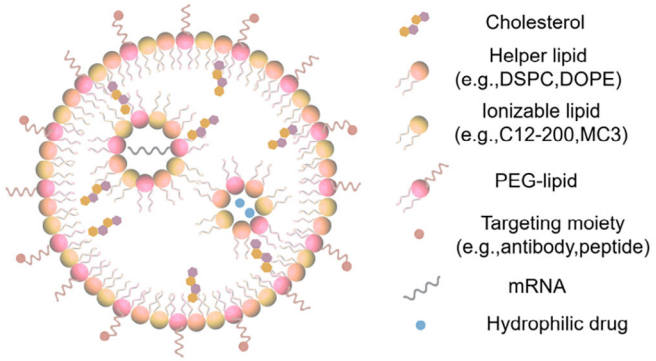

FIGURE 5 | Diagram of the LNP delivery system. DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; LNP, lipid nanoparticle; PEG, polyethylene glycol. *Source:* By Figdraw.

boosting colloidal stability and reducing reticuloendothelial system clearance. Liposomal formulations commonly incorporate esters of PEG like DSPE-PEG2000, DMG-PEG2000, and DSP-PEG2000. Enhanced with auxiliary lipids like 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), a conical configuration is formed, boosting the stability of LNPs' non-bilayer hexagonal HII phase. This is essential to increase the effectiveness of drug

delivery during membrane fusion and bilayer rupture. Currently, LNPs have been extensively studied as delivery carriers for proteins, peptides, and mRNAs.

Combinatorial libraries of lipid materials can be synthesized by employing different methods such as Michael addition, solid-phase synthesis, "click" chemistry, and epoxide-amine ring-opening reactions [94]. Among these, the Michael addition reaction offers several benefits, including solvent-free reaction conditions, high conversion rates, and simplicity in product purification when amine-containing hydrophilic head groups from commercial sources are coupled with new hydrophobic tail groups to create new lipid carriers. In addition, the physical characteristics of the lipid carriers, as well as the cargo protein delivery efficiency, can be changed by altering the composition and structure of the head group, linker group, and hydrophobic tail. To increase the effectiveness of cargo protein delivery, for instance, additional building blocks have been added to the lipid manufacturing process to change the length of the alkyl chain at the end of the hydrophobic region and insert a nonlinear cholesterol component into the tail structure. Li's research team partially integrated ethers, thiol ethers, and selenide ether moieties into the hydrophobic tails of lipids to investigate how heteroatoms impact the effectiveness of intracellular transport of genome-engineered proteins. The results showed that even with a different atom at the end of the hydrophobic tail, the combined lipids' intracellular delivery efficacy varied, with the combined lipid containing selenide ether surpassing the others in terms of protein delivery efficiency [95]. Combined lipid structures can integrate stimulus-responsive elements for cargo release upon specific intracellular cues, promoting accelerated cleavage and effective payload delivery upon cell entry. By loading the lipid tails with acid-degradable acetal groups, for instance, Li et al. successfully manufactured pH-responsive lipids that show extraordinary stability at physiological pH and break at mild acidity. A light-cleavable 2-nitrobenzyl ester group was incorporated into the tail structure to create light-responsive lipids, which further expand on stimuli-responsive lipids by permitting lipid breakdown in response to outside stimuli [96]. Using a one-step Michael addition procedure, Wang's group created a series of LNPs with disulfide bonds to deliver siRNAs for gene knockdown intracellularly. In a reducing intracellular environment containing glutathione (GSH), disulfide bonds in the lipid tails were disrupted, triggering the dissociation of LNP and the timely release of cargo [97]. Subsequently, they successfully mediated effective genome editing and protein delivery by electrostatic assembly of reducing lysable lipids with anionic Cas9: single-guide (sg) RNA complexes or negatively charged Cre recombinase. Additionally, in the presence of an intracellular reductive environment, proteins' ability to escape from endosomes to complete biological roles can be enhanced [11]. Surface modifications of lipid carriers with ligands can enhance their targeting precision. In summary, by altering lipid composition, size, and surface chemistry, lipid carriers can be developed into multifunctional structures to meet adjustable requirements, such as simultaneous detection, imaging, and targeting of malignant cells, which provides ideas for disease diagnosis and treatment.

Because the US Food and Drug Administration (FDA) authorized the use of two mRNA COVID-19 vaccines for emergency use, the potential of LNPs in drug delivery has been confirmed. Up to now, lipid-based drug carriers form the majority of FDA-approved nanomedical treatments. Nonetheless, proteins' intricacy regarding size, surface charge dynamics, and hydrophobic/hydrophilic equilibrium, coupled with the vital need to maintain their native three-dimensional structure for functionality, poses a substantial obstacle in the development of universally effective LNPs for protein transportation [93]. Currently, most combinatorial lipids are designed for gene delivery, with a limited number showing promise in delivering therapeutic proteins within cells.

4.2 | Polymer Nanoparticles

Polymer NPs frequently serve as efficient nanocarriers for the targeted intracellular transport of proteins. By modifying or adjusting the chemical structure of polymers, it is possible to design polymers that are cytocompatible and can maximize the efficiency of intracellular delivery while minimizing cytotoxicity. The physicochemical properties of the polymers, such as size, surface charge, shape, and mechanical strength, can be specifically designed for active or passive targeting. At the same time, protein delivery NPs should be designed to efficiently encapsulate cargo proteins, be easily degradable or separable, and be able to precisely trigger protein release from the corresponding sites.

Cargo proteins are primarily bound by carriers through electrostatic interactions, resulting in highly unstable complexes within plasma. Plasma's profuse serum proteins can potentially interfere with the binding process to the carrier before their cellular entry, resulting in the premature release of payload proteins. An urgent need exists for creating serum-tolerant protein delivery materials to boost protein delivery efficiency. Zhang et al. successfully synthesized a pH-responsive phase-change polymer by modifying 4-diethylaminophenyl isothiocyanate onto the fifth generation polyamide-amine dendrimers via a simple reaction. Hydrophobically modified dendrimers are insoluble at pH 7.4, can assemble into NPs with cargo proteins through electrostatic interactions as well as hydrophobic interactions, and are highly serum tolerant. When internalized into the cell, their internal tertiary amine groups are protonated at a low pH (pH 6.0), allowing the dendrimers to increase in their hydrophilicity and become completely soluble in water, allowing for the pH-responsive release of the protein [98]. Inspired by the intricate nature of high-density nucleic acid nanostructures, such as spherical nucleic acids, Distler et al. ingeniously created DNA dendrimers exhibiting controllable dimensions, length, and hierarchical design. These sophisticated structures can efficiently be uptaken by dendritic cells through type A scavenger receptor-mediated endocytosis. N-terminal cysteine residues of fluorescently labeled cargo peptides were coupled to dendrites by disulfide exchange reaction utilizing a pyridyl disulfide-containing cross-linker. The results demonstrated that the dense DNA clusters had no cytotoxicity and successfully carried the cargo peptide while also improving resistance to serum nuclease and spatially preventing enzymatic breakdown [99]. Currently, the most commonly used method for protein encapsulation is the water/oil/water double emulsion method. This method, along with others, employs chemicals or techniques that commonly cause protein denaturation, which is a drawback [100, 101]. In addition, this method tends to result in lower protein loadings. For example, after careful optimization by Varshochian et al., the maximum loading capacity of

bevacizumab can only reach 7.1% [102]. Therapeutic proteins that require more than 300 mg of delivery in a single dosage still require improvement in this loading capacity. In addition to guaranteeing high serum tolerance of the NPs, delivery mechanisms with high protein-loading capacity can be created to further boost the actual protein cell entrance. Protein desolvation may be a promising technique for concentrating proteins. The dropwise addition of a non-solvent protein (e.g., ethanol) to an aqueous solution of protein under gentle stirring can lead to protein supersaturation, resulting in the formation of protein NPs [103]. For example, Buzgo et al. performed antibody desolvation with acetonitrile to generate bioactive protein NPs, the size of which can be controlled by the addition of a low concentration of sodium chloride (<2 mM) [104]. Polymers can encapsulate protein NPs to increase protein loading. Morales-Cruz et al. nanoprecipitated proteins and then used a second polymer nanoprecipitation step to efficiently encapsulate protein NPs into poly(lactic-*co*-glycolic acid) (PLGA) nanomicrospheres. This resulted in >70% protein encapsulation while maintaining the proteins' high level of activity [105].

Conventionally administered intravenously, nanocarriers circulate within the bloodstream, where they face potential excretion, are influenced by blood flow dynamics, form coronae, and are encountered by phagocytic cells. The altered blood flow rate exerts a shear stress on the nanocarriers, which leads to damage and early release of therapeutic drugs. When nanocarriers interact with biomolecules (e.g., proteins, antibodies, lipids) and cells in the bloodstream, a corona layer forms around them, which affects their biodistribution and stability. In addition, rapid clearance by the mononuclear phagocyte system (MPS) is a major obstacle for protein delivery systems in vivo. MPS consists mainly of phagocytes, such as macrophages and dendritic cells from the spleen, lymphatic hubs, and liver, which readily remove NPs flowing in the bloodstream. In addition to eliminating nanocarriers from the blood, phagocytosis also triggers an immunological response via interleukin, necrosis factor, and interferon, which inevitably causes tissue damage and undesirable inflammation [106]. Nanoparticles' stealthiness is pivotal in evading protein adsorption, immune detection, and body clearance [107]. For NPs intended to be passively accumulated into target tissues using enhanced permeability and retention (EPR) effects, a sufficiently long circulation time contributes to their efficient accumulation in target tissues [108]. By covering the NPs with plasma proteins such as immunoglobulin or albumin, the phagocytic system can be prevented from identifying them [109]. Moreover, grafting uncharged hydrophilic polymers—of which PEG is the most widely used—onto the surface of particles produces so-called stealth particles, which lengthen the modified particles' plasma half-life by inhibiting renal excretion and obstructing efficient phagocytosis uptake [110]. Surface modification of liposomes with PEG reduces clearance by the reticuloendothelial system, and PEG-modified polymers can achieve the corresponding effect. The length, molecular weight, and grafting density of the PEG chains influence protein adsorption and stealth effects. In fact, an efficient MW ranges from 1500 to 3500 Da, whereas the most often used PEG-PEs are PEG 2000, 3000, or 5000 [111]. However, recent studies have shown that repeated administration of pegylated drug formulations can lead to the disappearance of invisible effects and the occurrence of adverse reactions [112]. For example, the non-biodegradable PEG has been observed to accumulate within renal cell cytoplasms, forming vacuoles, yet its implications on cellular and organ functioning remain unexplored. However, they may be important in the context of long-term application [113]. Furthermore, once pegylated medicines are administered, antibodies against PEG can be detected, suggesting that PEG may be more immunogenic than initially anticipated. Hypersensitivity reactions have been reported with PEG-containing treatments. Interestingly, amphiphilic ions and mixed-charge polymers have shown great potential in the fabrication of invisible delivery systems. For example, Qin et al. prepared a novel class of mixed-charge amphiphilic copolymers poly(aminoethyl methacrylate)–*co*-poly(methacrylic acid)–*co*-poly(*n*-butyl methacrylate) (CPMA) for efficient intracellular delivery of adriamycin (DOX). In a physiological environment (pH 7.4), the mixed-charge CPMA copolymers assembled into micelles with a zeta potential of −26 mV, significantly prolonging circulation time and enhancing tumor penetration. Under tumor extracellular acidic (pH 6.5) conditions, its zeta potential increased to −6 mV, promoting micelle retention in the tumor stroma and cancer cell uptake [114]. However, amphiphilic or mixed-charge polymers encounter certain challenges during chemical preparation, such as the insufficient water solubility of amphiphilic polymers and the difficulty in controlling the charge ratio of mixed-charge polymers [115]. Therefore, the design of an invisible nanocarrier is of great importance for drug delivery systems while remaining a great challenge. In order to solve this problem, Xu et al. constructed anionic dendritic lipopeptides (ADLs) with eight carboxyl groups in the terminal groups and cationic dendritic lipopeptides (CDLs) with eight amino groups in the terminal groups. The results demonstrated that equal amounts of ADLs and CDLs were co-assembled to generate neutral, invisible NPs with mixed charges, which successfully inhibited the adsorption of positive and negative proteins and prolonged blood transport [116].

Nanoparticles and polymers commonly adhere to cargo proteins via noncovalent bindings. Due to the intricate nature of protein chemistry and scarcity of binding sites, tailored NPs are often necessary for each protein, thus affecting their broad applicability. Therefore, it is essential to find a universal and biocompatible means of delivering proteins intracellularly. Wang's team constructed a novel supercharged polypeptide (SCP) with high cellular uptake, intracellular vesicle escape, and cytoplasmic localization capabilities. Introducing an unnatural amino acid containing a phenylboronic acid side chain at the N-terminus of each SCP can efficiently form nanocomplexes with different proteins through ionic interactions, nitrogen–boron coordination, and cation–π interactions, which can successfully deliver various proteins into the cytoplasm without affecting the protein bioactivity [117]. Although most nanoplatforms can achieve single protein delivery, it is more attractive to develop nanoplatforms that can deliver different proteins at the same time. Feng et al. proposed a lysine-modified cyclodextrin-based nanoplatform, which successfully achieved co-delivery of RNase A and deoxyribonuclease I into the cytoplasm with better anticancer effects [118]. However, the co-delivery of proteins acting at different sites is still challenging. Recently, Zhao et al. ingeniously developed a sophisticated magnetic protein nanocarrier, which not only exteriorly releases the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) but also precisely facilitates targeted intracellular delivery of glucose oxidase (GOx). The nanosystem's

design features a core–shell composition. The core comprises polyphenol-modified GOx and iron oxide NPs impregnated with boric acid, linked via pH-responsive borate bonds. The shell consisted of a polyethene glycolized polyethyleneimine polymer coupled to TRAIL via a matrix metalloproteinase-2 (MMP-2) responsive peptide. The peptide connecting TRAIL and PEI-PEG is broken by MMP-2, which is overexpressed in the tumor microenvironment when the nanosystem is magnetically directed to the tumor site. The released TRAIL then binds to receptors on the cell membrane, inducing intrinsic tumor apoptosis and improving the cellular uptake of the core. When pH-sensitive core structures enter a cell, they degrade quickly and set off a chain reaction that produces free radicals, which limit tumor growth. Experiments conducted in vitro and in vivo demonstrated that TRAIL and GOx have strong synergistic anticancer effects [119].

To enhance efficient passage across challenging hydrophobic cellular barriers, incorporating hydrophobic moieties like aliphatic lipids onto materials or biological compounds enhances membrane permeability and facilitates enhanced endocytic uptake. It has been demonstrated that polymers with hydrophobic lipids have superior delivery [120]. However, their lipophilic nature tends to make these ligands interact with membrane phospholipids and serum proteins, potentially resulting in premature release before entry into the cytoplasm. The introduction of ligands on polymers that are both hydrophobic and lipophobic is expected to solve this problem. For instance, the distinctive class of perfluorinated lipids (PFLs) possesses a unique combination of hydrophobic and lipophobic characteristics [121]. This property ensures effective cell membrane penetration and endosomal escape while preventing the early release of cargoes before they enter the cytoplasm. Lv et al. coupled fluorinated ligands with cationic polymers. This resulted in a significant improvement in the cationic polymers' serum tolerance and an increase in fluorination in multiple phases, including cellular uptake and endosomal escape [122]. Compared to nonfluorinated lipids, fluorous ones enhance polymer assembly, improve protein encapsulation, minimize protein denaturation, and boost endocytosis.

Most nanocarriers and protein complexes are readily lost during endocytosis due to the low pH of endosomes and lysosomes, as well as the presence of various degradative enzymes. To achieve the desired activity, endocytosed material needs to be released from the endosome into the cytoplasm promptly to avoid being broken down by lysosomal enzymes. However, the successful completion of endosomal escape is one of the main bottlenecks in the development of new macromolecular therapeutic agents [123]. Various innovative nanodevices for "endosomal escape" have emerged, encompassing mechanisms such as membrane fusion, proton sponge effect/osmotic lysis, swelling of NPs to rupture the endosomal membrane, and strategies to destabilize or disrupt the endosomal membranes [124]. Though numerous advancements have been made in facilitating endosomal escape, substantial enhancements are yet to be achieved. A prospective approach lies in the direct cytoplasmic delivery of large biomolecules, which bypass the endosomal route and thus elude capture and potential degradation by endosomes/lysosomes. For example, Zou et al. have successfully prepared INS-loaded ginsenoside (GS) NPs INS @ GS NPs by thin-film hydration. GS is an amphiphilic steroidal saponin that self-assembles into a shell layer to protect INS from hydrolysis. Unique interactions of GS with membrane components such as cholesterol and phospholipids result in transient pores on the membranes, thus allowing INS @ GS NPs to penetrate directly into cells within 5 min for ultrafast delivery of NPs [125]. Currently, there are two primary approaches for delivering biomolecules directly into the cytoplasm. One approach involves reversible binding of cargoes to carriers to form nanoscale colloidal assemblies and delivery of biomolecules via cholesterol-dependent lipid raft pathways [61, 126] or direct membrane permeation [127, 128]. Another approach is to design carrier molecules with particular chemical structures such as ammonium cations [129], guanidinium [61, 126], and lipophilic groups [127, 128], which induce temporary opening of the membrane upon interaction with the cell membrane. For instance, Mout et al. assembled arginine-functionalized gold NPs (AuNPs) with proteins containing oligo glutamate to create a spherical nano-assembly. The results demonstrated that such assemblies successfully integrated with the cell membrane and employed lipid raft-assisted mechanisms to deliver cargo proteins into the cytoplasmic environment. Five proteins with different charges, sizes, and functions were efficiently delivered into the cell and maintained their biologically active [61]. Nanoparticles with positively charged surfaces have shown a stronger tendency to be internalized through endocytosis involving membrane encapsulation, and larger particles above 10 nm significantly augment this mechanism [31, 130]. Thus, carrier-specific surface chemical groups, net zero charges, smaller sizes, and softer carrier-cargo assembly are essential for direct cytoplasmic delivery. For example, Sarkar et al. reported a guanidinium-terminated polyaspartic acid micelle that formed nano-assemblies with proteins/DNA and observed that the <200 nm colloidal assemblies exhibited a nearly negligible surface charge. The polymer has three different chemical groups, each corresponding to a specific function. The ammonium group provides a cationic charge for electrostatic binding with proteins/DNA; the hydrophobic oleyl group induces the formation of micelles; and the guanidinium group forms salt bridges with carboxylic acids/phosphates, which allows for specific interactions between the micelles and the cellular membranes, followed by a temporary deformation of the membranes and micelles, allowing the polymer micelles to enter the cell directly. The results showed that the polymer can deliver total protein/DNA directly into cells within 5 min [131].

With the rapid development of nanomaterials, there has been great interest in developing stimulus-sensitive polymeric vesicles to better control drug release. A series of novel smart delivery carriers, which include enzyme-responsive (based on matrix MMPs, GSH reductase, cathepsins, etc.) [132, 133]; pH-responsive (based on imine, hydrazone, amide bonds, ester (*ortho* ester and borate ester), etc.) [134]; redox-sensitive (based on disulfide and diselenide bonds, etc.) [135]; and temperature-sensitive (based on poly(nisopropylacrylamide), poly(ethylene oxide), poly(acrylic acid), etc.) [136] nanocarriers. Systems that respond to pH and redox potential are of special interest. For example, Wilson and others ingeniously designed a block copolymer that responds to stimuli with antigen/adjuvant blocks and pH-sensitive lysosomal blocks to enable the co-delivery of antigen and adjuvant into cells [137]. De Mel et al. introduced a biocompatible glycopolymer NP with a diameter of less than 100 nm, capable of complexing with proteins, reducing responsive antigen release, and pH-triggered lysosomal degradation and adjuvant release [138]. Kudruk et al.

co-assembled peptides and amphiphilic β-cyclodextrins (β-CDs) to form the framework of a liposome-like cyclodextrin vesicle model and modified the surface of the reducible peptide shell. The complex successfully delivered the peptide to the cytoplasm and tightly regulated peptide release by reduction and endosomal peptidase-triggered vesicle disassembly [139].

The effectiveness of a polymer's cargo-transportation system is crucial. In addition, the biodegradability and biocompatibility of the polymer are important prerequisites for constructing a protein or peptide delivery system that can be used in clinical therapy. Various biodegradable polymers, such as PLGA, chitosan (CS), and hyaluronic acid (HA), have demonstrated exceptional potential as precursor materials for nanocarriers in vitro, showcasing their inherent biocompatibility and efficacy in drug delivery applications. For example, PLGA can hydrolyze into lactic and glycolic acids, which can then be transformed into carbon dioxide and water. These compounds can then be easily removed from the body by regular metabolic processes. PLGA, a popular biodegradable and biocompatible copolymer, is a versatile drug delivery platform. The FDA and the European Medical Association (EMA) have approved it for numerous clinical uses [140]. CS is a natural, biodegradable, nontoxic polysaccharide that is biosafe. CS NPs (CS-NPs) can effectively cross tumor cell membranes through electrostatic interactions or endocytosis and are considered to be effective carriers of proteins and peptides due to their nontoxicity and ease of internalization by cells [141]. Turky et al., for instance, used an ionic gel technique to create CS-NPs loaded with an antimicrobial peptide (NRC-07). CS-NPs loaded with NRC-07 significantly reduced the viability of both bacteria and cancer cells in a concentration-dependent manner as compared to free antimicrobial peptides [142]. Furthermore, various other biocompatible and biodegradable materials, such as poly(amino acids), poly(lactic acid) (PLA), poly(glutamic acid) (PGA), poly(caprolactone) (PCL), *N*-(2-hydroxypropyl)-methacrylate (HPMA), provide a secure and non-harmful platform for in vivo application [143].

4.3 | Inorganic Nanoparticles

Extensively researched inorganic NPs, such as silica NPs, metal-based NPs, and carbon-based NPs, have demonstrated significant potential for protein transportation [144]. On the basis of their morphological characteristics, silica NPs can be categorized as hollow silica nanoparticles (HSNs), dendritic silica nanoparticles (DMSNs), mesoporous silica nanoparticles (MSNs), and solid silica NPs. HSNs are a class of nanocarriers with degradable shells, which can effectively protect drug molecules from the risk of enzymatic degradation, in addition to their advantages of large specific surface area, low density, and adjustable pore size. DMSNs, which are porous nanomaterials with large pore sizes, are especially good at loading large molecule drugs and so on and have attracted much attention in the field of protein drug delivery in recent years. Owing to its inherent properties, such as large surface area, tunable pore size, and facile surface modification, MSN is widely recognized as an ideal nanocarrier for efficient delivery [145]. In contrast to solid silica NPs, MSNs are hollow and can trap proteins in their tortuous channels. Current studies have demonstrated that MSNs can be used as carriers for the loading of these cargoes, ranging from small molecules of drugs to biomolecules such as sugars, nucleic acids, peptides, and proteins [146]. Due to their high specific surface area, MSNs exhibit high protein loading. The precise tuning of the porosity, pore size, and wall thickness of MSNs allows these particles to accommodate a wide range of proteins, such as cytochrome c [147], RNase A [148], IgG [149], and ovalbumin [150]. However, the low biodegradability of MSNs severely restricts their clinical applications. Chen et al. added $CaCl_2$ and Na_2HPO_4 to the MSN framework in order to address this issue. They replaced some Si–O–Si bonds in the original structure with Si–O–Ca and P–O–Ca bonds, which are easily hydrolyzed under acidic conditions. The results demonstrated that the newly synthesized MSNs could be efficiently internalized by macrophages and degraded in acidic lysosomes or nuclear endosomes to release cargo peptides, thus possessing acid-responsive degradation properties [151]. Some silica NPs were designed to easily accomplish endosomal escape. According to Yu et al., utilizing octadecyl-functionalized "rough" silica NPs (C18-RSN) to deliver the therapeutic protein RNase A significantly induced apoptosis in cancer cells. By rupturing endolysosomal membranes, hydrophobic alterations, including octadecyl groups, facilitate endosomal egress in comparison to RSNs lacking such modifications. In contrast to RSN without the hydrophobic modification, the hydrophobic modification with the octadecyl group disrupted the endolysosomal membranes, aiding in endosomal escape [152].

Metal-based NPs exhibit exceptional potential as a drug delivery platform thanks to their diminutive size, stable structure, and distinctive optical characteristics. Extensively studied AuNPs, renowned for their low toxicity, have emerged as prominent carriers for protein delivery. AuNPs can often be shaped with empty internal spaces to increase encapsulation effectiveness. In addition, protein delivery via AuNPs necessitates surface functionalization with a binding ligand for cargo protein attachment or adsorption. For example, Mout et al. used arginine-functionalized AuNPs to successfully transport a variety of protein drugs with different sizes, charges, and functions, all of which can be efficiently transported into the cytoplasm or nucleus [61]. Morales et al. encapsulated DNA in AuNPs to provide amine termini that could bind to protein chelators and successfully deliver cargo proteins into the cytosol via endocytosis [153].

Carbon-based NPs, including carbon nanotubes (CNTs), nanodiamonds (NDs), GOx, and carbon dots (C-dots), offer a wide range of biomedical applications. CNTs, often referred to as CNTs, have the remarkable properties of high surface area-to-volume ratio and nanoscale stability. By functionalizing CNTs, they can be designed as carriers for efficient delivery of various cargo molecules such as carboplatin, paclitaxel, nucleotides, and proteins. Functionally modified CNTs can permeate biological membranes, facilitate the entry of biomolecules into the cytoplasm, and shield the anchored substances from enzymatic breakdown. For example, Batista de Faria et al. designed an anticancer vaccine containing multi-walled CNTs (MWCNTs) for delivery of the Toll-like receptor agonist, CpG, and the testis antigen, NY-ESO-1. They discovered that vaccination produced a potent immune response mediated by CD4+ T and CD8+ T cells, which considerably slowed the growth of the tumor and increased the mice's survival period [154]. Other carbon-based nanomaterials with high hydrophilicity and high specific

surface area can also be used as nanocarriers. For example, Jiang and colleagues reported a graphene-based nanocarrier for the co-delivery of TRAIL and DOX, which can be internalized into cells to mediate DNA damage-mediated cytotoxicity [155]. Zhang et al. prepared C-dots/enhanced green fluorescent protein (EGFP) nanocomplexes with a size of about 200 nm by mixing C-dots with an EGFP. This delivery system outperforms free proteins by safeguarding them from enzymatic degradation and successfully transferring EGFP into HeLa cells [156].

In summary, inorganic NPs offer several beneficial properties for performing intracellular protein delivery, including controlled size and surface functionalization, long cycle time, efficient cellular uptake, targeting ability, and minimal toxicity. Notably, protein loading and absorption efficiency are significantly influenced by the size, surface functionalization, and inorganic core selection of NPs [53]. Employing diverse preparation strategies, functionalized nanomaterials can be formed with enhanced properties like prolonged blood circulation and targeted binding, minimized side effects from controlled release, and accelerated development of safer medications.

5 | Subcellular Delivery of Protein/Peptide

More specific sites can produce more therapeutic effects and fewer adverse drug reactions. Even after being safely delivered into the cytoplasm, protein agents must still find the specific site, where they are expected to exert their therapeutic potential. To maximize therapeutic effects and minimize unwanted effects, the target destinations of protein therapeutics have been narrowed from organs/tissues to cells and further narrowed from the cytoplasm to organelles. There are various organelles within cells, including the nucleus, mitochondria, endoplasmic reticulum (ER), Golgi apparatus (GA), and lysosomes. The total number of protein species within these organelles is nearly equivalent to that of the cytoplasm.

Currently, the subcellular targeting delivery of therapeutic proteins has gradually attracted people's interest, and its development could greatly stimulate the advancement of protein/peptide-based therapies. In general, organelle-targeting strategies can be summarized into two main types: passive targeting and active targeting. The former typically utilizes concentration and size effects, whereas the latter depends on the presence of localization signals. For example, the nuclear targeting delivery of therapeutic agents can promote gene editing, regulation, and expression. Nuclear pores allow small molecular weight proteins (<60 kDa) to enter the nucleus through passive diffusion. Model cargo proteins, like GFP (238 residues, 27 kDa), can passively diffuse through nuclear pore into the nucleus and illuminate the nucleus [157]. For proteins larger than the nuclear pore, passive nuclear diffusion is greatly reduced, and they usually require the assistance of active nuclear targeting elements. Active targeting of the nucleus can be achieved through NLS. NLS is a short peptide sequence primarily derived from eukaryotic and viral nuclear proteins, such as nucleoplasmin, simian virus 40 (SV-40) T antigen, and NF-KB [158]. For example, Kong et al. conjugated SV-40 T (nuclear localization sequence is PKKKRKV) with ferrocene diphenylalanine peptide to create a co-assembly system. The system autonomously formed multifunctional peptide-based virus-like NPs capable of targeting the nucleus [159].

Creating strategies for active targeting can also be applied to various other subcellular organelles. In cases where the cytosol is not the intended target, the targeting element can relocate the conjugates/carriers to the target organelle. Typically, organelle-targeting components are classified into various categories, such as ligands and peptide signals. Mitochondrial malfunction is associated with a range of diseases, including cancer, metabolic disorders, and immune diseases [160]. Consequently, targeting mitochondria is crucial for drug delivery. To effectively target and deliver therapeutic proteins to the mitochondria, bioactive molecules can be utilized, such as mitochondrial targeting sequences (MTSs) [161], mitochondrial penetrating peptides [162], SS peptides, cysteine-rich peptides, and mRNA [163], as well as small molecules like triphenylphosphine (TPP) [164], guanine, biguanides, rhodamine, and transition metal complexes [165]. MTSs are typically natural peptides composed of 20–40 residues, forming amphipathic α-helical structures which can be identified by mitochondrial outer membrane receptors and import systems. Furthermore, peptidases in the mitochondrial matrix can precisely split MTS to liberate the cargo. For example, the fusion with MTS peptide enables the restriction endonuclease small to target mitochondrial DNA mutants [166]. TPP is a widely used organic cation that can penetrate the mitochondrial membrane and locates within the mitochondria due to the negative potential of the mitochondrial lining. For example, Jiao et al. specifically delivered lipophilic TPP-modified therapeutic recombinant P53 proteins (TPP/P53) through extracellular vesicles (EVs) derived from breast cancer cells. After the vesicles are effectively captured by cancer cells, the loaded TPP/P53 becomes capable of precisely aiming at the mitochondria after cellular internalization [167].

The ER or GA, as a central organelle for the processing and distribution of proteins, lipids, and ions, is closely related to diseases. It was found that grp78, grp94, and protein disulfide isomerase have ER-targeting function, because their C-terminals have ER-targeting peptide KDEL responsible for ER localization [158]. For example, in order to locate the HaloTag protein in the ER, Murrey et al. conjugated the ER-targeting peptide KDEL to the HaloTag protein fusion by bioorthogonal click chemistry [168]. GA-based biomolecule delivery is mainly focused on peptide delivery. As an illustration, Yu and colleagues described an example of self-sorting peptide assembly capable of, respectively, targeting ER and GA [169]. Xu et al. reported the EISA of ALP cleavable peptides capable of targeting GA [170]. Certain diseases related to the deficiency of specific lysosomal enzymes (so-called storage diseases) can only be treated by administering exogenous enzymes. The acidic internal environment of lysosomes (pH ~5.0) favors the accumulation of weakly basic molecules, enabling the passive targeting of small molecules containing basic amines and lipophilic components to lysosomes [171]. Moreover, it is also possible to actively target lysosomes through targeted signals. Mannose 6-phosphate (M6P) serves as a notable targeting signal, capable of attaching to the M6P receptor (M6PR) within the lysosome targeting receptors (LTRs) group, identified by the GA, and can transport cargos to lysosomes. As an illustration, the facilitation of enzyme replacement therapy (ERT) mainly occurs through the interplay of M6P residues on external enzymes with

M6PR on most cell surfaces, facilitating endocytosis to effectively target lysosomes [172].

Delivering therapeutic agents to treat organelle-related diseases is crucial. Despite progress in recent years, the delivery of organelle-targeting proteins/peptides remains challenging. Many organelle-targeting domains also have unknown or unclear targeting mechanisms, which are currently being gradually explored.

6 | Conclusion and Perspective

With the development of molecular biology and recombinant technology to promote the development of new proteins, it is urgent to design an efficient protein intracellular delivery vector. Intracellularly targeted protein formulations would represent an exciting new class of drugs for use in many therapeutic areas, including oncology, infectious diseases, and genetic disorders. Although cytoplasmic protein delivery has enormous therapeutic promise, no FDA-approved medications have been created with this technique to date. The creation of efficient and scalable intracellular delivery techniques is now a significant obstacle to the clinical application of protein therapies.

Over the past decades, many strategies for cytoplasmic protein delivery have been proposed. Unlike nucleic acids containing similar repeating units, therapeutic proteins/peptides are often fundamentally different in size, structure, surface charge, hydrophilicity/hydrophobicity, and oligomerization state. On the basis of the functional, structural, and physical characteristics of the protein pharmaceuticals, each of the increasing number of cytoplasmic delivery techniques has certain benefits and drawbacks. Initially, CPPs attracted considerable interest as possible cellular transduction domains that may effectively transfer proteins by linking proteins covalently or noncovalently. It is important to keep in mind that CPP-based delivery strategies must address insufficient cell selectivity, unwanted toxicity, internalization efficiency, and endosomal escape. Furthermore, researchers are beginning to use nanocarriers for protein transfer. Functional tunability, high loading capacity, lowered immunological response, and defense against enzymatic degradation are only a few of the numerous desirable qualities of NPs. The creation of a universal nano-delivery vehicle for cytoplasmic protein transport, such as those employed for nucleic acid delivery, has the potential to transform biotechnology and improve human health. Regardless of the delivery method, the main focus is to maintain cargo function through all stages of the purification, modification, and delivery process. The integration of highly charged or hydrophobic CPPs can significantly impact the folding, expression, and functionality of the intended therapeutic proteins. Proteins may become denatured when exposed to high salt concentrations, harsh pH levels, or organic solvents over a long period. Furthermore, the frequent freeze–thaw cycles typically employed in liposome encapsulation can cause permanent damage to the encapsulated substance. Therefore, basic research is required to better comprehend how proteins interact with peptides, polymers, lipids, detergents, and solvents to enhance cargo stability and performance. In addition, multiple challenges regarding in vivo targeting efficiency, specificity, stability, biocompatibility, systemic toxicity, and immunogenicity of delivery carriers still need to be properly addressed. At the same time, they must be sufficiently straightforward in their design and formulation to enable large-scale manufacturing. In general, additional advancements are required to convert these fundamental studies on intracellular protein transport into preclinical or clinical investigations and, ultimately, into commercial products. Moreover, the targeted delivery of proteins/peptides to organelles has great clinical application prospects, but it remains challenging. Therefore, by understanding the latest strategies and issues in the subcellular targeted delivery of proteins/peptides, we can develop and study advanced and innovative organelle-targeting systems that can open a new era. With continuous optimization, these subcellular targeted delivery strategies will make a significant contribution to advanced drug delivery systems in the future.

Author Contributions

Wenyan Zhang wrote the manuscript. Wen Li and Xue Han selected the topic and designed the framework of the article. Jiaojiao Fu and Renjie Luo collected and downloaded all references. Huiling Liu and Bingdong Zhu revised the manuscript. Haiyan Wang and Jinxia Wang contributed to the revision and graphics of the article.

Acknowledgments

The authors thank the National Natural Science Foundation of China (82260557), Lanzhou Science and Technology Plan Project (2023-ZD-21), and Gansu Provincial People's Hospital Fund (22GSSYD-39) for financial support.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

References

1. J. Lv, Q. Fan, H. Wang, and Y. Cheng, "Polymers for Cytosolic Protein Delivery," *Biomaterials* 218 (2019): 119358.

2. E. Cho, G. H. Nam, Y. Hong, et al., "Comparison of Exosomes and Ferritin Protein Nanocages for the Delivery of Membrane Protein Therapeutics," *Journal of Controlled Release* 279 (2018): 326–335.

3. S. Mitragotri, P. A. Burke, and R. Langer, "Overcoming the Challenges in Administering Biopharmaceuticals: Formulation and Delivery Strategies," *Nature Reviews Drug Discovery* 13 (2014): 655–672.

4. H. Acar, J. M. Ting, S. Srivastava, J. L. LaBelle, and M. V. Tirrell, "Molecular Engineering Solutions for Therapeutic Peptide Delivery," *Chemical Society Reviews* 46 (2017): 6553–6569.

5. A. Fu, R. Tang, J. Hardie, M. E. Farkas, and V. M. Rotello, "Promises and Pitfalls of Intracellular Delivery of Proteins," *Bioconjugate Chemistry* 25 (2014): 1602–1608.

6. W. Han, Z. Chen, P. Niu, X. Ren, C. Ding, and S. Yu, "Development of a Colloidal Gold Immunochromatographic Strip for Rapid Detection of *Riemerella anatipestifer* in Ducks," *Poultry Science* 99 (2020): 4741–4749.

7. C. J. Smoyer and S. L. Jaspersen, "Breaking Down the Wall: The Nuclear Envelope During Mitosis," *Current Opinion in Cell Biology* 26 (2014): 1–9.

8. J. Li, J. J. Røise, M. He, R. Das, and N. Murthy, "Non-Viral Strategies for Delivering Genome Editing Enzymes," *Advanced Drug Delivery Reviews* 168 (2021): 99–117.

9. M. P. Stewart, A. Sharei, X. Ding, G. Sahay, R. Langer, and K. F. Jensen, "In Vitro and Ex Vivo Strategies for Intracellular Delivery," *Nature* 538 (2016): 183–192.

10. C. Yu, E. Tan, Y. Xu, J. Lv, and Y. Cheng, "A Guanidinium-Rich Polymer for Efficient Cytosolic Delivery of Native Proteins," *Bioconjugate Chemistry* 30 (2019): 413–417.

11. M. Wang, J. A. Zuris, F. Meng, et al., "Efficient Delivery of Genome-Editing Proteins Using Bioreducible Lipid Nanoparticles," *Proceedings of the National Academy of Sciences of the United States of America* 113 (2016): 2868–2873.

12. N. M. Nguyen Le, S. Zsák, B. Le-Vinh, et al., "Charge-Converting Nanoemulsions as Promising Retinal Drug and Gene Delivery Systems," *ACS Applied Materials & Interfaces* 14 (2022): 44981–44991.

13. X. Liu, Z. Zhao, F. Wu, Y. Chen, and L. Yin, "Tailoring Hyperbranched Poly(*β*-Amino Ester) as a Robust and Universal Platform for Cytosolic Protein Delivery," *Advanced Materials* 34 (2022): e2108116.

14. L. Fu, X. Hua, X. Jiang, and J. Shi, "Multistage Systemic and Cytosolic Protein Delivery for Effective Cancer Treatment," *Nano Letters* 22 (2022): 111–118.

15. B. Liu, W. Ejaz, S. Gong, et al., "Engineered Interactions With Mesoporous Silica Facilitate Intracellular Delivery of Proteins and Gene Editing," *Nano Letters* 20 (2020): 4014–4021.

16. E. A. Prasetyanto, A. Bertucci, D. Septiadi, R. Corradini, P. Castro-Hartmann, and L. De Cola, "Breakable Hybrid Organosilica Nanocapsules for Protein Delivery," *Angewandte Chemie (International Edition in English)* 55 (2016): 3323–3327.

17. A. Harizaj, F. Van Hauwermeiren, S. Stremersch, et al., "Nanoparticle-Sensitized Photoporation Enables Inflammasome Activation Studies in Targeted Single Cells," *Nanoscale* 13 (2021): 6592–6604.

18. T. C. Südhof and J. E. Rothman, "Membrane Fusion: Grappling With SNARE and SM Proteins," *Science* 323 (2009): 474–477.

19. F. Heitz, M. C. Morris, and G. Divita, "Twenty Years of Cell-Penetrating Peptides: From Molecular Mechanisms to Therapeutics," *British Journal of Pharmacology* 157 (2009): 195–206.

20. R. Todorova, "Comparative Analysis of the Methods of Drug and Protein Delivery for the Treatment of Cancer, Genetic Diseases and Diagnostics," *Drug Delivery* 18 (2011): 586–598.

21. K. B. Johnsen, J. M. Gudbergsson, M. N. Skov, L. Pilgaard, T. Moos, and M. Duroux, "A Comprehensive Overview of Exosomes as Drug Delivery Vehicles—Endogenous Nanocarriers for Targeted Cancer Therapy," *Biochimica Et Biophysica Acta* 1846 (2014): 75–87.

22. T. Y. Yoon, B. Okumus, F. Zhang, Y. K. Shin, and T. Ha, "Multiple Intermediates in SNARE-Induced Membrane Fusion," *Proceedings of the National Academy of Sciences of the United States of America* 103 (2006): 19731–19736.

23. W. C. Wimley, "Describing the Mechanism of Antimicrobial Peptide Action With the Interfacial Activity Model," *ACS Chemical Biology* 5 (2010): 905–917.

24. W. B. Kauffman, T. Fuselier, J. He, and W. C. Wimley, "Mechanism Matters: A Taxonomy of Cell Penetrating Peptides," *Trends in Biochemical Sciences* 40 (2015): 749–764.

25. J. B. Rothbard, T. C. Jessop, and P. A. Wender, "Adaptive Translocation: The Role of Hydrogen Bonding and Membrane Potential in the Uptake of Guanidinium-Rich Transporters Into Cells," *Advanced Drug Delivery Reviews* 57 (2005): 495–504.

26. M. Vazdar, J. Heyda, P. E. Mason, et al., "Arginine "Magic": Guanidinium Like-Charge Ion Pairing From Aqueous Salts to Cell Penetrating Peptides," *Accounts of Chemical Research* 51 (2018): 1455–1464.

27. C. Allolio, K. Baxova, M. Vazdar, and P. Jungwirth, "Guanidinium Pairing Facilitates Membrane Translocation," *Journal of Physical Chemistry B* 120 (2016): 143–153.

28. P. P. Di Fiore and M. von Zastrow, "Endocytosis, Signaling, and Beyond," *Cold Spring Harbor Perspectives in Biology* 6 (2014): a016865.

29. M. V. Park, A. M. Neigh, J. P. Vermeulen, et al., "The Effect of Particle Size on the Cytotoxicity, Inflammation, Developmental Toxicity and Genotoxicity of Silver Nanoparticles," *Biomaterials* 32 (2011): 9810–9817.

30. Z. Krpetić, F. Porta, E. Caneva, V. Dal Santo, and G. Scarì, "Phagocytosis of Biocompatible Gold Nanoparticles," *Langmuir* 26 (2010): 14799–14805.

31. S. Zhang, H. Gao, and G. Bao, "Physical Principles of Nanoparticle Cellular Endocytosis," *ACS Nano* 9 (2015): 8655–8671.

32. G. Sahay, J. O. Kim, A. V. Kabanov, and T. K. Bronich, "The Exploitation of Differential Endocytic Pathways in Normal and Tumor Cells in the Selective Targeting of Nanoparticulate Chemotherapeutic Agents," *Biomaterials* 31 (2010): 923–933.

33. G. Sahay, D. Y. Alakhova, and A. V. Kabanov, "Endocytosis of Nanomedicines," *Journal of Controlled Release* 145 (2010): 182–195.

34. J. A. Zuris, D. B. Thompson, Y. Shu, et al., "Cationic Lipid-Mediated Delivery of Proteins Enables Efficient Protein-Based Genome Editing In Vitro and In Vivo," *Nature Biotechnology* 33 (2015): 73–80.

35. M. Kaksonen and A. Roux, "Mechanisms of Clathrin-Mediated Endocytosis," *Nature Reviews Molecular Cell Biology* 19 (2018): 313–326.

36. L. M. Traub and J. S. Bonifacino, "Cargo Recognition in Clathrin-Mediated Endocytosis," *Cold Spring Harbor Perspectives in Biology* 5 (2013): a016790.

37. M. Sousa de Almeida, E. Susnik, B. Drasler, P. Taladriz-Blanco, A. Petri-Fink, and B. Rothen-Rutishauser, "Understanding Nanoparticle Endocytosis to Improve Targeting Strategies in Nanomedicine," *Chemical Society Reviews* 50 (2021): 5397–5434.

38. L. Xu, S. L. Kuan, and T. Weil, "Contemporary Approaches for Site-Selective Dual Functionalization of Proteins," *Angewandte Chemie (International Edition in English)* 60 (2021): 13757–13777.

39. Y. Wang, Y. Xie, J. Li, et al., "Tumor-Penetrating Nanoparticles for Enhanced Anticancer Activity of Combined Photodynamic and Hypoxia-Activated Therapy," *ACS Nano* 11 (2017): 2227–2238.

40. E. Gros, S. Deshayes, M. C. Morris, et al., "A Non-Covalent Peptide-Based Strategy for Protein and Peptide Nucleic Acid Transduction," *Biochimica Et Biophysica Acta* 1758 (2006): 384–393.

41. M. Akishiba, T. Takeuchi, Y. Kawaguchi, et al., "Cytosolic Antibody Delivery by Lipid-Sensitive Endosomolytic Peptide," *Nature Chemistry* 9 (2017): 751–761.

42. K. Sakamoto, M. Akishiba, T. Iwata, et al., "Optimizing Charge Switching in Membrane Lytic Peptides for Endosomal Release of Biomacromolecules," *Angewandte Chemie (International Edition in English)* 59 (2020): 19990–19998.

43. W. M. Chien, Y. Liu, and M. T. Chin, "Genomic DNA Recombination With Cell-Penetrating Peptide-Tagged Cre Protein in Mouse Skeletal and Cardiac Muscle," *Genesis (New York, N.Y.: 2000)* 52 (2014): 695–701.

44. S. Ramakrishna, A. B. Kwaku Dad, J. Beloor, R. Gopalappa, S. K. Lee, and H. Kim, "Gene Disruption by Cell-Penetrating Peptide-Mediated Delivery of Cas9 Protein and Guide RNA," *Genome Research* 24 (2014): 1020–1027.

45. S. Fawell, J. Seery, Y. Daikh, et al., "Tat-Mediated Delivery of Heterologous Proteins Into Cells," *Proceedings of the National Academy of Sciences of the United States of America* 91 (1994): 664–668.

46. H. Harada, M. Hiraoka, and S. Kizaka-Kondoh, "Antitumor Effect of TAT-Oxygen-Dependent Degradation-Caspase-3 Fusion Protein Specifically Stabilized and Activated in Hypoxic Tumor Cells," *Cancer Research* 62 (2002): 2013–2018.

47. X. Chen, F. Sa'adedin, B. Deme, P. Rao, and J. Bradshaw, "Insertion of TAT Peptide and Perturbation of Negatively Charged Model Phospholipid Bilayer Revealed by Neutron Diffraction," *Biochimica Et Biophysica Acta* 1828 (2013): 1982–1988.

48. E. Vivès, P. Brodin, and B. Lebleu, "A Truncated HIV-1 Tat Protein Basic Domain Rapidly Translocates Through the Plasma Membrane and Accumulates in the Cell Nucleus," *Journal of Biological Chemistry* 272 (1997): 16010–16017.

49. T. K. Kim and J. H. Eberwine, "Mammalian Cell Transfection: The Present and the Future," *Analytical and Bioanalytical Chemistry* 397 (2010): 3173–3178.

50. A. Fu, Y. Wang, L. Zhan, and R. Zhou, "Targeted Delivery of Proteins Into the Central Nervous System Mediated by Rabies Virus Glycoprotein-Derived Peptide," *Pharmaceutical Research* 29 (2012): 1562–1569.

51. D. Jo, D. Liu, S. Yao, R. D. Collins, and J. Hawiger, "Intracellular Protein Therapy With SOCS3 Inhibits Inflammation and Apoptosis," *Nature Medicine* 11 (2005): 892–898.

52. J. Mueller, I. Kretzschmar, R. Volkmer, and P. Boisguerin, "Comparison of Cellular Uptake Using 22 CPPs in 4 Different Cell Lines," *Bioconjugate Chemistry* 19 (2008): 2363–2374.

53. P. Lönn and S. F. Dowdy, "Cationic PTD/CPP-Mediated Macromolecular Delivery: Charging Into the Cell," *Expert Opinion on Drug Delivery* 12 (2015): 1627–1636.

54. R. Glogau, A. Blitzer, F. Brandt, M. Kane, G. D. Monheit, and J. M. Waugh, "Results of a Randomized, Double-Blind, Placebo-Controlled Study to Evaluate the Efficacy and Safety of a Botulinum Toxin Type A Topical Gel for the Treatment of Moderate-to-Severe Lateral Canthal Lines," *Journal of Drugs in Dermatology* 11 (2012): 38–45.

55. S. Yu, H. Yang, T. Li, et al., "Efficient Intracellular Delivery of Proteins by a Multifunctional Chimaeric Peptide In Vitro and In Vivo," *Nature Communications* 12 (2021): 5131.

56. J. C. LeCher, S. J. Nowak, and J. L. McMurry, "Breaking in and Busting Out: Cell-Penetrating Peptides and the Endosomal Escape Problem," *Biomolecular Concepts* 8 (2017): 131–141.

57. N. S. El-Sayed, T. Miyake, A. N. Shirazi, et al., "Design, Synthesis, and Evaluation of Homochiral Peptides Containing Arginine and Histidine as Molecular Transporters," *Molecules* 23 (2018): 1590.

58. M. Amoura, F. Illien, A. Joliot, et al., "Head to Tail Cyclisation of Cell-Penetrating Peptides: Impact on GAG-Dependent Internalisation and Direct Translocation," *Chemical Communications (Cambridge, England)* 55 (2019): 4566–4569.

59. G. Lättig-Tünnemann, M. Prinz, D. Hoffmann, et al., "Backbone Rigidity and Static Presentation of Guanidinium Groups Increases Cellular Uptake of Arginine-Rich Cell-Penetrating Peptides," *Nature Communications* 2 (2011): 453.

60. J. Yin, Q. Wang, S. Hou, L. Bao, W. Yao, and X. Gao, "Potent Protein Delivery Into Mammalian Cells via a Supercharged Polypeptide," *Journal of the American Chemical Society* 140 (2018): 17234–17240.

61. R. Mout, M. Ray, T. Tay, K. Sasaki, G. Yesilbag Tonga, and V. M. Rotello, "General Strategy for Direct Cytosolic Protein Delivery via Protein–Nanoparticle Co-Engineering," *ACS Nano* 11 (2017): 6416–6421.

62. Z. Qian, T. Liu, Y. Y. Liu, et al., "Efficient Delivery of Cyclic Peptides Into Mammalian Cells With Short Sequence Motifs," *ACS Chemical Biology* 8 (2013): 423–431.

63. Z. Qian, J. R. LaRochelle, B. Jiang, et al., "Early Endosomal Escape of a Cyclic Cell-Penetrating Peptide Allows Effective Cytosolic Cargo Delivery," *Biochemistry* 53 (2014): 4034–4046.

64. B. R. Liu, S. Y. Lo, C. C. Liu, et al., "Endocytic Trafficking of Nanoparticles Delivered by Cell-Penetrating Peptides Comprised of Nona-Arginine and a Penetration Accelerating Sequence," *PLoS ONE* 8 (2013): e67100.

65. J. S. Appelbaum, J. R. LaRochelle, B. A. Smith, D. M. Balkin, J. M. Holub, and A. Schepartz, "Arginine Topology Controls Escape of Minimally Cationic Proteins From Early Endosomes to the Cytoplasm," *Chemistry & Biology* 19 (2012): 819–830.

66. A. Erazo-Oliveras, N. Muthukrishnan, R. Baker, T. Y. Wang, and J. P. Pellois, "Improving the Endosomal Escape of Cell-Penetrating Peptides and Their Cargos: Strategies and Challenges," *Pharmaceuticals (Basel)* 5 (2012): 1177–1209.

67. Y. J. Chen, Q. W. Deng, L. Wang, et al., "GALA Peptide Improves the Potency of Nanobody–Drug Conjugates by Lipid-Induced Helix Formation," *Chemical Communications (Cambridge, England)* 57 (2021): 1434–1437.

68. J. Kuehne and R. M. Murphy, "Synthesis and Characterization of Membrane-Active GALA-OKT9 Conjugates," *Bioconjugate Chemistry* 12 (2001): 742–749.

69. M. Li, Y. Tao, Y. Shu, et al., "Discovery and Characterization of a Peptide That Enhances Endosomal Escape of Delivered Proteins In Vitro and In Vivo," *Journal of the American Chemical Society* 137 (2015): 14084–14093.

70. T. R. Kyriakides, C. Y. Cheung, N. Murthy, P. Bornstein, P. S. Stayton, and A. S. Hoffman, "pH-Sensitive Polymers That Enhance Intracellular Drug Delivery In Vivo," *Journal of Controlled Release* 78 (2002): 295–303.

71. W. Du, S. Du, X. Dong, et al., "Biodegradable Silica Nanocapsules Enable Efficient Nuclear-Targeted Delivery of Native Proteins for Cancer Therapy," *Biomaterials* 294 (2023): 122000.

72. K. Sakamoto, H. Furukawa, J. V. V. Arafiles, M. Imanishi, K. Matsuura, and S. Futaki, "Artificial Nanocage Formed via Self-Assembly of β-Annulus Peptide for Delivering Biofunctional Proteins Into Cell Interiors," *Bioconjugate Chemistry* 33 (2022): 311–320.

73. S. B. Gentry, S. J. Nowak, X. Ni, et al., "A Real-Time Assay for Cell-Penetrating Peptide-Mediated Delivery of Molecular Cargos," *PLoS ONE* 16 (2021): e0254468.

74. M. C. Morris, J. Depollier, J. Mery, F. Heitz, and G. Divita, "A Peptide Carrier for the Delivery of Biologically Active Proteins Into Mammalian Cells," *Nature Biotechnology* 19 (2001): 1173–1176.

75. A. Lesniak, A. Salvati, M. J. Santos-Martinez, M. W. Radomski, K. A. Dawson, and C. Åberg, "Nanoparticle Adhesion to the Cell Membrane and Its Effect on Nanoparticle Uptake Efficiency," *Journal of the American Chemical Society* 135 (2013): 1438–1444.

76. N. Chen, Y. He, M. Zang, et al., "Approaches and Materials for Endocytosis-Independent Intracellular Delivery of Proteins," *Biomaterials* 286 (2022): 121567.

77. M. Yu, J. Wu, J. Shi, and O. C. Farokhzad, "Nanotechnology for Protein Delivery: Overview and Perspectives," *Journal of Controlled Release* 240 (2016): 24–37.

78. Y. B. Kim, K. T. Zhao, D. B. Thompson, and D. R. Liu, "An Anionic Human Protein Mediates Cationic Liposome Delivery of Genome Editing Proteins Into Mammalian Cells," *Nature Communications* 10 (2019): 2905.

79. W. Wang, S. Wu, J. Wang, et al., "Superoxide Dismutase Transcellular Shuttle Constructed From Dendritic MOF and Charge Reversible Protein Derivatives," *Chemical Science* 10 (2019): 4476–4485.

80. H. Cui, L. Zhang, S. Zeng, et al., "Charge-Reversible Pro-Ribonuclease Enveloped In Virus-Like Synthetic Nanocapsules for Systemic Treatment of Intractable Glioma," *ACS Applied Materials & Interfaces* 14 (2022): 30493–30506.

81. M. Wang, K. Alberti, S. Sun, C. L. Arellano, and Q. Xu, "Combinatorially Designed Lipid-Like Nanoparticles for Intracellular Delivery of Cytotoxic Protein for Cancer Therapy," *Angewandte Chemie (International Edition in English)* 53 (2014): 2893–2898.

82. J. Li, J. Yu, J. Zhao, et al., "Palladium-Triggered Deprotection Chemistry for Protein Activation in Living Cells," *Nature Chemistry* 6 (2014): 352–361.

83. J. Zhao, S. Lin, Y. Huang, J. Zhao, and P. R. Chen, "Mechanism-Based Design of a Photoactivatable Firefly Luciferase," *Journal of the American Chemical Society* 135 (2013): 7410–7413.

84. M. Wang, S. Sun, C. I. Neufeld, B. Perez-Ramirez, and Q. Xu, "Reactive Oxygen Species-Responsive Protein Modification and Its Intracellular Delivery for Targeted Cancer Therapy," *Angewandte Chemie (International Edition in English)* 53 (2014): 13444–13448.

85. S. Pal, B. Reshma G, F. P. Mohny, et al., "Albumin Nanoparticles Surface Decorated With a Tumor-Homing Peptide Help in Selective Killing of Triple-Negative Breast Cancer Cells," *ACS Applied Materials & Interfaces* 15 (2023): 46721–46737.

86. Y. Li, Y. Zhang, and B. Yan, "Nanotoxicity Overview: Nano-Threat to Susceptible Populations," *International Journal of Molecular Sciences* 15 (2014): 3671–3697.

87. A. D. Bangham, M. M. Standish, and J. C. Watkins, "Diffusion of Univalent Ions Across the Lamellae of Swollen Phospholipids," *Journal of Molecular Biology* 13 (1965): 238–252.

88. J. T. Granados-Riveron and G. Aquino-Jarquin, "Engineering of the Current Nucleoside-Modified mRNA-LNP Vaccines Against SARS-CoV-2," *Biomedicine & Pharmacotherapy* 142 (2021): 111953.

89. Z. Gu, A. Biswas, M. Zhao, and Y. Tang, "Tailoring Nanocarriers for Intracellular Protein Delivery," *Chemical Society Reviews* 40 (2011): 3638–3655.

90. H. H. Wang and A. Tsourkas, "Cytosolic Delivery of Inhibitory Antibodies With Cationic Lipids," *Proceedings of the National Academy of Sciences of the United States of America* 116 (2019): 22132–22139.

91. X. Wei, B. Shao, Z. He, et al., "Cationic Nanocarriers Induce Cell Necrosis Through Impairment of Na+/K+-ATPase and Cause Subsequent Inflammatory Response," *Cell Research* 25 (2015): 237–253.

92. Y. Hirai, R. Saeki, F. Song, et al., "Charge-Reversible Lipid Derivative: A Novel Type of pH-Responsive Lipid for Nanoparticle-Mediated siRNA Delivery," *International Journal of Pharmaceutics* 585 (2020): 119479.

93. Y. Hirai, H. Hirose, M. Imanishi, T. Asai, and S. Futaki, "Cytosolic Protein Delivery Using pH-Responsive, Charge-Reversible Lipid Nanoparticles," *Scientific Reports* 11 (2021): 19896.

94. S. Altınoglu, M. Wang, and Q. Xu, "Combinatorial Library Strategies for Synthesis of Cationic Lipid-Like Nanoparticles and Their Potential Medical Applications," *Nanomedicine (London)* 10 (2015): 643–657.

95. Y. Li, T. Yang, Y. Yu, et al., "Combinatorial Library of Chalcogen-Containing Lipidoids for Intracellular Delivery of Genome-Editing Proteins," *Biomaterials* 178 (2018): 652–662.

96. Y. Li, R. Li, A. Chakraborty, et al., "Combinatorial Library of Cyclic Benzylidene Acetal-Containing pH-Responsive Lipidoid Nanoparticles for Intracellular mRNA Delivery," *Bioconjugate Chemistry* 31 (2020): 1835–1843.

97. M. Wang, K. Alberti, A. Varone, D. Pouli, I. Georgakoudi, and Q. Xu, "Enhanced Intracellular siRNA Delivery Using Bioreducible Lipid-Like Nanoparticles," *Advanced Healthcare Materials* 3 (2014): 1398–1403.

98. S. Zhang, J. Lv, P. Gao, Q. Feng, H. Wang, and Y. Cheng, "A pH-Responsive Phase-Transition Polymer With High Serum Stability in Cytosolic Protein Delivery," *Nano Letters* 21 (2021): 7855–7861.

99. M. E. Distler, M. H. Teplensky, K. E. Bujold, C. D. Kusmierz, M. Evangelopoulos, and C. A. Mirkin, "DNA Dendrons as Agents for Intracellular Delivery," *Journal of the American Chemical Society* 143 (2021): 13513–13518.

100. H. S. Yoo, H. K. Choi, and T. G. Park, "Protein–Fatty Acid Complex for Enhanced Loading and Stability Within Biodegradable Nanoparticles," *Journal of Pharmaceutical Sciences* 90 (2001): 194–201.

101. K. A. Black, D. Priftis, S. L. Perry, J. Yip, W. Y. Byun, and M. Tirrell, "Protein Encapsulation via Polypeptide Complex Coacervation," *ACS Macro Letters* 3 (2014): 1088–1091.

102. R. Varshochian, M. Jeddi-Tehrani, A. R. Mahmoudi, et al., "The Protective Effect of Albumin on Bevacizumab Activity and Stability in PLGA Nanoparticles Intended for Retinal and Choroidal Neovascularization Treatments," *European Journal of Pharmaceutical Sciences* 50 (2013): 341–352.

103. A. O. Elzoghby, W. M. Samy, and N. A. Elgindy, "Albumin-Based Nanoparticles as Potential Controlled Release Drug Delivery Systems," *Journal of Controlled Release* 157 (2012): 168–182.

104. L. C. Nelemans, V. A. Melo, M. Buzgo, E. Bremer, and A. Simaite, "Antibody Desolvation With Sodium Chloride and Acetonitrile Generates Bioactive Protein Nanoparticles," *PLoS ONE* 19 (2024): e0300416.

105. M. Morales-Cruz, G. M. Flores-Fernández, M. Morales-Cruz, et al., "Two-Step Nanoprecipitation for the Production of Protein-Loaded PLGA Nanospheres," *Results in Pharma Sciences* 2 (2012): 79–85.

106. C. von Roemeling, W. Jiang, C. K. Chan, I. L. Weissman, and B. Y. S. Kim, "Breaking Down the Barriers to Precision Cancer Nanomedicine," *Trends in Biotechnology* 35 (2017): 159–171.

107. B. Wang, X. He, Z. Zhang, Y. Zhao, and W. Feng, "Metabolism of Nanomaterials In Vivo: Blood Circulation and Organ Clearance," *Accounts of Chemical Research* 46 (2013): 761–769.

108. Y. Matsumoto, J. W. Nichols, K. Toh, et al., "Vascular Bursts Enhance Permeability of Tumour Blood Vessels and Improve Nanoparticle Delivery," *Nature Nanotechnology* 11 (2016): 533–538.

109. Y. Liu, Z. Wang, Y. Liu, et al., "Suppressing Nanoparticle-Mononuclear Phagocyte System Interactions of Two-Dimensional Gold Nanorings for Improved Tumor Accumulation and Photothermal Ablation of Tumors," *ACS Nano* 11 (2017): 10539–10548.

110. H. Ren, Y. He, J. Liang, et al., "Role of Liposome Size, Surface Charge, and PEGylation on Rheumatoid Arthritis Targeting Therapy," *ACS Applied Materials & Interfaces* 11 (2019): 20304–20315.

111. M. L. Immordino, F. Dosio, and L. Cattel, "Stealth Liposomes: Review of the Basic Science, Rationale, and Clinical Applications, Existing and Potential," *International Journal of Nanomedicine* 1 (2006): 297–315.

112. P. Zhang, F. Sun, C. Tsao, et al., "Zwitterionic Gel Encapsulation Promotes Protein Stability, Enhances Pharmacokinetics, and Reduces Immunogenicity," *Proceedings of the National Academy of Sciences of the United States of America* 112 (2015): 12046–12051.

113. E. Wenande and L. H. Garvey, "Immediate-Type Hypersensitivity to Polyethylene Glycols: A Review," *Clinical and Experimental Allergy* 46 (2016): 907–922.

114. Z. Qin, T. Chen, W. Teng, Q. Jin, and J. Ji, "Mixed-Charged Zwitterionic Polymeric Micelles for Tumor Acidic Environment Responsive Intracellular Drug Delivery," *Langmuir* 35 (2019): 1242–1248.

115. L. D. Blackman, P. A. Gunatillake, P. Cass, and K. E. S. Locock, "An Introduction to Zwitterionic Polymer Behavior and Applications in Solution and at Surfaces," *Chemical Society Reviews* 48 (2019): 757–770.

116. Q. Xu, C. Shu, Y. Li, R. Zhuang, L. Jiang, and X. Xu, "Controllable Mixed-Charged Co-Assembly of Dendritic Lipopeptides Into Invisible Capsid-Like Nanoparticles as Potential Drug Carriers," *Chemical Communications (Cambridge, England)* 57 (2021): 4859–4862.

117. Q. Wang, Y. Yang, D. Liu, et al., "Cytosolic Protein Delivery for Intracellular Antigen Targeting Using Supercharged Polypeptide Delivery Platform," *Nano Letters* 21 (2021): 6022–6030.

118. J. Tang, Y. Liu, D. Qi, et al., "Nucleus-Targeted Delivery of Multi-Protein Self-Assembly for Combined Anticancer Therapy," *Small* 17 (2021): 2101219.

119. Y. Zhao, K. Feng, G. Lei, et al., "A Smart Magnetic Nanosystem for Sequential Extracellular and Intracellular Release of Proteins for Cancer Therapy," *Nanoscale* 15 (2023): 11280–11289.

120. E. Klein, C. Leborgne, M. Ciobanu, et al., "Nucleic Acid Transfer With Hemifluorinated Polycationic Lipids," *Biomaterials* 31 (2010): 4781–4788.

121. C. Zhang, K. Yan, C. Fu, H. Peng, C. J. Hawker, and A. K. Whittaker, "Biological Utility of Fluorinated Compounds: From Materials Design

to Molecular Imaging, Therapeutics and Environmental Remediation," *Chemical Reviews* 122 (2022): 167–208.

122. J. Lv, H. Wang, G. Rong, and Y. Cheng, "Fluorination Promotes the Cytosolic Delivery of Genes, Proteins, and Peptides," *Accounts of Chemical Research* 55 (2022): 722–733.

123. S. A. Smith, L. I. Selby, A. P. R. Johnston, and G. K. Such, "The Endosomal Escape of Nanoparticles: Toward More Efficient Cellular Delivery," *Bioconjugate Chemistry* 30 (2019): 263–272.

124. K. I. Cupic, J. J. Rennick, A. P. Johnston, and G. K. Such, "Controlling Endosomal Escape Using Nanoparticle Composition: Current Progress and Future Perspectives," *Nanomedicine (London)* 14 (2019): 215–223.

125. J. J. Zou, J. Q. Le, B. C. Zhang, et al., "Accelerating Transdermal Delivery of Insulin by Ginsenoside Nanoparticles With Unique Permeability," *International Journal of Pharmaceutics* 605 (2021): 120784.

126. Y. W. Lee, D. C. Luther, R. Goswami, et al., "Direct Cytosolic Delivery of Proteins Through Coengineering of Proteins and Polymeric Delivery Vehicles," *Journal of the American Chemical Society* 142 (2020): 4349–4355.

127. Y. Guo, E. Terazzi, R. Seemann, J. B. Fleury, and V. A. Baulin, "Direct Proof of Spontaneous Translocation of Lipid-Covered Hydrophobic Nanoparticles Through a Phospholipid Bilayer," *Science Advances* 2 (2016): e1600261.

128. W. Tai, P. Zhao, and X. Gao, "Cytosolic Delivery of Proteins by Cholesterol Tagging," *Science Advances* 6 (2020): eabb0310.

129. W. Shen, Q. Wang, Y. Shen, et al., "Green Tea Catechin Dramatically Promotes RNAi Mediated by Low-Molecular-Weight Polymers," *ACS Central Science* 4 (2018): 1326–1333.

130. H. M. Ding and Y. Q. Ma, "Theoretical and Computational Investigations of Nanoparticle–Biomembrane Interactions in Cellular Delivery," *Small* 11 (2015): 1055–1071.

131. A. K. Sarkar, K. Debnath, H. Arora, P. Seth, N. R. Jana, and N. R. Jana, "Direct Cellular Delivery of Exogenous Genetic Material and Protein via Colloidal Nano-Assemblies With Biopolymer," *ACS Applied Materials & Interfaces* 14 (2022): 3199–3206.

132. Q. Hu, P. S. Katti, and Z. Gu, "Enzyme-Responsive Nanomaterials for Controlled Drug Delivery," *Nanoscale* 6 (2014): 12273–12286.

133. M. Shahriari, M. Zahiri, K. Abnous, S. M. Taghdisi, M. Ramezani, and M. Alibolandi, "Enzyme Responsive Drug Delivery Systems in Cancer Treatment," *Journal of Controlled Release* 308 (2019): 172–189.

134. H. Ding, P. Tan, S. Fu, et al., "Preparation and Application of pH-Responsive Drug Delivery Systems," *Journal of Controlled Release* 348 (2022): 206–238.

135. H. Zheng, L. Yin, X. Zhang, et al., "Redox Sensitive Shell and Core Crosslinked Hyaluronic Acid Nanocarriers for Tumor-Targeted Drug Delivery," *Journal of Biomedical Nanotechnology* 12 (2016): 1641–1653.

136. G. Ma, N. Kostevšek, I. Monaco, et al., "PD1 Blockade Potentiates the Therapeutic Efficacy of Photothermally-Activated and MRI-Guided Low Temperature-Sensitive Magnetoliposomes," *Journal of Controlled Release* 332 (2021): 419–433.

137. J. T. Wilson, S. Keller, M. J. Manganiello, et al., "pH-Responsive Nanoparticle Vaccines for Dual-Delivery of Antigens and Immunostimulatory Oligonucleotides," *ACS Nano* 7 (2013): 3912–3925.

138. J. De Mel, M. Hossain, O. Shofolawe-Bakare, et al., "Dual-Responsive Glycopolymers for Intracellular Codelivery of Antigen and Lipophilic Adjuvants," *Molecular Pharmaceutics* 19 (2022): 4705–4716.

139. S. Kudruk, S. Pottanam Chali, A. L. Linard Matos, et al., "Biodegradable and Dual-Responsive Polypeptide-Shelled Cyclodextrin-Containers for Intracellular Delivery of Membrane-Impermeable Cargo," *Advanced Science (Weinheim)* 8 (2021): 2100694.

140. S. M. Masloub, M. H. Elmalahy, D. Sabry, W. S. Mohamed, and S. H. Ahmed, "Comparative Evaluation of PLGA Nanoparticle Delivery System for 5-Fluorouracil and Curcumin on Squamous Cell Carcinoma," *Archives of Oral Biology* 64 (2016): 1–10.

141. H. Zhang, F. Wu, Y. Li, et al., "Chitosan-Based Nanoparticles for Improved Anticancer Efficacy and Bioavailability of Mifepristone," *Beilstein Journal of Nanotechnology* 7 (2016): 1861–1870.

142. N. O. Turky, N. A. Abdelmonem, S. N. Tammam, M. Z. Gad, H. G. Breitinger, and U. Breitinger, "Antibacterial and In Vitro Anticancer Activities of the Antimicrobial Peptide NRC-07 Encapsulated in Chitosan Nanoparticles," *Journal of Peptide Science* 30 (2024): e3550.

143. P. A. Gunatillake and R. Adhikari, "Biodegradable Synthetic Polymers for Tissue Engineering," *European Cells & Materials* [Electronic Resource] 5 (2003): 1–16. discussion 16.

144. F. Scaletti, J. Hardie, Y. W. Lee, D. C. Luther, M. Ray, and V. M. Rotello, "Protein Delivery Into Cells Using Inorganic Nanoparticle–Protein Supramolecular Assemblies," *Chemical Society Reviews* 47 (2018): 3421–3432.

145. R. R. Castillo, M. Colilla, and M. Vallet-Regí, "Advances in Mesoporous Silica-Based Nanocarriers for Co-Delivery and Combination Therapy Against Cancer," *Expert Opinion on Drug Delivery* 14 (2017): 229–243.

146. R. Narayan, U. Y. Nayak, A. M. Raichur, and S. Garg, "Mesoporous Silica Nanoparticles: A Comprehensive Review on Synthesis and Recent Advances," *Pharmaceutics* 10 (2018): 118.

147. I. I. Slowing, B. G. Trewyn, and V. S. Lin, "Mesoporous Silica Nanoparticles for Intracellular Delivery of Membrane-Impermeable Proteins," *Journal of the American Chemical Society* 129 (2007): 8845–8849.

148. J. Zhang, S. Karmakar, M. Yu, N. Mitter, J. Zou, and C. Yu, "Synthesis of Silica Vesicles With Controlled Entrance Size for High Loading, Sustained Release, and Cellular Delivery of Therapeutical Proteins," *Small* 10 (2014): 5068–5076.

149. A. K. Meka, P. L. Abbaraju, H. Song, et al., "A Vesicle Supra-Assembly Approach to Synthesize Amine-Functionalized Hollow Dendritic Mesoporous Silica Nanospheres for Protein Delivery," *Small* 12 (2016): 5169–5177.

150. B. G. Cha, J. H. Jeong, and J. Kim, "Extra-Large Pore Mesoporous Silica Nanoparticles Enabling Co-Delivery of High Amounts of Protein Antigen and Toll-Like Receptor 9 Agonist for Enhanced Cancer Vaccine Efficacy," *ACS Central Science* 4 (2018): 484–492.

151. L. Qiao, M. Chen, S. Li, et al., "A Peptide-Based Subunit Candidate Vaccine Against SARS-CoV-2 Delivered by Biodegradable Mesoporous Silica Nanoparticles Induced High Humoral and Cellular Immunity in Mice," *Biomaterials Science* 9 (2021): 7287–7296.

152. Y. Niu, M. Yu, A. Meka, et al., "Understanding the Contribution of Surface Roughness and Hydrophobic Modification of Silica Nanoparticles to Enhanced Therapeutic Protein Delivery," *Journal of Materials Chemistry B* 4 (2016): 212–219.

153. D. P. Morales, G. B. Braun, A. Pallaoro, et al., "Targeted Intracellular Delivery of Proteins With Spatial and Temporal Control," *Molecular Pharmaceutics* 12 (2015): 600–609.

154. P. C. de Faria, L. I. dos Santos, J. P. Coelho, et al., "Oxidized Multiwalled Carbon Nanotubes as Antigen Delivery System to Promote Superior CD8 + T Cell Response and Protection Against Cancer," *Nano Letters* 14 (2014): 5458–5470.

155. T. Jiang, W. Sun, Q. Zhu, et al., "Furin-Mediated Sequential Delivery of Anticancer Cytokine and Small-Molecule Drug Shuttled by Graphene," *Advanced Materials* 27 (2015): 1021–1028.

156. J. Zhang, M. Zheng, and Z. Xie, "Co-Assembled Hybrids of Proteins and Carbon Dots for Intracellular Protein Delivery," *Journal of Materials Chemistry B* 4 (2016): 5659–5663.

157. B. L. Timney, B. Raveh, R. Mironska, et al., "Simple Rules for Passive Diffusion Through the Nuclear Pore Complex," *Journal of Cell Biology* 215 (2016): 57–76.

158. H. Su, G. Rong, L. Li, and Y. Cheng, "Subcellular Targeting Strategies for Protein and Peptide Delivery," *Advanced Drug Delivery Reviews* 212 (2024): 115387.

159. J. Kong, Y. Wang, J. Zhang, W. Qi, R. Su, and Z. He, "Rationally Designed Peptidyl Virus-Like Particles Enable Targeted Delivery of Genetic Cargo," *Angewandte Chemie (International Edition in English)* 57 (2018): 14032–14036.

160. "Focusing on Mitochondrial Form and Function," *Nature Cell Biology* 20 (2018): 735, https://doi.org/10.1038/s41556-018-0139-7.

161. D. Ma, Q. Zong, Y. Du, et al., "Sequential Enzyme-Activated Macrotheranostic Probe for Selective Tumor Mitochondria Targeting," *Acta Biomaterialia* 135 (2021): 628–637.

162. E. K. Lei and S. O. Kelley, "Delivery and Release of Small-Molecule Probes in Mitochondria Using Traceless Linkers," *Journal of the American Chemical Society* 139 (2017): 9455–9458.

163. R. M. Chin, T. Panavas, J. M. Brown, and K. K. Johnson, "Optimized Mitochondrial Targeting of Proteins Encoded by Modified mRNAs Rescues Cells Harboring Mutations in mtATP6," *Cell Reports* 22 (2018): 2818–2826.

164. S. Yang, G. L. Wu, N. Li, et al., "A Mitochondria-Targeted Molecular Phototheranostic Platform for NIR-II Imaging-Guided Synergistic Photothermal/Photodynamic/Immune Therapy," *Journal of Nanobiotechnology* 20 (2022): 475.

165. X. Shao, C. Meng, W. Song, T. Zhang, and Q. Chen, "Subcellular Visualization: Organelle-Specific Targeted Drug Delivery and Discovery," *Advanced Drug Delivery Reviews* 199 (2023): 114977.

166. A. Kukat, C. Kukat, J. Brocher, et al., "Generation of ρ^0 Cells Utilizing a Mitochondrially Targeted Restriction Endonuclease and Comparative Analyses," *Nucleic Acids Research* 36 (2008): e44.

167. Y. Jiao, Y. Tang, Y. Li, et al., "Tumor Cell-Derived Extracellular Vesicles for Breast Cancer Specific Delivery of Therapeutic P53," *Journal of Controlled Release* 349 (2022): 606–616.

168. H. E. Murrey, J. C. Judkins, C. W. Am Ende, et al., "Systematic Evaluation of Bioorthogonal Reactions in Live Cells With Clickable HaloTag Ligands: Implications for Intracellular Imaging," *Journal of the American Chemical Society* 137 (2015): 11461–11475.

169. X. Liu, M. Li, J. Liu, et al., "In Situ Self-Sorting Peptide Assemblies in Living Cells for Simultaneous Organelle Targeting," *Journal of the American Chemical Society* 144 (2022): 9312–9323.

170. W. Tan, Q. Zhang, M. C. Quiñones-Frías, et al., "Enzyme-Responsive Peptide Thioesters for Targeting Golgi Apparatus," *Journal of the American Chemical Society* 144 (2022): 6709–6713.

171. A. K. Gilbert and M. D. Pluth, "Subcellular Delivery of Hydrogen Sulfide Using Small Molecule Donors Impacts Organelle Stress," *Journal of the American Chemical Society* 144 (2022): 17651–17660.

172. J. Paulk, "Lysosome-Targeting Chimeras Evolve," *Nature Chemical Biology* 17 (2021): 931–933.