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Advancing cancer gene therapy: the emerging role of nanoparticle delivery systems

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Abstract

Gene therapy holds immense potential due to its ability to precisely target oncogenes, making it a promising strategy for cancer treatment. Advances in genetic science and bioinformatics have expanded the applications of gene delivery technologies beyond detection and diagnosis to potential therapeutic interventions. However, traditional gene therapy faces significant challenges, including limited therapeutic efficacy and the rapid degradation of genetic materials in vivo. To address these limitations, multifunctional nanoparticles have been engineered to encapsulate and protect genetic materials, enhancing their stability and therapeutic effectiveness. Nanoparticles are being extensively explored for their ability to deliver various genetic payloads—including plasmid DNA, messenger RNA, and small interfering RNA—directly to cancer cells. This review highlights key gene modulation strategies such as RNA interference, gene editing systems, and chimeric antigen receptor (CAR) technologies, alongside a diverse array of nanoscale delivery systems composed of polymers, lipids, and inorganic materials. These nanoparticle-based delivery platforms aim to improve targeted transport of genetic material into cancer cells, ultimately enhancing the efficacy of cancer therapies.

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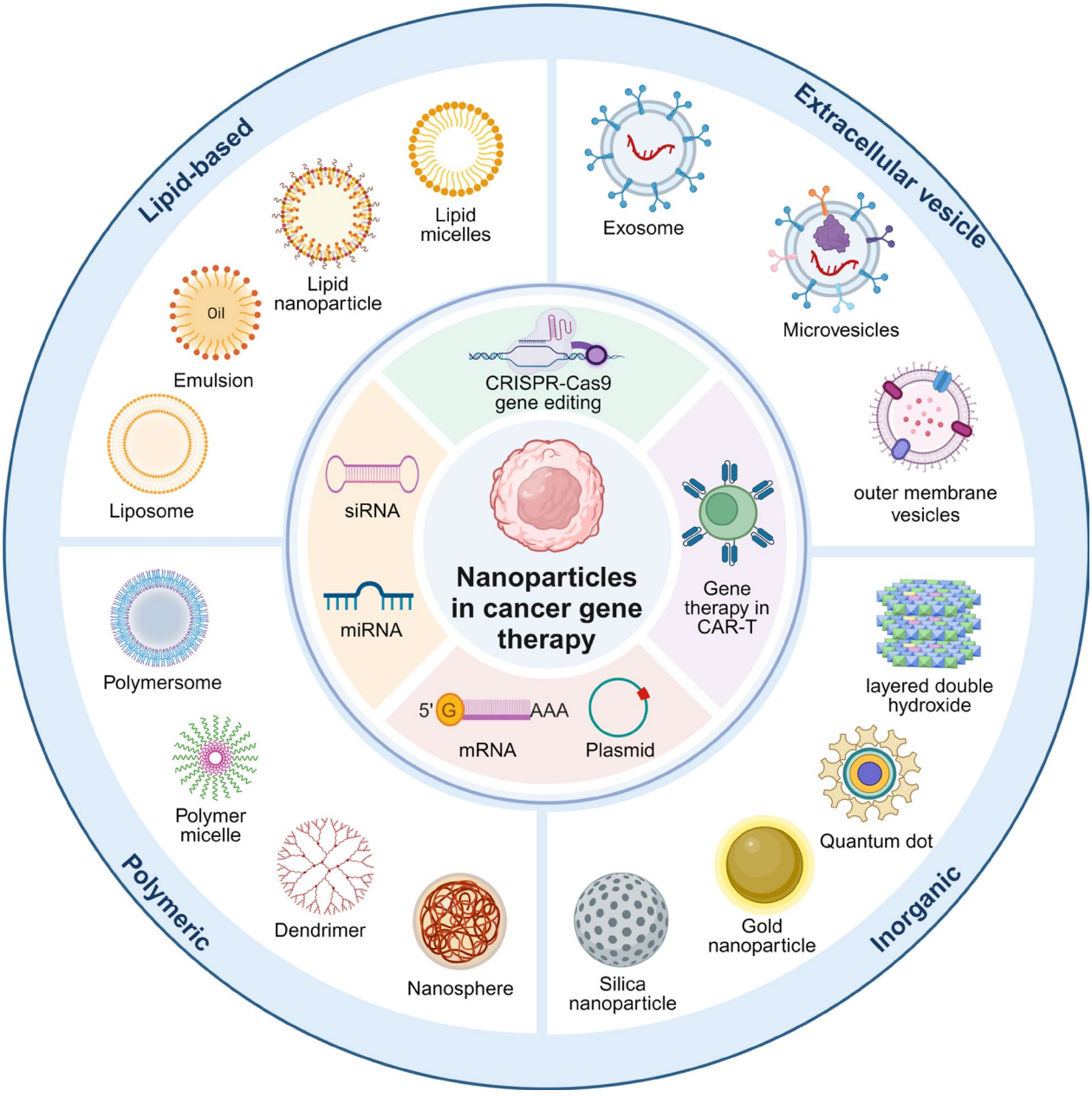
Graphical abstract



Keywords Cancer gene therapy, Nanoparticles, Gene editing, CAR technologies

Introduction

Gene therapy has emerged as a transformative approach in oncology, offering new strategies to address the genetic and molecular abnormalities underlying cancer. As a result, it has gained significant attention in pharmacology and biotechnology [1]. Advances in genetic engineering, a deeper understanding of tumor biology, and rapid developments in nanotechnology have collectively driven substantial progress in this field [2]. One of the most critical breakthroughs has been the development of safe and efficient gene delivery vehicles that can transport therapeutic genes directly into cancer cells, correct genetic mutations, and modulate tumor-specific signaling pathways [3].

In recent years, nanotechnology has gained prominence as an alternative to traditional viral vectors, which are increasingly limited by genotoxicity and adverse side effects [4]. As promising candidates for non-viral gene

delivery, nanoparticles (NPs) offer an innovative platform for targeted delivery of therapeutic agents. Their nanoscale size, stable physiological properties, customizable structural modifications, and high surface-to-volume ratio make them highly advantageous for gene therapy applications [5, 6]. NPs can be carefully engineered to enhance therapeutic efficacy through surface modifications, such as the attachment of cross-linkers and the integration of stimuli-responsive systems. These features facilitate targeted accumulation at specific sites while reducing off-target toxicity [7]. Moreover, NPs protect therapeutic cargo from enzymatic degradation in circulation, extend its half-life, and improve cellular and nuclear uptake, ultimately enhancing biodistribution and therapeutic outcomes [8, 9].

Recent advancements in NP-based gene delivery have led to the development of both organic (e.g., lipid and polymeric NPs) and inorganic (e.g., gold NPs, carbon dots, mesoporous silica NPs, clay NPs) systems [10, 11]. In this review, we discuss recent progress in the use of NPs as delivery vehicles for gene therapy, including nucleic acid cargo delivery, CRISPR-based gene editing, and chimeric antigen receptor (CAR) therapy (Fig. 1). We explore various gene therapy strategies, emphasizing the impact of nanosystem modifications on therapeutic efficacy. Additionally, we examine different types of genetic cargo, their relative advantages, and therapeutic potential. Finally, we highlight current challenges in nanosystem engineering and discuss future perspectives in the field.

NPs as gene delivery system

Organic NPs

Organic NPs, including polymeric NPs (PNPs) and lipid-based NPs (LNPs), are widely utilized for gene delivery through chemical bonding or physical embedding [12]. PNPs, made from biodegradable and biocompatible polymers such as polyethylenimine (PEI), polyethylene glycol (PEG), and poly(lactic-co-glycolic acid) (PLGA), have found extensive applications in gene therapy [13]. At physiological pH, these polymers can encapsulate nucleic acids, forming polymeric complexes that enhance gene delivery efficiency [14]. PNPs offer several advantages, including controlled release, protection of genetic material from degradation, and ease of functionalization for targeted tumor therapy [15, 16]. Lipid-based NPs have emerged as one of the most successful and versatile gene delivery platforms, particularly for messenger RNA (mRNA) vaccines and gene-editing systems [17, 18]. Their ability to encapsulate fragile nucleic acids, protect them from enzymatic degradation, and enhance cellular uptake via endocytosis makes them highly effective [19]. Surface modifications with targeting ligands, such as peptides or antibodies, further improve their specificity for cancer cells [20]. Lipid-based NP delivery systems primarily include liposomes, micelles, emulsions, and LNPs. Among these, LNPs constitute the dominant platform for NP-mediated mRNA delivery and have been widely used in preclinical and clinical applications for various diseases [21]. The mechanisms and structural optimization strategies of these systems will be detailed in the mRNA delivery section.

Apart from mRNA vaccines, LNPs are also used for delivering CRISPR components such as ribonucleoproteins (RNPs), avoiding the genomic integration risks of viral vectors. Editas Medicine's AGN-151,587 used LNPs to deliver Cas9 RNPs to retinal pigment epithelial cells, achieving precise gene editing in Leber congenital amaurosis type 10 patients. In preclinical primates, subretinal injection of LNPs resulted in > 20% editing efficiency with no off-target mutations detected with whole-genome sequencing [22]. Moreover, LNPs have been widely used in RNAi therapeutics. For example, LNPs delivered siRNA to oncogenic pathways (e.g., KRAS G12D in pancreatic cancer) and showed promising preclinical results, with combinatorial therapy (siRNA + chemotherapy) reducing tumor volume by 55% in orthotopic models [23]. Mechanistically, LNPs were co-encapsulated with siRNA and doxorubicin and achieved synergistic effects by reversing multidrug resistance (MDR1 silencing) and enhancing drug accumulation in the nucleus [24].

Inorganic NPs

Inorganic NPs, including gold NPs (AuNPs), graphene quantum dots (GQDs), silver NPs (AgNPs), mesoporous silica NPs (MSNs), and layered double hydroxides (LDHs), have been extensively explored for gene therapy due to their small size, tunable surface properties, and ability to facilitate ligand binding [25, 26]. AuNPs, ranging from 1 to 100 nm in size, are widely used for their high surface-to-volume ratio, excellent biocompatibility, and low toxicity [27]. Variations in morphology, size, PEGylation, and surface charge influence their drug-loading capacity [28]. AuNPs have been successfully integrated into cancer therapies, often in combination with chemotherapy or photothermal therapy [29, 30]. Various optimization strategies, such as surface functionalization, have been developed to extend circulation time, enhance tumor accumulation, and regulate intracellular release [30]. Similarly, AgNPs (1–100 nm) are another class of inorganic NPs with promising biomedical applications [31]. Beyond their well-known antibacterial properties, L-cysteine silver complexes have been investigated as potential drug carriers with excellent biocompatibility [32].

MSNs, characterized by their porous silica-based structures, offer high chemical stability and tunable pore sizes that improve drug dissolution and encapsulation

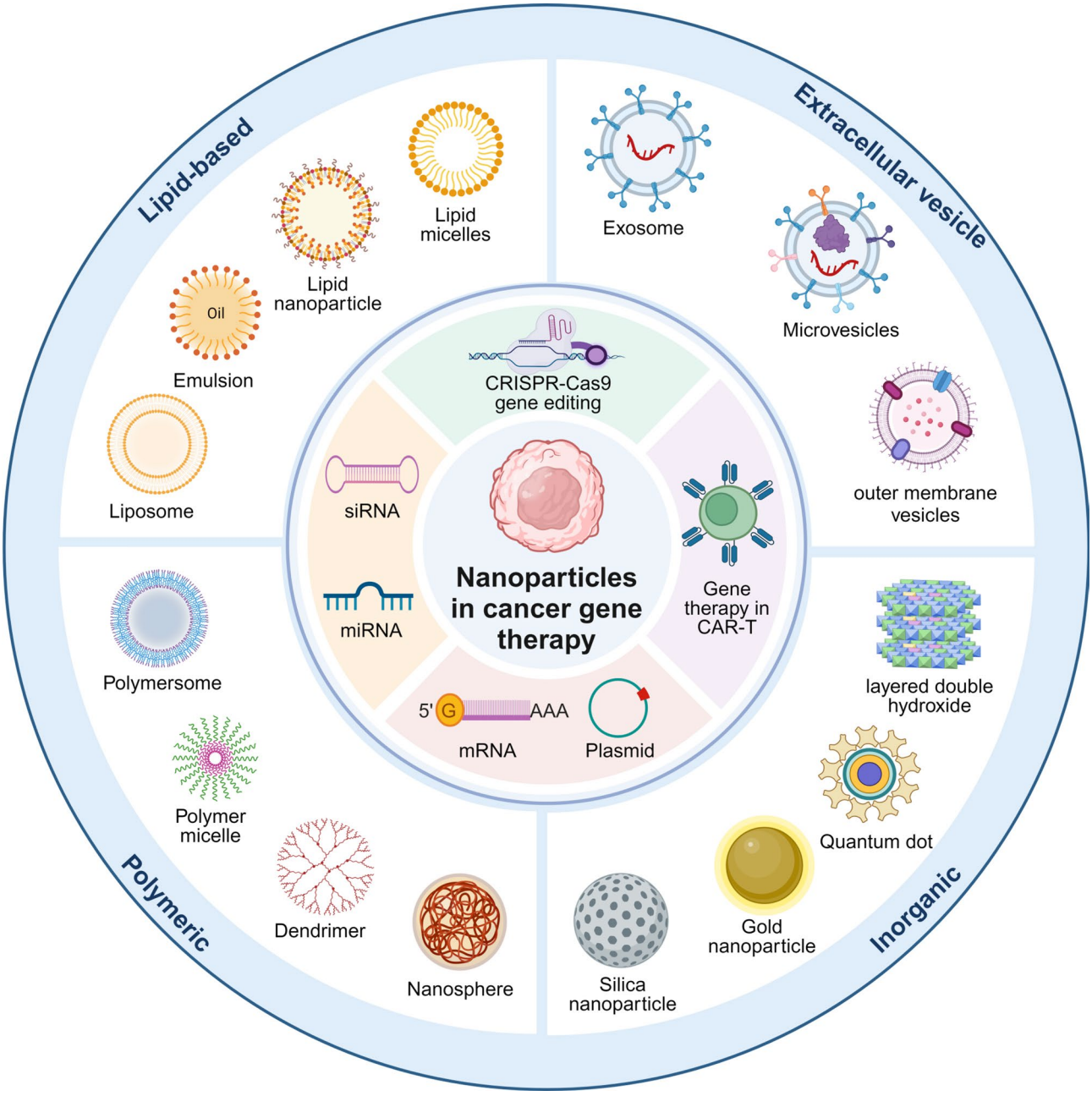


Fig. 1 Schematic illustration of various nanoparticles utilized for cancer gene therapy. Created with BioRender.com

efficiency [33]. Their exceptional chemical, thermal, and mechanical stability across various physiological conditions makes them ideal for gene delivery. Recent advancements have focused on surface modifications to enhance therapeutic efficacy and enable controlled pharmacokinetics. For example, structure-optimized MSNs co-loaded with a Toll-like receptor 9 (TLR9) agonist and an antigen demonstrated increased lymph node accumulation, leading to a stronger antigen-specific B and T cell response [34].

GQDs, an evolutionary class of semiconductor quantum dots, have gained attention as nanocarriers due to their high stability and low cytotoxicity [35]. Additionally, various two-dimensional materials serve as efficient drug carriers, including LDHs, which possess a layered structure formed through electrostatic interactions and hydrogen bonding [36–38]. Recent efforts have focused on optimizing LDHs for enhanced stability, selective accumulation, and improved gene delivery efficiency [39, 40].

Extracellular vesicles

Extracellular vesicles (EVs) are nanoscale, membrane-bound particles secreted by all cell types, naturally encapsulating proteins and nucleic acids during their formation [41]. They play a vital role in intercellular communication by delivering their molecular cargo to target cells, thereby influencing various cellular processes [42]. EVs offer key advantages, including biocompatibility, non-immunogenicity, and low toxicity.

More importantly, EVs can be engineered to carry specific surface or internal payloads, making them an appealing platform for delivering various therapeutic agents [43]. The incorporation of cargo into EVs can be achieved through two main approaches: either by overexpressing the desired cargo in the producer cells so that it is included during EV biogenesis, or by modifying the vesicles physically or chemically after they have been collected [44]. Additionally, genetically engineered cells can be implanted to produce functionalized EVs continuously within the body [45]. Although post-collection modification offers greater flexibility in cargo loading, it also necessitates more rigorous purification processes and poses challenges in manufacturing and regulatory compliance. Using EVs as biomimetic nanovesicles might be an effective strategy for gene therapy. Another novel nanomaterial worth introducing is DNA nanostructure-based vectors. The straightforward synthesis process and exceptional biocompatibility of different shape-based DNA nanocarriers have been fabricated in a broad spectrum of biomedical delivery [46], such as tetrahedrons [47], prisms [48], nanotubes [49] and planar origami [50, 51].

Intracellular trafficking of NPs

NPs navigate intracellular barriers to access specific cellular compartments and organelles. Within the cell, motor proteins and cytoskeletal structures facilitate the movement of NPs through intricate trafficking pathways, directing them toward various intracellular destinations (Fig. 2). Following attachment onto the cell membrane, NPs are typically enclosed in a membrane-bound vesicle known as an early endosome, which may undergo a maturation process over time. These early endosomes, characterized by a pH of approximately 6.5, can merge with other vesicles and transport their contents to targeted locations within the cell, such as the cytosol, nucleus membrane, mitochondria, Golgi apparatus, or endoplasmic reticulum. However, some of the transported materials (e.g., proteins and lipids) are returned to the plasma membrane via recycling endosomes [52]. The remaining cargo is sorted into intraluminal vesicles, forming multivesicular bodies, also referred to as late endosomes, which have a more acidic environment (pH 5.5). Through further processing, the cargo is directed toward one of several potential fates: (i) delivery to specific organelles, (ii) fusion with lysosomes (pH ~5) to create endolysosomal vesicles (pH 4.5), where hydrolytic enzymes (such as proteases, lipases, phosphatases, and nucleases) degrade part of the trapped NPs as well as some cargo, (iii) secretion outside the cell via exosomes, or (iv) recycling back to the plasma membrane through reverse fusion events [53, 54]. While the lysosomal network represents the predominant pathway for intracellular trafficking and metabolism of NPs, certain NPs can bypass this system by early endosomal escape or escape into the cytoplasm through mechanisms such as membrane fusion, destabilization of the vesicle membrane, particle swelling, or osmotic rupture, thereby gaining access to the cytosolic environment [16].

Nanogene for cancer therapy

Plasmid DNA (pDNA) and messenger RNA (mRNA) are widely used to enhance the expression of target genes, while small interfering RNA (siRNA), microRNA (miRNA), and antisense oligonucleotide (ASO) function as nucleic-acid therapeutics by selectively binding to target RNAs to inhibit gene expression. Additionally, the CRISPR/CRISPR-associated protein 9 (Cas9) system has emerged as a powerful tool for gene modulation, enabling gene activation, repression, or precise correction of genetic sequences. This section explores recent advancements in nanogene therapy, which integrates NPs with various nucleic acid-based therapeutics to enhance cancer treatment. We discuss the different types of genetic cargo utilized in nanogene therapy, their mechanisms of action, and how nanoparticle-based delivery systems improve stability, targeting, and therapeutic outcomes.

Direct overexpression of a gene

DNA

In the DNA-based nanoplatform, cells were transfected with plasmid or chemically synthesized DNA to trigger immune responses against the encoded antigen. Among them, pDNA is currently receiving extensive attention as a vehicle for gene therapy applications. pDNA is generally isolated from recombinant Escherichia coli and subsequently purified by removing RNA, proteins, and endotoxins. The essential elements of pDNA comprise components that are crucial for bacterial maintenance and replication, as well as sequences required for mammalian expression. pDNA leverages the transcriptional apparatus of the host cell to produce proteins and maintains its activity for an extended duration compared to protein-based therapies. Compared to mRNA, pDNA offers benefits in cost-effectiveness, ease of transportation, and stability. However, due to its larger size, pDNA has a higher risk of introducing unintended elements, and developing a safer and more efficient vector

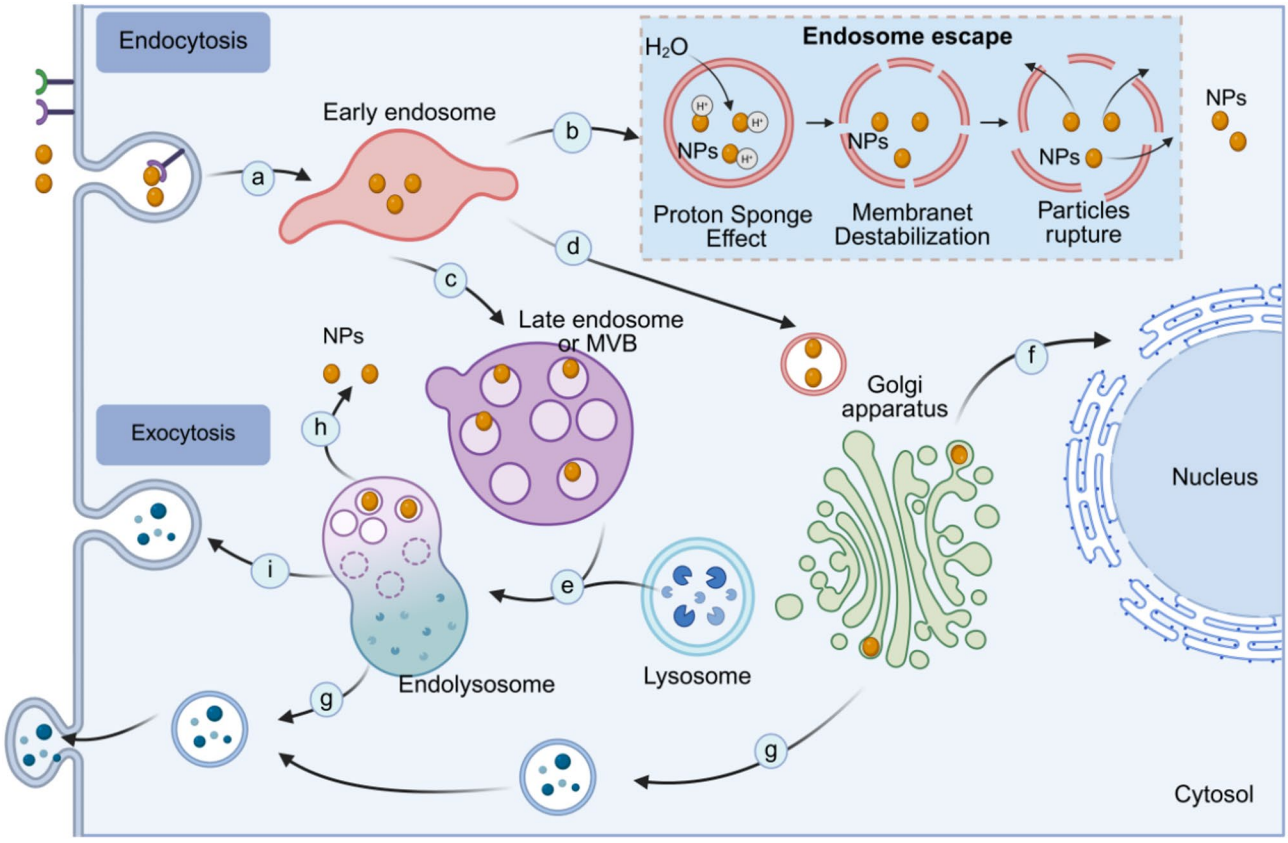


Fig. 2 Schematic representation of NPs' intracellular trafficking. **a**, After cellular internalization, nanoparticles are confined in early endosomes. **b**, Some NPs can escape from the endosome and be released into the cytoplasm via proton sponge effect, membrane destabilization, particle rupture to reach the cytosol, **c**, Some NPs is sorted into the late endosome. **d**, They fuse with vesicles and ferry the cargo to the desired cellular destination (i.e. cytosol, nucleus, mitochondria, Golgi apparatus). Then, the cargo will be directed to one of the following possible fates: **f**, specific cell organelles, **e**, form endolysosomes, **g**, secretion in exosomes, **h**, recycling to the plasma or **i**, secreted extracellularly. Created with BioRender.com

for systemic gene delivery remains a significant challenge [55]. For expression, pDNA must traverse the cell's plasma membrane and the nuclear envelope. Once inside the nucleus, it can be transcribed into mRNA, which can be translated into the desired protein.

LNPs have emerged as a promising adeno-associated virus (AAV) vector's alternative. Several studies have utilized LNPs to deliver pDNA for the treatment of monogenic disorders. Hashida et al. [56] introduced histidinylation of a galactosylated cholesterol derivative to enhance gene transport to hepatocytes through the "proton sponge effect". Akita et al. [57] conducted a novel hepatic gene transfer system, which utilizes a new derivative of ssPalm. This ssPalm is a lipid-like material that can be cleaved by disulfide bonds and activated at low pH levels, in combination with the anti-inflammatory medication dexamethasone. This ssPalm-based LNP delivery nanosystem mitigated the inflammatory responses caused by pDNA transfection and enhanced the target gene expression in mice. Despite the availability of various lipid nanoparticle systems for pDNA delivery, achieving therapeutically effective transgene expression in body remains a significant challenge.

mRNA

mRNA-based therapies offer a potential alternative to DNA-based approaches, primarily because they carry a reduced risk of causing mutations and enable simpler, temporary expression [58]. Unlike pDNA, mRNA can be directly translated in the cytoplasm. Furthermore, the production and purification of mRNA in vitro could avoid host protein and virus-derived pollution [59, 60]. However, the large size, unstable structure combined with the negative charges of naked mRNA impede the capability of reaching and entering the target site [61]. To solve the above barriers, LNPs are stereotyped as self-assembled nanocarriers for encapsulating, protecting and delivering nucleus acids currently, especially for mRNA delivery [62]. Since the successful application of nucleoside-modified mRNA-LNPs in the Pfizer/BioNTech and Moderna SARS-CoV-2 vaccines, the lipid-based system has attracted more attention in this field [63, 64]. More and more mRNA-LNP therapeutic systems have been

widely tested in both preclinical and clinical trials for the treatment of cancers [65], infectious diseases [66], rare genetic disorders [67], neurodegenerative diseases [68] and various diseases treated through protein substitution therapy [69]. Despite these advancements, the optimal mRNA-LNP delivery system remains elusive because of the flexible linear structure of the single-stranded mRNA.

LNPs usually consist of four key elements: cationic or ionizable lipids, cholesterol, phospholipids, and poly(ethylene glycol) linked to a lipid anchor [70]. Served as the core element of LNPs, cationic or ionizable lipids are crucial for facilitating the interaction with mRNA molecules. These lipids enable the rapid endosome escape of mRNA by engaging in pH-induced electrostatic interactions with the negatively charged endo/lysosomal membrane [71]. Moreover, substituting the zwitterionic phospholipid with the cationic lipid shifted the accumulation of LNPs from liver to lung [72]. This replacement enhanced the positive surface charge of LNPs by five times and shifted protein expression from liver to lung from 36:1 to 1:56. Likewise, substituting zwitterionic phospholipids with phosphatidylserine reduced the positive charge by 50% and decreased the ratio of protein expression from liver to spleen. Other studies also noted that incorporating a fifth cationic component into LNPs can redirect their delivery to the lung [73]. The uptake of particles by cells can be influenced by various properties of NPs, including their dimensions, morphology, surface charge, and composition [74]. LNPs have been designed and developed to prepare mRNA delivery tools with different characteristics. One important discovery is about the structural characteristics of cholesterol which have a potential relationship with efficient intracellular delivery and gene transfection [75]. Data showed that C-24 alkyl phytosterols improves the LNPs' gene transfection efficiency. The length of the alkyl chain, the flexibility of the sterol ring, and the polarity are essential factors for sustaining high transfection performance. On the other hand, the change of characteristics of cholesterol also influence non-hepatic delivery in a manner distinct from that of charged helper lipids. Radmand et al. [76] found that tropism of positively charged cholesterol is differ from that of cationic helper lipid. They also observed that positively charged cholesterol resulted in a distinct lung-to-liver delivery ratio compared to charged helper lipids. However, there are worries about utilizing cationic lipids due to the cell toxicity caused by their positively charged nature [77]. Besides, recent research demonstrated a new mechanism underlying the reduction of mRNA functionality in the LNP delivery system. Impurities with electrophilic characteristics, stemming from ionizable cationic lipids, are improved to play an important role by oxidizing and then hydrolyzing the tertiary amine [78]. However, efforts to reduce the cytotoxicity of cationic lipids by lowering their positive charge appear counterproductive, as this can lead to decreased genetic material encapsulation efficiency and transfection efficiency. Therefore, when designing lipid-based delivery systems, it is crucial to achieve a balance between minimizing toxicity, enhancing immune response, and ensuring therapeutic efficacy.

Rational design and combinatorial synthesis have facilitated the creation of biodegradable and efficient ionizable lipids. However, systematic methods for refining the structure of ionizable lipids through iterative optimization remain underdeveloped [62]. Historically, the discovery of optimal ionizable lipid structures has depended on trial-and-error screening experiments. However, extensive testing requires considerable time, large amounts of materials, extensive animal testing, and advanced equipment such as combinatorial chemical methods and high-throughput techniques [79]. Even with the allocation of significant resources, the efficiency and success rates of experiments continue to be low because of the extensive chemical space associated with ionizable lipids. On the other hand, designing lipids based exclusively on human intuition is constrained by personal experience and the limited ability to effectively utilize accumulated data. Recently, the integration of artificial intelligence (AI) models has shown promise in addressing these challenges. AI is adept at identifying patterns within large datasets and applying these insights to predict new outcomes. In the realm of optimal lipid discovery, AI has made notable advancements. Additionally, AI models have been designed to forecast multiple properties of pharmaceuticals across various formulations, such as solid dispersions, cyclodextrin complexes, and NPs [80]. Wang et al. [81] systematically compiled diverse structures of ionizable lipids from literature and patents with the goal of constructing AI models. These models are capable of predicting apparent pKa values and mRNA delivery efficiency for lipid nanoparticles. They offer valuable insights into lipid design and have been utilized to predict lipid properties, thereby speeding up the screening process (Fig. 3a). Through this methodology, several ionizable lipids were successfully identified and confirmed through experimental validation to exhibit strong performance (Fig. 3b). All newly developed lipids achieved high levels of mRNA expression in the liver. Notably, LQ089 performed exceptionally well, rivaling the efficacy of SM-102 (Fig. 3c). This study reinforces the promise of AI techniques in designing ionizable lipids through enhancing the efficiency of screening and elucidating the structure-activity relationship. Besides, Xu et al. [82] presented the AI-guided platform (AGILE), which integrates deep learning with combinatorial chemistry. This platform is trained using a large dataset that includes both virtual simulations and experimental wet-lab results

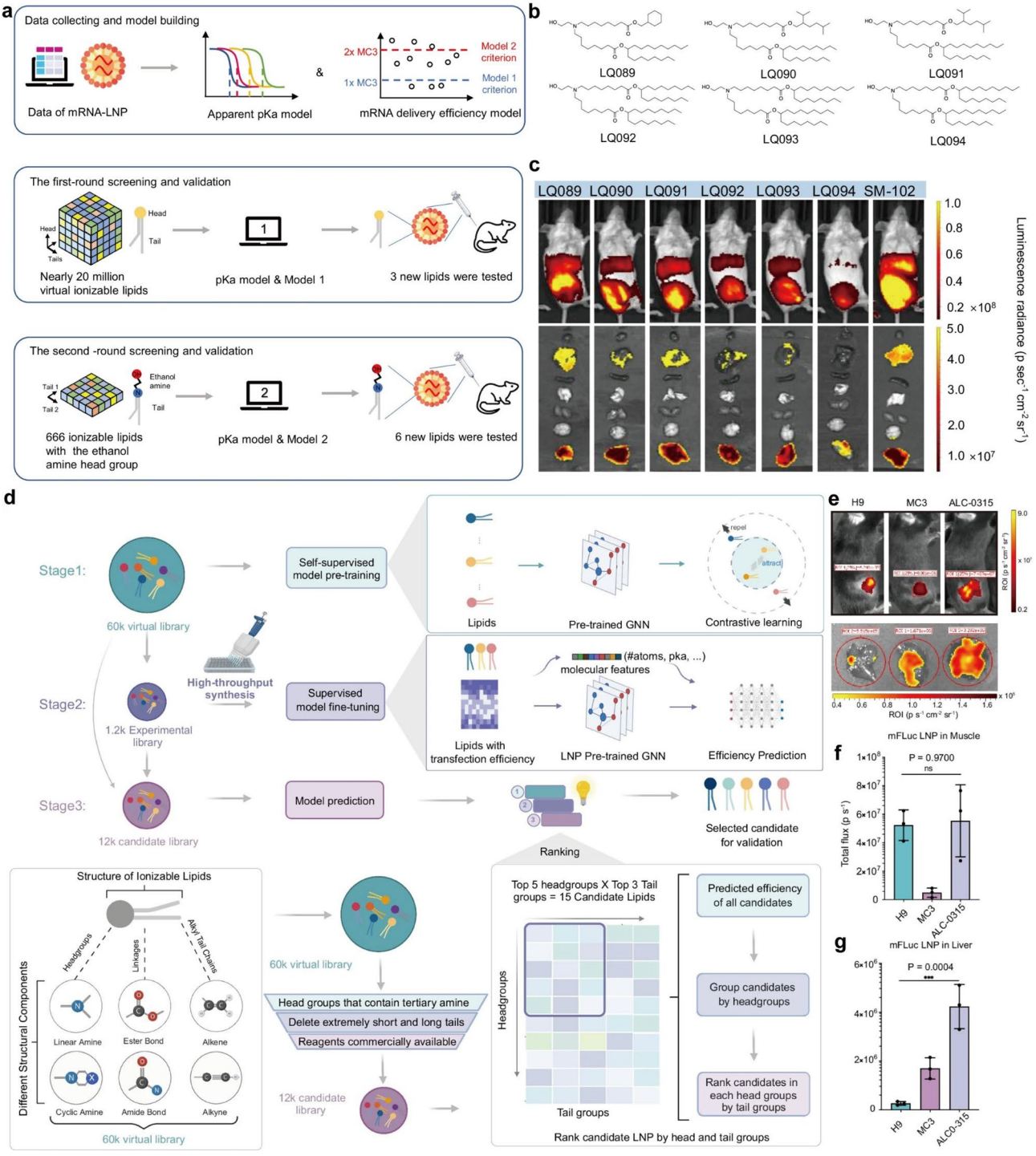


Fig. 3 Optimization of ionizable lipids through AI-powered approaches. **a**, Schemetic of AI-powered development of ionizable lipids. **b**, The optimal ionizable lipids chosen from the initial screening phase. **c**, Representative images of the luminescence. Reproduced with permission [81]. Copyright© 2024, The Author(s). **d**, Overview of the AGILE platform design pipeline. **e**, Imaging of injection sites. **g**, **h**, Quantification analysis of luminescent intensity in mice **g** and their livers **h**. Reproduced with permission [82]. Copyright© 2024, The Author(s)

(Fig. 3d). AGILE optimizes the development of ionizable lipids through efficient library design, screening using deep neural networks, and adaptability across various cell lines. With AGILE, they can swiftly design and assess ionizable lipids for LNP formulation by leveraging an extensive library. It was observed that H9-LNPs transport mRNA to muscle cells with an efficiency 7.8 times higher than MC3, rivaling the performance of ALC-0315

(Fig. 3e, f). Notably, H9-LNPs exhibited significant tissue specificity, resulting in markedly lower mRNA expression in liver compared to other ionizable lipids-LNPs (Fig. 3g). AGILE signifies a pioneering integration of chemistry and deep learning, shedding light on the complex dynamics involved in LNP system design and expanding these insights for a wide range of applications. Importantly, AGILE provides a practical methods to the costly and time-consuming challenges in lipid synthesis and screening. This AI-driven approach exemplifies the transformative impact of combining high-throughput screening with advanced computational methods, providing ideas for overcoming conventional hurdles in nanomedicine studies.

The integration of machine learning (ML) into nanoparticle development has emerged as a transformative paradigm, extending far beyond the structure-activity relationship modeling. ML optimizes critical stages of nanoparticle engineering by bridging computational modeling, high-throughput experimentation, and iterative refinement, enabling rational design, efficient synthesis and formulation, and predictive performance assessment. ML accelerates the identification of novel nanomaterials with tailored properties through generative algorithms, such as generative adversarial networks and diffusion models, which design chemically feasible structures (e.g., metal, polymer, or hybrid nanoparticles) with target attributes including size, surface charge, surface properties and biodegradability [83, 84]. Moreover, ML algorithms, including Bayesian optimization and reinforcement learning, streamline synthesis and formulation by optimizing parameters such as temperature, concentration, reagent ratio, and reaction time to achieve monodisperse nanoparticles with narrow size distributions and high yields [85]. ML models characterize how nanoparticle features (size, surface chemistry, coating) influence targeted delivery and pharmacokinetics, such as blood circulation time (*via* stealth PEGylation), tissue accumulation (e.g., enhanced permeability and retention effect in tumors), and intracellular trafficking (e.g., endosomal escape kinetics). ML acts as a catalyst for closed-loop innovation, enabling predictive engineering across the entire lifecycle of nanoparticle development from nanomaterial screening to clinical translation. By decoding complex relationships among structure, synthesis, formulation and function, ML not only accelerates innovation but also fosters a paradigm shift toward rational, data-driven design, echoing the transformative impact of AGILE for LNPs while expanding its reach to diverse nanomaterial systems. This synergy between ML and experimental science paves the way for breakthroughs in drug delivery, diagnostics, and clinical applications, underscoring the pivotal role of computational tools in modern nanomedicines and materials science.

In addition to LNP, other NPs for mRNA delivery are also ongoing, such as polymer-based polyplexes, lipopolyplexes coated with lipid shells, NPs assisted by cationic lipids, inorganic NPs, and nanoemulsions [86, 87]. Among them, LDH NPs are a family of inorganic NPs that have been examined as an effective vector for drug delivery [88]. Recently, modified LDH has been demonstrated to significantly enhance mRNA delivery efficiency in antitumor applications. For example, Zhang et al. [89] presented a dual-functional immunomodulator (MO@NAL). This system was conducted by incorporating ovalbumin mRNA into LDH coated with lysozyme (Fig. 4a). After intratumoral administration, MO@NAL rapidly counteracted the excess acidity within the tumor microenvironment (TME), elevating the pH from around 6.5 to 7.0 (Fig. 4b). This change was attributed to the swift degradation of MO@NAL in the TME, as evidenced by the loss of fluorescence intensity at the administration site after one day (Fig. 4c). Remarkably, a second administration at tumor tissue resulted in significantly higher fluorescence intensity at 60 and 72 h post-second administration compared to the corresponding time points following the initial administration (Fig. 4c). Strong OVA expression was observed in the tumor 48 h following the initial administration (Fig. 4d). Intriguingly, in tumor-bearing mice that had got prior vaccination with an OVA vaccine, the administration of MO@NAL resulted in a notable enhancement in the infiltration of cytotoxic T cells while concurrently diminishing the presence of immunosuppressive Tregs (Fig. 4e). The combination of MO@NAL with either the OVA or OVA-specific adoptive T cell transfer resulted in significantly inhibited tumor growth in non-pre-vaccinated mouse models (Fig. 4f, g). These results indicated that these LDH-based nanosystem successfully deliveryed OVA mRNA to tumor cell, thereby offering targets that can recruit and guide cytotoxic T cells to eliminate tumor cells.

Apart from the effect of LDH in anti-tumor yield, these NPs also possess significant potential as an affordable and non-toxic delivery system for introducing biomolecules into plants [90, 91]. Yong et al. [92] advanced LDH NPs by coating with lysozyme. Lysozyme-coated LDH is able to cross the plant cell wall and translocate through the plant. Through the application of lysozyme-coated LDH nanosheets, researchers observed enhanced uptake, especially in critical root areas. Roots possess the capability to absorb nucleic acids, which subsequently function in plant tissues. For example, externally small RNAs can modulate target gene expression via RNA interference mechanisms. Notably, these small RNAs can also travel through hydroponic systems from one plant's roots to another, influencing gene expression in adjacent plants [93]. Lysozyme-coated LDH significantly improved the plants' uptake process. Their research demonstrated that

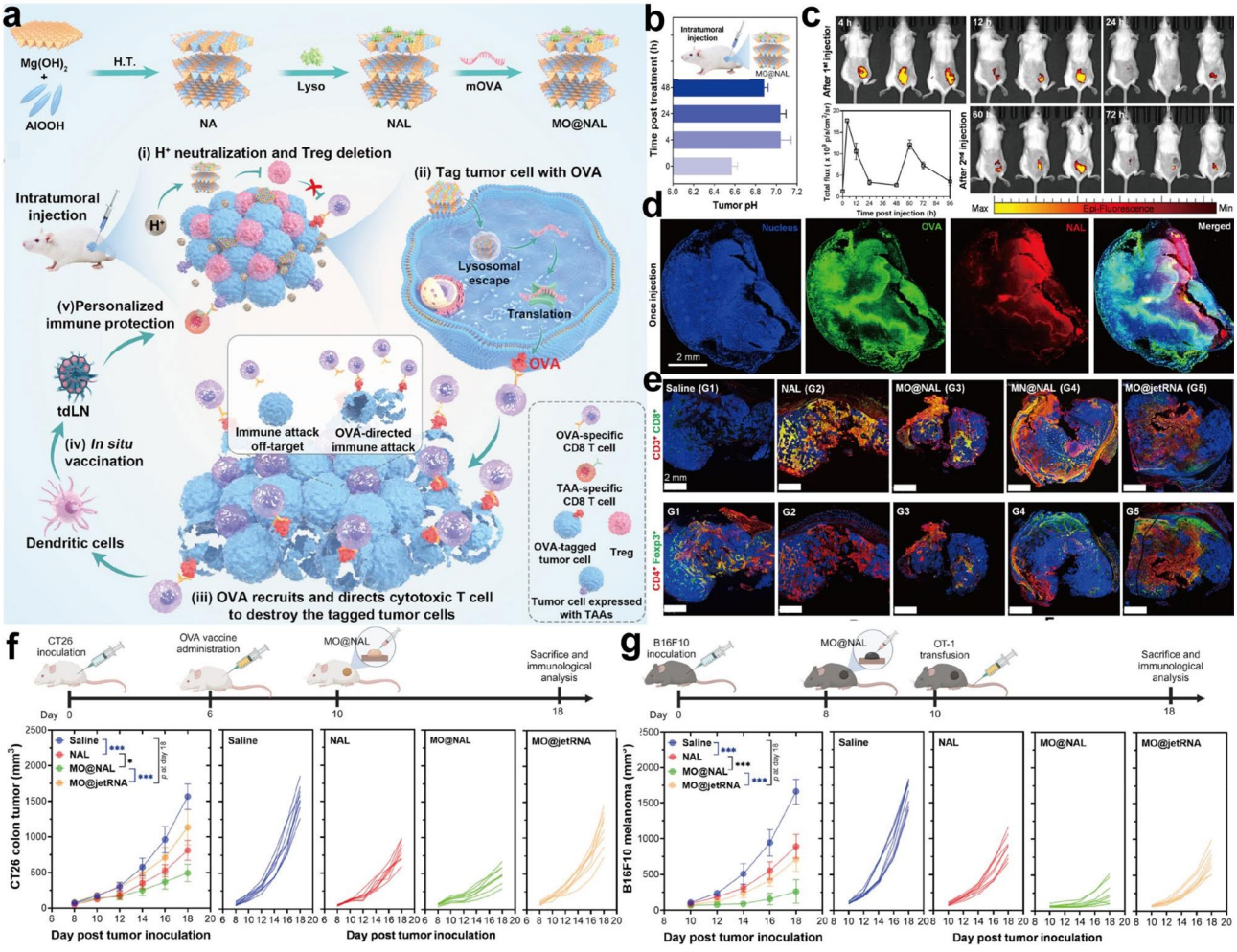


Fig. 4 Tagging tumors with LDH/mRNA to enhance cancer immunotherapy. **a**, Schematic of preparation progress of MO@NAL. **b**, The alteration in pH within tumor tissue after a single administration of MO@NAL. **c**, Retention of RITC-MO@NAL after the first administration and second administration. **d**, Distribution and OVA's expression in tumor tissues after intratumoral administration of MO@NAL. **e**, Distribution of cytotoxic T cells ($CD3^+$ $CD8^+$, yellow) and Tregs ($CD4^+$ $Foxp3^+$, yellow) within tumor tissues. **f**, **g**, MO@NAL directs inhibit CT26 **g** and B16F10 **h** tumor progression. Reproduced with permission [89]. Copyright© 2025, The Authors. Published by Elsevier Ltd

lysozyme, an antimicrobial enzyme with a mild ability to cleave β-1,4-glycosidic bonds in cellulose, enhances the absorption of nanosheets by relaxing the cell wall structure. Through the use of lysozyme, the researchers demonstrated that active enzyme function is crucial for improved root uptake. Lysozyme-coated LDH showed superior performance compared to normal LDH in transporting mRNA into the root tips. As a result, gene expression was efficient in root cells. A significant advantage of this study lies in its detailed examination of the journey of lysozyme-coated LDH through plant tissues following root absorption. This advancement provides a potent new method for modifying plant metabolic processes and traits via nucleic acid-mediated approaches. This enhancement in clay NPs formulation raises several important follow-up questions. While lysozyme-coated LDHs have demonstrated support for nucleic acid uptake in root tips, their effectiveness is temporary, leading to doubts about their potential to replace existing transformation methods. Furthermore, the safety profile of LDHs in agricultural settings warrants further research. While similar formulations have been deemed safe for medical applications, their use in agriculture may require additional evaluations concerning effects on non-target organisms, stability within soil environments, and the necessary dosages under field conditions, as well as potential risks to consumers.

Although there are variations in the NPs used for mRNA delivery, the effectiveness of mRNA expression remains insufficient to meet clinical requirements. Looking ahead, it will be important to investigate ways to fully leverage the intrinsic advantages of these materials, balance the toxicity, immunity and therapeutic effectiveness

to prepare mRNA delivery system will remain crucial research directions.

Gene inactivation or knockdown

Served as an endogenous pathway for post-transcriptional gene silencing which is triggered by double-strand RNA, RNA interference contains siRNA and miRNA [94]. This part focuses on the application of nanoparticles in siRNA delivery, especially in brain targeted delivery, and briefly introduces the situation of miRNA and ASO delivery.

SiRNA

siRNA-based RNA interference therapy designed for silencing or downregulating the expression of disease-associated genes has been wildly-applied in many disease types ranging from viral infections to neurodegenerative diseases and cancers giving its unexplored potential in treating undruggable diseases, extending well beyond what is achievable with conventional small-molecule drug therapies [95].

Highly specific targeting of pathogenic elements makes it fill the defect of chemotherapy. Thus the combination of siRNA and chemotherapeutic drugs seems to offer more promising therapeutic solutions for some types of cancer with enhanced efficacy. At present, a variety of lipid-based delivery platforms have been employed to simultaneously transport siRNA and drugs. These systems, particularly cationic liposomes, can safeguard nucleic acid payloads against enzymatic degradation and reduce the renal clearance of siRNA. A complex nanosystem combined the chemotherapeutic drugs with siRNA, research showed the great potential of co-delivery strategy for the synergistic treatment of gastric cancer. The novel integration of As_2O_3 with HER2-siRNA showed remarkable antitumor efficacy in the orthotopic gastric tumor model. In this approach, As_2O_3 effectively induced apoptosis and curtailed tumor metastasis, while the administration of HER2-siRNA inhibited the expression of HER2, thereby reducing tumor metastatic potential [96]. It is evident that the nanocarrier modified with the cRGD peptide can accomplish pH-induced drug release. This occurs because the pH-responsive layer rapidly dissolves in the acidic endo/lysosomal environment, facilitating escape from the lysosome. In fact, pH-responsive nanosystems are wild-applied in precise-targeting deliveries, enhancing the efficacy of cytosolic siRNA delivery by the pH-activated nano-bomb effect that induces endo/lysosomal escape [97, 98]. There are many other strategies which also combined with anti-tumor drugs. For example, Chen et al. [99] prensented cationic micelles coated with a blend of chondroitin sulfate (CS) and PEGlated CS to delivere both oxoplatin and Xkr8-siRNA (Fig. 5a). CS, which possesses a significant negative charge, has the ability to diminish the positive charge of the resultant NPs [100]. CS functions as a natural ligand for CD44 (overexpressed in various cancer cells and tumor-associated endothelial cells). Both CS-based and hyaluronic acid-based NPs have been served as delivery vehicles for targeted tumor therapy. These investigations have consistently shown substantial inhibition of tumor development and significant enhancement of antitumor immune responses. Besides LNP, many other nanomaterials also can be served as promising platforms for the combination of chemotherapy and siRNA therapy. A micro/nanocomposite constructed from porous silicon serves as a promising carrier for concurrently delivering and concentrating multiple therapeutic agents within the lung [101]. Another versatile co-delivery platform was designed for triple-negative breast cancer treatment (Fig. 5b) [102]. The nanoplatform is functionalized with hyaluronic acid to selectively target CD44 receptors on triple-negative breast cancer (TNBC) cells. By co-delivering cabazitaxel and IKBKE(an oncogene inhibitor in TNBC) siRNA, this formulation demonstrated significant tumor accumulation and enhanced antitumor efficacy.

The advancement of siRNA targeting the lung, kidney, and brain to clinical stages has been limited due to numerous challenges associated with delivering siRNA to these organs. Among them, the most significant challenge for brain-targeted siRNA therapy is overcoming the blood-brain barrier (BBB), which restricts molecular transport, as well as achieving specific targeting of diseased tissues within the brain. Advances in nanotechnology have improved the development of engineered NPs encapsulating siRNA, specifically designed to penetrate biological barriers and enhance the efficacy of brain disease treatments [103]. Receptor-mediated transcytosis (RMT), cell-mediated transport, carrier-facilitated transport, adsorption-mediated transcytosis, and techniques that temporarily disrupt tight junction integrity are employed as strategies to facilitate nanoparticle passage across the BBB. The majority of siRNA NPs that have successfully traversed the BBB utilize the RMT approach, which leverages the vesicular trafficking mechanisms within brain endothelial cells to transport a diverse array of proteins. Among these, transferrin (Tf) and rabies viral glycoprotein (RVG) tags are the most commonly employed. Tf binds to the transferrin receptor, while RVG targets the nicotinic acetylcholine receptor (nAChR), both receptors being present in brain endothelial cells. Although Tf receptor is widely distributed throughout the body, nAChR expression is restricted to the brain, enabling specific targeting. This RVG-based strategy has been utilized to target genes linked to ischemic stroke, Huntington's disease, and Alzheimer's disease, respectively [104]. Zhang et al. [105] showed that LDH NPs modified with

low-density lipoprotein receptor 1 ligand angiopep-2 and nAChR ligand RVG29 effectively traversed the BBB and entered the brain tissue. Zhao et al. [106] developed a novel delivery system known as polymer-locked fusogenic liposomes (Plofsomes). These liposomes can effectively traverse the BBB and release siRNA directly into the cytoplasm of glioblastoma multiforme (GBM) cells (Fig. 5c). Besides RVG and Tf, other promising candidates for RMT include leptin, apolipoprotein E3-reconstituted high-density lipoprotein (ApoE), and T7 peptides [107]. Zhang et al. [108] designed an innovative temozolomide nanocapsule to simultaneously deliver pyruvate kinase M2 siRNA (siPKM2) along with temozolomide (Fig. 5d). This delivery system is designed with siPKM2 encapsulated in its core and a shell composed of methacrylate-temozolomide, inhibiting energy metabolism while improving the cytotoxic efficacy of temozolomide. By incorporating ApoE, the nanocapsules achieve dual-targeting specificity towards both the BBB and GBM. The inclusion of a glutathione-responsive crosslinker with disulfide bonds ensures precise cleavage and release of methacrylate-temozolomide and siPKM2 in the high glutathione milieu characteristic of GBM cells. Furthermore, in vivo studies confirm that the ApoE-based siRNA delivery system exhibits effective targeting capabilities and significantly prolongs the survival of nude mice bearing tumors.

Collectively, appropriate modifications or engineering of nanocarriers offer the potential for effective siRNA delivery, addressing challenges related to circulation, brain penetration, and specific tissue targeting, thus

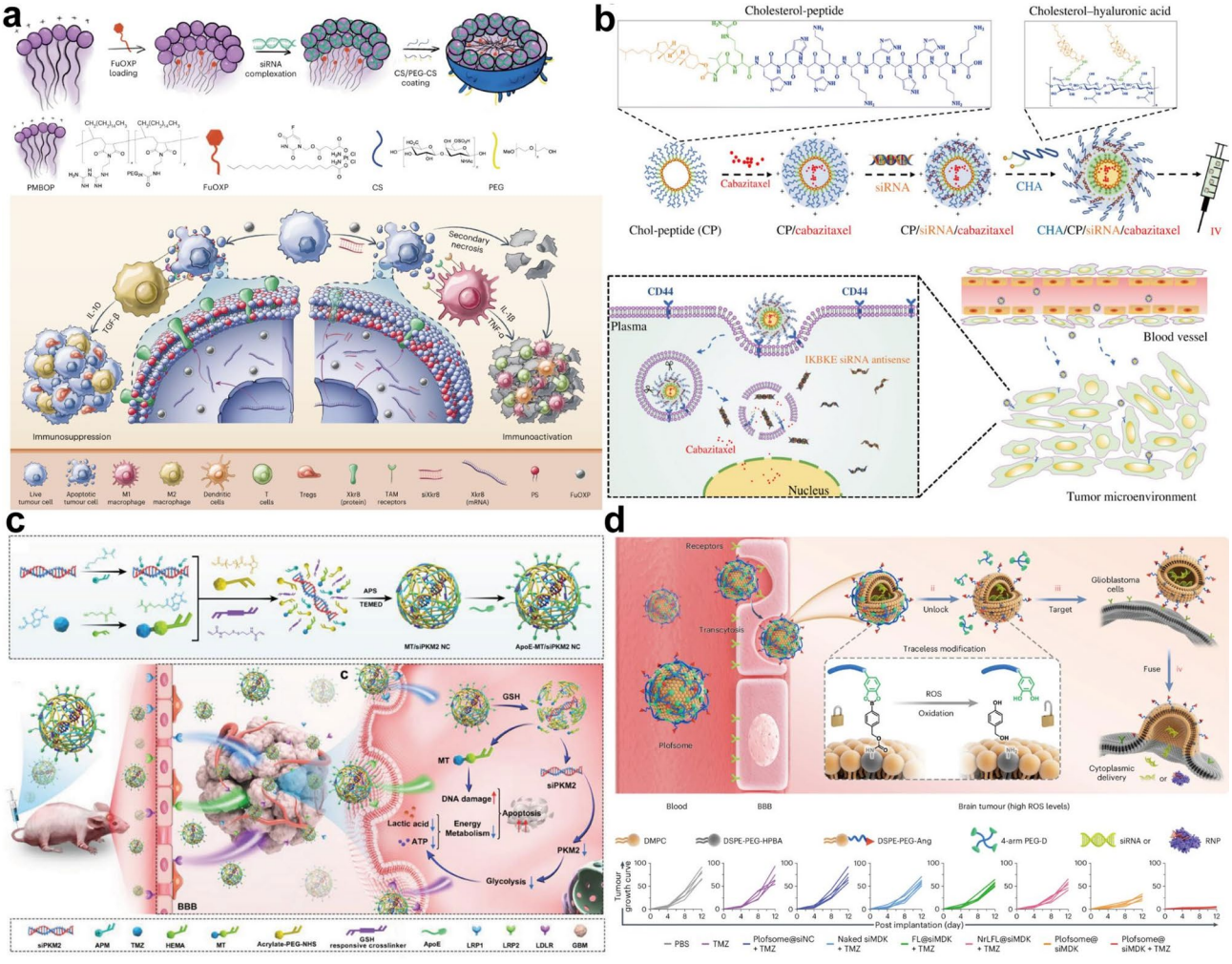


Fig. 5 Schematic illustration of NPs in siRNA delivery. **a**, Proposed approach to counteract chemotherapy-drug-induced activation of Xkr8. Reproduced with permission [99]. Copyright© 2022, Springer Nature. **b**, Schematic of hybrid siRNA nanocomplex in antitumor. Reproduced with permission [102]. Copyright© 2020, The American Association for the Advancement of Science. **c**, Schematic diagram of platform for GBM-targeted siRNA delivery. Reproduced with permission [106]. Copyright© 2024, Springer Nature. **d**, Schematic illustration of the preparation of ApoE-MT/siPKM2 NC and their effectiveness in inhibiting GBM through targeted drug release following successful penetration of BBB. Reproduced with permission [108]. Copyright© 2024 Wiley-VCH GmbH

strengthening the applicability of siRNAs to disease treatments.

MiRNA

Emerging evidence points to the potential of certain miRNAs in cancer therapy by influencing tumor development and altering immune evasion mechanisms [109]. However, the exploration of effective miRNA delivery methods remains limited. The tumor suppressor gene p53 is either absent or mutated in approximately half of non small cell lung cancer cases. Mouse double minute 2 (MDM2) serves as a key regulator of p53 function. Morro et al. [110] prepared the CCL660 system, which encapsulates miR-660 targeting MDM2 within cationic LNPs. In lung cancer mouse models, systemic administration of CCL660 led to increased miRNA levels in tumors and inhibited tumor growth in both wild-type and mutant p53 tumors. This effect was achieved by restoring p53 activity and reducing MDM2 expression. Importantly, this treatment did not affect surrounding normal tissues and also suppressed tumor metastasis. Another significant miRNA in NSCLC is miR-200c, whose lower expression levels correlate with poorly differentiated tumors. Studies have shown that miR-200c can inhibit cancer growth. Like other gene therapies, miR-200c requires an efficient delivery system. Peng et al. [111] designed an amphiphilic polyphosphazene polymersome using monomethoxy poly(ethylene glycol) and ethyl-p-aminobenzoate. The electroneutral nature of the system minimized systemic toxicity and extended the circulation stability of miR-200c. Additionally, Maryna et al. [112] developed a delivery vehicle for miRNA-29b aimed at cells that overexpress mucin-1. To ensure stability, they incorporated IgG along with poloxamer-188 in the system and conjugated mucin-1 aptamers to the surface of the particles to facilitate targeted delivery.

ASO

ASOs are another wildly studied approach for knockdown or silencing the expression level of the disease-related gene. Unlike the double-stranded siRNA, ASO is a synthetic single-stranded nucleic acid that generally contains 12–30 nucleotides in length. After entering the cell, ASO selectively binds to the target mRNA, and the resulting DNA-RNA hybrid subsequently recruits RNase H. This enzyme recognizes the heteroduplex structure and catalyzes the cleavage of the RNA strand, leading to a decrease in mRNA levels [113]. Compared with siRNA, the single-strand structure of ASO leads to clear targeting and strong specificity via binding interaction. Moreover, chemical modifications can enhance the stability of ASO by increasing its resistance to intracellular DNase [114]. Nevertheless, the limited cell membrane permeability and the absence of nuclear-targeting capabilities of ASOs have constrained their broad application in silencing nuclear RNAs. Various ASO-conjugated nanocarriers have been designed for more precise and efficient delivery.

Cheng et al. [115] employed a gapmer-based approach for ASO design, introducing 2′-O-methyl modifications at the termini of the oligonucleotides. They also preserved complete sequence modifications through the use of phosphorothioate bonds throughout the molecules. These modifications serve to safeguard the ASOs against nuclease degradation and improve cleavage efficiency mediated by RNase H. To enhance stability, LNPs were utilized to deliver dual-targeting ASOs that simultaneously address both Bcl-2 and Akt-1. In a separate ASO-based gene therapy designed for the treatment of homozygous familial hypercholesterolemia, modifications with O-methyl groups were introduced at the ribose termini [116]. The ASO drug was then loaded on the Au nanoparticle with a nucleus-targeting TAT peptide. Research demonstrated the chosen ASO-Au-TAT NPs that specifically target lncRNA MALAT1 have been developed to a promising platform for controlling cancer metastasis [117]. Targeted mitochondrial delivery of ASO-loaded NPs seems to be regarded as a potential therapy for treating mitochondrial diseases. With the help of liposomal nanocarrier system MITO-Porter, the packaging efficiency showed 10-folds higher compared with conventional methods, which encapsulate ASO with following steps:1) PEI was conjugated with ASO via electrostatic interaction 2) Endosome escape device (chol-GALA), cellular uptake and mitochondrial targeting device (R8 peptides) were modified on the mitochondrial fusogenic lipid envelope for nano complex encapsulation. The delivery of Darm ASO has mitochondrial targeting activity, achieving mitochondria targeting with less toxicity and high specificity [118].

The co-delivery strategy of ASOs with siRNA or other drugs is considered as more potential gene therapy for synergistic effects. siRNA/ASO-complexed nanomedicine is loaded on superparamagnetic iron oxide NPs for directing differentiation of transplanted neural stem cells by silencing Pnky lncRNA, which has been shown to have a strong inhibitory effect on the neuronal differentiation [119]. Another co-delivery of siRNA and ASO strategy was designed to be near-infrared radiation (NIR)-responsive [120]. Once under NIR stimulation, the oxygen-sensitive linker connecting the siRNA and pASO facilitates the endo/lysosomal escape of the released siRNA and pASO, enabling them to reach their cytosolic targets. Consequently, this system serves as a promising self-delivery nanoplatform for cancer therapy. As for co-delivery of chemotherapeutic drugs, Pan's design provided a promising therapy [51], which constructed a multifunctional DNA origami-based nanovector. This

nanovectors was designd to co-deliver dual-targeted ASOs and doxorubicin for enhanced therapy in drug-resistant cells. Further investigations demonstrated that Apt-DOA is capable of markedly reducing the expression of Bcl-2 and P-gp proteins concurrently, leading to significant cell apoptosis and a notable improvement in therapeutic efficacy [51].

CRISPR-based gene editing systems

The advancement of gene editing systems beyond Cas9, including Cas12, Cas13, and Cas14, has expanded the toolkit for precise genetic manipulation in tumor therapy, necessitating in-depth exploration of their unique mechanisms, advantages, and translational potentials [121]. Cas9 is the most extensively studied and utilized, known for its ability to introduce targeted double-strand breaks in DNA guided by a single-guide RNA (sgRNA) [122]. Cas12, a type V endonuclease, is characterized by its dual activity: specific cleavage of target DNA followed by non-specific single-stranded DNA (ssDNA) degradation through trans-cleavage [123]. This property could be leveraged in tumors to disrupt oncogenic pathways by not only inactivating primary target genes but also amplifying genomic disruption in cancer-specific sequences. However, uncontrolled trans-cleavage poses risks of off-target genomic damage, requiring careful optimization of delivery systems to restrict activity to tumor cells. In contrast, Cas13, a type VI RNA-targeting nuclease, offers a transcriptome-editing approach, enabling reversible silencing of oncogenic mRNAs without permanent genomic alteration [124]. This is particularly advantageous for targeting genes whose protein products are critical for tumor progression but whose DNA sequences are difficult to modify safely. Cas13's RNA specificity reduces concerns about irreversible genomic changes, aligning with the need for transient therapeutic effects in certain contexts. Cas14, a compact type V nuclease, stands out for its small size, which may facilitate more efficient intracellular delivery, especially in NPs where payload size impacts encapsulation and trafficking efficiency [125]. Its potential for precise DNA targeting with minimized steric hindrance could address delivery challenges associated with larger Cas proteins, though its off-target profile and therapeutic applications remain under investigation. Compared to Cas9, these systems exhibit trade-offs in target preference (DNA vs. RNA), cleavage mechanisms (specific vs. promiscuous), and structural features that influence NP-mediated delivery. For instance, Cas12 and Cas9 share DNA-targeting capabilities but differ in cleavage specificity and bystander activity, whereas Cas13's RNA focus expands the scope of gene regulation to post-transcriptional levels. NP design for these systems must account for their molecular properties: Cas12 and Cas14, as DNA nucleases, may require similar strategies to Cas9 for nuclear localization, such as cationic lipid or polymer-based carriers that enhance endosomal escape and nuclear entry. In contrast, Cas13, acting in the cytoplasm, could benefit from LNPs optimized for mRNA delivery, ensuring cytosolic release of the nuclease or its encoding transcripts.

In tumor therapy, Cas12's trans-cleavage might be harnessed to disrupt multiple oncogenic drivers within a cell, while Cas13 could silence non-coding RNAs or oncogenic mRNAs that are pivotal for tumor survival and metastasis. Cas14's compactness may enable targeted delivery to hard-to-reach tumor niches or cells with low transfection efficiency. However, challenges such as optimizing NP formulations for each enzyme's activity, minimizing immune responses to exogenous nucleases, and ensuring spatiotemporal control over editing remain critical. Future research should focus on integrating these systems with advanced NPs, leveraging stimuli-responsive materials to trigger enzyme release in the tumor microenvironment, and developing predictive models to assess off-target risks specific to each nuclease. By capitalizing on their unique attributes, these advanced gene editing systems, in conjunction with optimized NP delivery, hold promise for enhancing the precision and efficacy of cancer gene therapy, paving the way for more tailored and safer therapeutic strategies.

Next, taking Cas9 as an example, we further introduce the CRISPR gene editing system. Two key elements of the CRISPR/Cas9 system are Cas9 protein and sgRNA. Guided by sgRNA, Cas9 can precisely locate any specific genomic region and function as 'molecular scissors' to generate a double-strand break (DSB) (Fig. 6a). Cells would then activate the repair process via non-homologous end-joining or homology-directed repair (HDR) [126]. Although the CRISPR/Cas9 system could artificially insert, delete, replace or modify specific target genes served as a powerful toolbox in multiple preclinical and clinical studies, there are still many obstacles that emerged in traditional delivery strategies for therapeutic application, especially those defects in cellular damage, restricted packaging capability, and immune system activation. Therefore, NPs systems have been standing out by virtue of their tunable structure. The delivery models of the CRISPR/Cas nanosystem could be divided into three main categories: DNA, mRNA, and protein according to the type of cargo (Fig. 6b).

DNA-based

The pDNA encoding the Cas9 gene together with the sgRNA gene was broadly used at the laboratory level. Compared with the delivery strategy of mRNA or protein, this delivery model exhibited higher stability and easy availability (Fig. 6b). Furthermore, there is no strict requirement for the purity of pDNA, which packaged both Cas9 or sgRNA cassettes. However, the expending

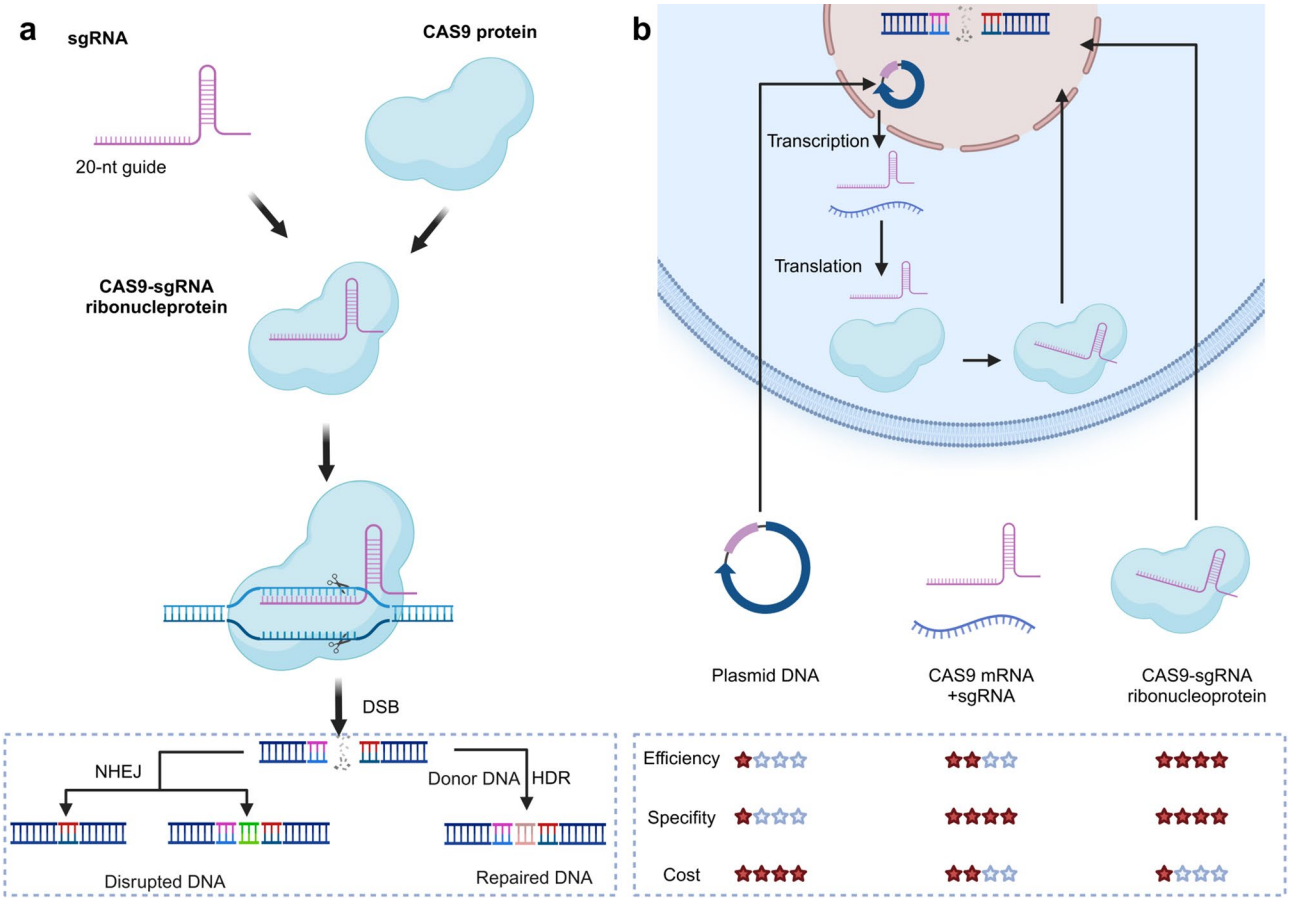


Fig. 6 Operation principle and cellular delivery methods of CRISPR-Cas9 system. **a**, Two main components: the Cas9 protein and the sgRNA. The sgRNA functions as a molecular guide, identifying the target DNA sequence via its 20-nucleotide guide sequence. **b**, The delivery formats for CRISPR components. The cargo format include pDNA, Cas9 mRNA with sgRNA, and the Cas9-sgRNA RNP. Created with BioRender.com

application of this pDNA-based delivery strategy was limited by its large transgene size, especially those Cas9 derived from *Streptococcus pyogenes* [127, 128]. The delivery vehicles showed reduced genome editing efficiency and higher off-target potential because of the much longer period it might take through during the penetration of both cell membrane and nuclear membrane [129]. Lipid systems demonstrate significant potential for delivering DNA-based vesicles, owing to their natural bilayer structure that provides protection against nuclease degradation and facilitates efficient endosomal escape. However, numerous strategies remain to be explored to enhance transfection efficiency and optimize cellular internalization. A new polyethylene glycol phospholipid-modified LNP was engineered to efficiently condense and encapsulate pDNA while preserving its sequence length. The cationic lipids on the particle's surface enhance endocytosis-mediated cellular uptake, while the DSPE-PEG surface modification mitigates potential toxicity and immunogenicity concerns [130]. Similar optimization strategies were applied to another cationic lipid-mediated PEG-b-PLGA NPs for macrophage-specific gene editing. The alteration of the native chicken β-actin promoter to macrophage-specific promoter called CD68 has driven the targeted expression of Cas9 in macrophages and their precursor monocytes, thus settling the safety concerns as a result of the off-target effect [131]. This kind of delivery strategy is also tried for restoring autoantigen-specific immune tolerance, ultimately preventing the development of type 1 diabetes. The NPs are simultaneously encapsulated autoimmune peptide, CRISPR-Cas9 pDNA and sgRNAs targeting co-stimulatory molecules, which could strongly trigger the expansion of peptide-specific Treg cells through the knockdown of co-stimulatory molecules on engineered dendritic cells achieved by designed CRISPR/Cas9 system [132].

The nonspecific distribution of Cas9 remains the major concern of genotoxicity. The selective activation of CRISPR/Cas9 complex expression in specific tissues or organs is aimed to achieve maximal therapeutic effect together with minimal systemic toxicity. To address this, many pDNA-based intelligent responsive NPs delivery systems are designed to achieve specific spatiotemporal

control over Cas9 activities. The biomimetic macrophage membrane can help address the above issues by guiding NanoCas9 system to the inflammatory lesion. In response to inflammatory cues, the precursor molecule sensitive to reactive oxygen species (ROS) becomes activated and subsequently releases trimethoprim in its active state. This ROS-responsive target nanosystem ensures inflammation-specific genome editing because dCas9 could only be stably expressed under the ROS stimuli (Fig. 7a) [133]. Other pDNA-based precise genome-editing rely on thermal power [134], magnet [135], blue light [136] and infrared light [137]. Recently, photothermal therapy has been actively explored to change TME from "cold" to "hot", which has been demonstrated to facilitate inducing immunogenic cell death [138]. Combining the hyperthermia-induced activation with disruption of immune checkpoint blockade mediated by CRISPR/Cas9 seems more appealing for cancer immunotherapy (Fig. 7b) [138]. Previous research has demonstrated that semiconducting polymers (SPs) might be excellent candidates for responding to the second near-infrared window -triggered gene editing according to its photothermal conversion characteristic, which enabled the NPs to escape from the endo/lysosomal and facilitated the release of CRISPR/Cas9. With the further chemical modification towards the initial backbone of SPs, this strategy opens the avenue for remote control of precise gene therapy [137]. In addition to photothermal conversion, another research converts NIR into locally visible blue light with the help of upconversion NPs, then interacts with the photosensitive protein to achieve subcellular localization, which solves the issue of limited penetration depth associated with the original blue light [136]. The use of these delivery systems responds to temperature, light, electromagnetic field or pH facilitate the effective enrichment of Cas9 in target tissues or organs, thereby reducing the potential risk of carcinogenesis.

mRNA-based

The long-term existence of Cas9 may lead to potential off-target genotoxicity. One effective alternative strategy is to utilize Cas9 mRNA for the expression of the Cas9 protein. This strategy introduces mRNA encoding Cas9 with guide RNA into the host cell for gene editing, which leads to the transient translation in the cytosol without the process of transcription, substantially minimizing the frequency of off-target effects in genome editing within cells. And once they are used up they would not regenerate, Thus the induced gene editing is much faster and safer compared with pDNA-based delivery [139]. However, the shortened expression time might reduce the efficiency of editing. More importantly, the inherent characteristics of mRNA have brought many obstacles to this delivery strategy. mRNA is a single-stranded molecule that can easily be degraded by RNases in serum. In addition, serving as a potential pathogen-associated molecule, exogenous mRNA would be recognized by TLRs to produce immune response, further leading to mRNA degradation and translation inhibition [140]. Appropriate chemical modifications are designed to inhibit innate immunity while their function as a natural adjuvant molecule is necessary for some specific immune gene therapy. Therefore how to balance the contradictions between stability and immunogenicity of mRNA remains a big paradox.

Recently, several novel mRNA-based delivery nanosystems have shown efficient yet safe transport of mRNA, enabling the effective expression of functional Cas9. Biodegradable amino-ester nanomaterials make the control of biodegradability rate possible. By turning the functional groups on the easter chains those lipids existing in plasma and tissues could be controllably eliminated, substantially improving their tolerability [141]. To ensure the effective and precise transport of these elements to specific cells or organs, chemical modifications are frequently necessary. For example, to achieve specific delivery of Cas9 mRNA and sgRNA to tissues, multi-component LNPs were engineered using ionizable phospholipids that can disrupt cellular membranes [142]. Cheng et al. [143] presented a novel approach called selective organ targeting (SORT). SORT involves the comprehensive engineering of various types of lipid nanoparticles to specifically modify tissues outside the liver by integrating an extra SORT molecule. The SORT LNPs, designed specifically for targeting specific organ, have been developed to selectively modify clinically important cell types. SORT can be utilized with a diverse range of gene editing methods, such as mRNA delivery, Cas9 mRNA/sgRNA. It is anticipated that SORT will facilitate the advancement of protein replacement and genome correction therapy in targeted tissues (Fig. 8a). Lung-targeted genome editing was accomplished by screening a 720 biodegradable ionizable lipids library, utilizing an inhalable delivery method for CRISPR-Cas9 gene-editing tools (Fig. 8b) [79]. And Qiu et al. [144] utilized the FDA-approved liver-targeted LNP formulation MC3, which contains ionizable lipids, to deliver Cas9 mRNA and sgRNA in liver specific genome editing. Zhao et al. [145] reported a copolymer system for delivery of Cas9 mRNA and sgRNA. This system employs carboxylesterase-responsive cationic triad copolymeric NPs, which are targeted toward proprotein convertase subtilisin/kexin type 9 to address hyperlipidemia (Fig. 8c). These carriers are designed to respond to hepatocyte carboxylesterase, thereby facilitating the targeted release of RNA for genome editing applications. The results indicated that Cas9 mRNA/sgRNA copolymer system efficiently accumulate within hepatocytes, resulting in the inhibition of subtilisin/kexin type 9 and

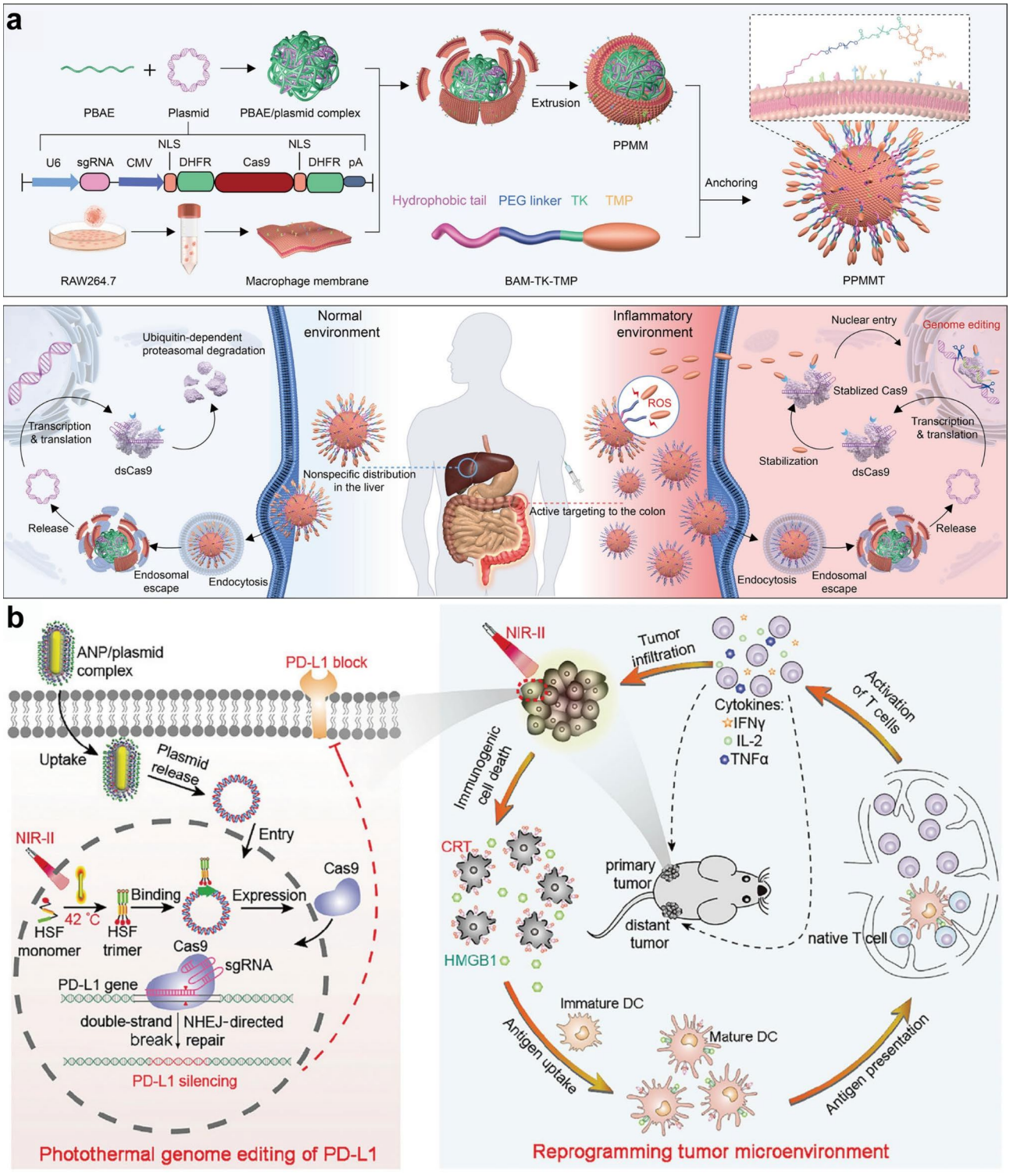
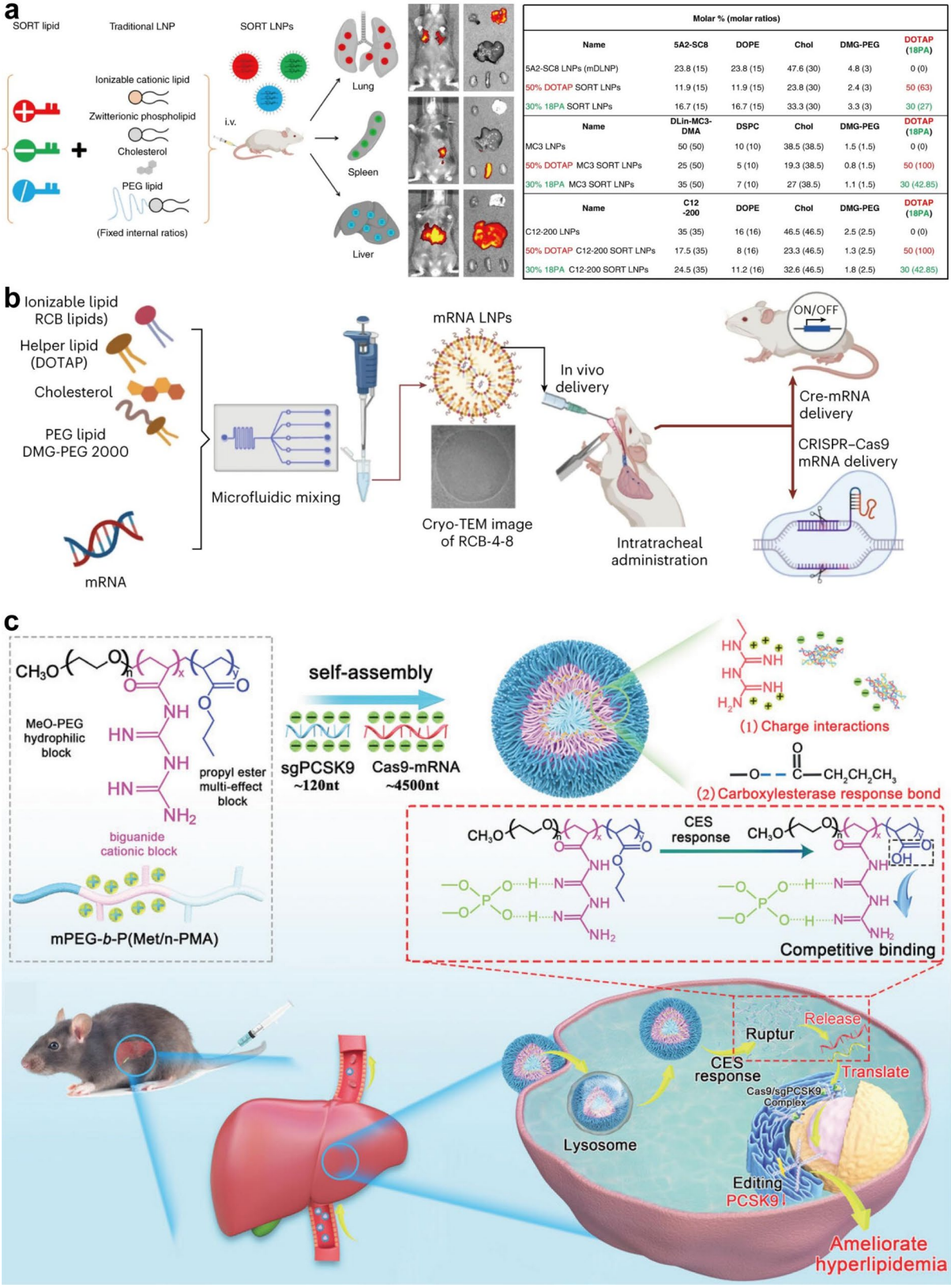


Fig. 7 Schematic diagram of targeted delivery and responsive genome editing facilitated by pDNA-based CRISPR/Cas9 nanosystems. **a**, Schematic illustration of ROS-responsive NanoProCas9 system in the inflammatory colon lesion. Reproduced with permission [133]. Copyright© 2021, the American Association for the Advancement of Science. **b**, Schematic diagram of the photothermal CRISPR/Cas9 strategy for PD-L1 gene editing in tumor. Reproduced with permission [138]. Copyright© 2021 Wiley-VCH GmbH



Molar % (molar ratios)

Name	5A2-SC8	DOPE	Chol	DMG-PEG	DOTAP (18PA)
5A2-SC8 LNPs (mDLNP)	23.8 (15)	23.8 (15)	47.6 (30)	4.8 (3)	0 (0)
50% DOTAP SORT LNPs	11.9 (15)	11.9 (15)	23.8 (30)	2.4 (3)	50 (63)
30% 18PA SORT LNPs	16.7 (15)	16.7 (15)	33.3 (30)	3.3 (3)	30 (27)
Name	**DLin-MC3-DMA**	**DSPC**	**Chol**	**DMG-PEG**	**DOTAP (18PA)**
MC3 LNPs	50 (50)	10 (10)	38.5 (38.5)	1.5 (1.5)	0 (0)
50% DOTAP MC3 SORT LNPs	25 (50)	5 (10)	19.3 (38.5)	0.8 (1.5)	50 (100)
30% 18PA MC3 SORT LNPs	35 (50)	7 (10)	27 (38.5)	1.1 (1.5)	30 (42.85)
Name	**C12 -200**	**DOPE**	**Chol**	**DMG-PEG**	**DOTAP (18PA)**
C12-200 LNPs	35 (35)	16 (16)	46.5 (46.5)	2.5 (2.5)	0 (0)
50% DOTAP C12-200 SORT LNPs	17.5 (35)	8 (16)	23.3 (46.5)	1.3 (2.5)	50 (100)
30% 18PA C12-200 SORT LNPs	24.5 (35)	11.2 (16)	32.6 (46.5)	1.8 (2.5)	30 (42.85)

Fig. 8 Schematic illustration of mRNA-based CRISPR/Cas9 nanosystems applied in gene therapy. **a**, Schematic illustration of selective organ targeting (SORT) and details of SORT molecule formulations. Reproduced with permission [143]. Copyright© 2022 Springer Nature. **b**, Schematic diagram of the formulation for pulmonary delivery of CRISPR/Cas9 system. Reproduced with permission [79]. Copyright© 2023 Springer Nature. **c**, Schematic of Cas9-mRNA delivery nanosystem for hyperlipidemia amelioration. Reproduced with permission [145]. Copyright© 2023 Wiley-VCH GmbH

a significant reduction in low-density lipoprotein cholesterol and overall cholesterol levels in mouse serum, with reductions of approximately 80% relative to untreated groups. This finding suggests a promising strategy for targeted gene therapy and cholesterol management. Other research has also concentrated on developing ionizable lipids or optimizing the lipid formulation in LNP to enhance the delivery of Cas9 mRNA and sgRNA, thereby improving the efficiency of genome editing [146]. Moreover, chemical modifications have been introduced to ionizable lipids to address concerns regarding inadequate biodegradability of LNPs [147]. Besides research on ionizable lipids, Gautam et al. [148] showed that incorporating PEG lipids modified with carboxy esters or carboxylic acids into LNPs can significantly enhance mRNA expression in retinal photoreceptors as opposed to unmodified LNPs. This discovery enhanced the effectiveness of ocular CRISPR-Cas9 system. More precisely, when Cas9 mRNA and sgRNA were co-encapsulated in carboxy ester-modified PEG LNPs resulted in a targeted editing efficiency of 27% in the retinal pigment epithelium.

Delivery timing has become another new issue. For the co-delivery of Cas9 mRNA and sgRNA with the same vesicle, the time for in-situ translation of mRNA to functional protein must be taken into consideration to ensure the Cas9 protein and complete sgRNA be present in the cell simultaneously. To solve this problem, systemic delivery of Cas9 mRNA by LNPs together with sgRNA and HDR template delivered by AAV was developed to activate repair of disease-related genes in animals [149]. sgRNA was not co-delivered with the Cas9 mRNA, which narrowed the time window of targeted cleavage. The improvement of sgRNA stability through optimization of delivery time and chemical modification helps increase the editing efficiency for systemic delivery [149]. However, another research demonstrated that simultaneous delivery of Cas9 mRNA and sgRNA using a unified NPs system would guarantee delivery to the same individual cells, thus driving the in vivo utility to a maximal level [150]. To achieve optimal editing efficiency, further research is necessary to ensure the coordination between staged delivery and co-delivery.

Ribonucleoprotein-based

The most direct and fastest way for delivering is to co-deliver Cas9 protein and sgRNA(Cas9 and sgRNA ribonucleoprotein, RNP). This protein-based strategy avoids the process of transcription and translation, therefore providing the most transient expression time and significantly improving the efficiency of gene editing. On the other hand, this strategy does not exit the risks associated with the insertion and disruption of the exogenous genome, thus offering functionally deliveryed both increased safety and broader applicability compared with DNA or mRNA-based delivery. Additionaly, the purity of RNP needs to take into consideration for those unwanted bacterial proteins would induce immunotoxicity and a further threat to health, which means a higher economic cost of RNP-based delivery was required for the purification step compared with deliveries based on DNA or RNA levels. The poor internalization of RNPs also provides big challenges for loading on the vehicles. Therefore, many carrier-based deliveries based on lipid-based material [134, 151, 152], peptides [153, 154], polymers [155], inorganic NPs [156], dendrimers [157] and other nanomaterials [158] had been applied for optimizing the efficiency and stability of Cas9/gRNA RNP delivery. However, Cas9 RNP essentially as protein, its poor stability, large size and high cytotoxicity make many traditional delivery systems ineffective. Thus some novel nanomaterials or strategy optimizations have been implemented and reported to solve these obstacles.

Experimental results showed that anionic charge and chain length of polymers are key factors for RNP enhancement. Besides, the addition of polyglutamic acid further stabilizes the nanopolymers by preventing aggregation into micron-sized particles [159]. This strategy not only ensures stability and activity but also improves the efficiency of gene editing and reduces toxicity in vivo. Another study demonstrated the inclusion of phosphorothioate-modified DNA oligonucleotides could offer both physical and chemical protective benefits by interaction with polymer-derived RNP complex to prolong its activity [160]. In addition to forming condensed RNP, the strategy also promotes internalization into the cell through strong interaction towards target cell-membrane. Interestingly, the method of surface immobilizing could also enhance the interaction with the host cell membrane. Leong et al. [161] designed a scaffold-mediated CRISPR/Cas9 system that uses nanofibrils coated with mesenchymal stem cell membranes to mimic the bone marrow microenvironment, for increasing the retention time at the injection site, thus could be regarded as an efficient editing method for local delivery to bone marrow (Fig. 9a).

Recently, combinatorial therapy has been introduced into Cas9 RNP delivery with the rapid development of the gene delivery system, greatly reducing the undesired side effects by narrowing the difference of pharmacokinetics and biodistribution between Cas9 RNP and small molecular drugs. This co-delivery strategy has been widely used in the therapy of immune-related diseases, cancer and other diseases. In the treament of inflammatory skin disorders, Wan et al. [162] reported a dissolvable microneedle engineered for transdermal co-delivery of Cas9 RNP and dexamethasone. Once inserted into the skin, the microneedle rapidly dissolved to deliver these formulations. These formulations are taken up by

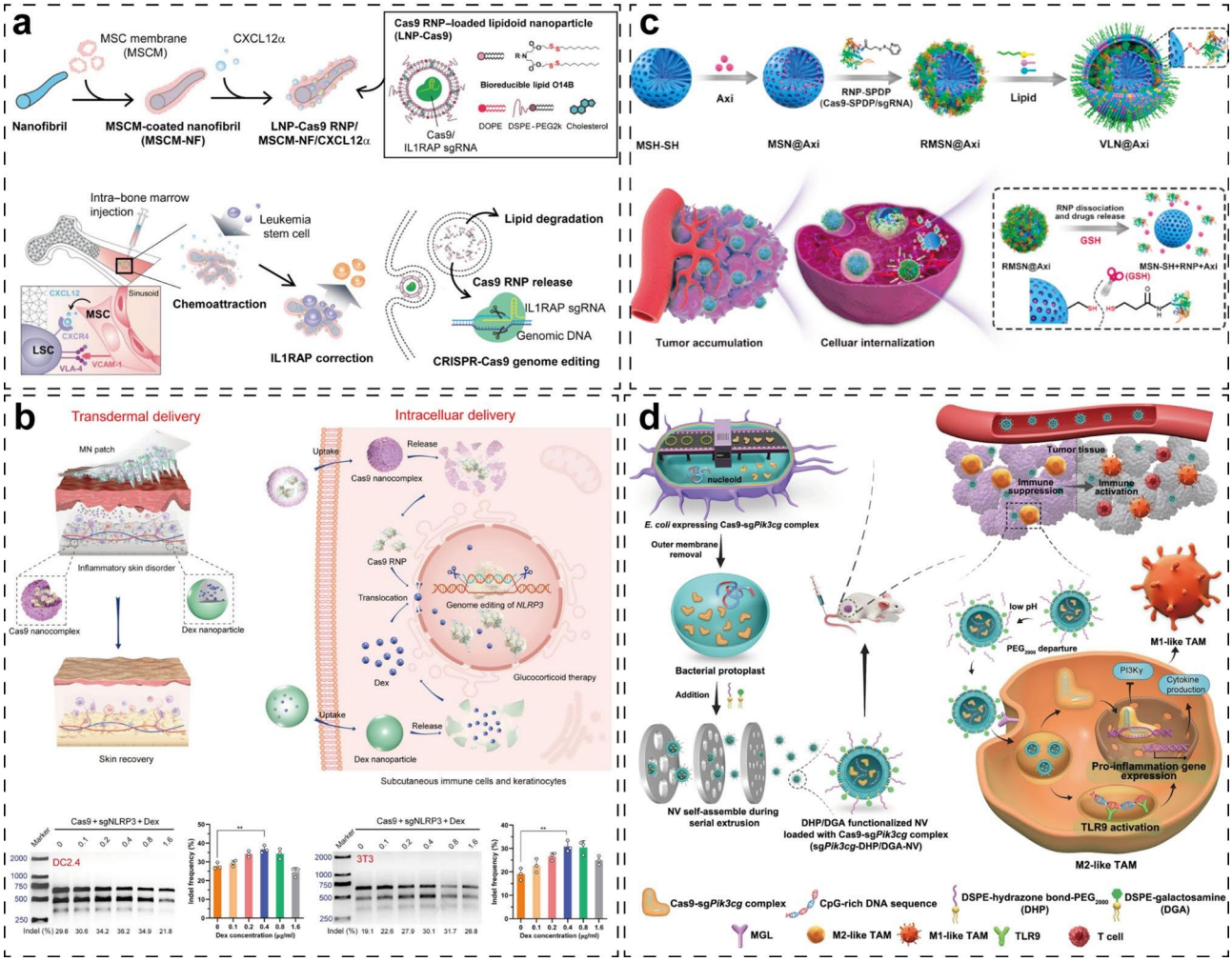


Fig. 9 Schematic diagram of RNP-based CRISPR/Cas9 nanosystems for gene therapy. **a**, Schematic diagram of the RNP-LNP nanosystems. Reprinted with permission [161]. Copyright© 2021, The American Association for the Advancement of Science. **b**, Schematic of transdermal and intracellular delivery of Cas9 and glucocorticoids. Reprinted with permission [162]. Copyright© 2021, The American Association for the Advancement of Science. **c**, Schematic diagram for the synthesis and delivery process of VLN@Axi for efficient cancer immunotherapy. Reproduced with permission [163]. Copyright© 2020, Elsevier. **d**, Schematic of the preparation and application of NIR-sensitive and reducing agent-sensitive NPs. Reproduced with permission [168]. Copyright© 2024, The Author(s)

keratinocytes and nearby immune cells, thereby enabling targeted therapeutic outcomes within the inflamed subcutaneous tissues. Therefore, this transdermal co-delivery system led to the disruption of subcutaneous NOD-like receptor, LRR- and pyrin domain-containing protein 3 inflammasomes (Fig. 9b). MSNs were coated with lipid layers to form virus-mimicking NPs, thereby protecting the RNP against enzymatic degradation and prolonging the circulation in vivo (Fig. 9c) [163]. The versatile strategy for gene editing, when combined with synergistic drug effects, can be further designed as stimulus-responsive nanosystems. NIR-responsive and reducing agent-responsive NPs were used to co-deliver the RNP and antitumor photosensitizer. Under this photodynamic therapy, photosensitizer would generate ROS upon NIR irradiation, helping facilitate the release of RNP and enhance the tumor cell sensitivity to ROS [158]. Another research also proved the potentiality of the NIR light-triggered system [164, 165]. Apart from these traditional drug delivery systems, outer membrane vesicles (OMVs) derived from bacteria have recently served as promising delivery carriers [166]. OMVs modified by target genes have demonstrated the distinctive ability to integrate the targeting protein, rendering them highly potent for protein delivery applications [167]. Zhao et al. [168] developed nanovesicles derived from bacterial protoplasts, which were modified with pH-responsive PEG-linked phospholipid derivatives and galactosamine-linked phospholipid derivatives, specifically designed for tumor-associated macrophage targeting. Using this modified platforms, they effectively encapsulated EVs with two key elements: a RNP complex targeting macrophage

polarization gene Pik3cg and DNA fragments rich in CpG motifs from bacteria, which serve as TLR9 agonists. This EV-based self-assembling method held potential for scalable clinical manufacturing and reshaped the TME by maintaining an M1-like phenotype in tumor-associated macrophages (Fig. 9d).

Although targeting different oncogenes, these remote-control RNP delivery platforms all specifically inhibited the proliferation of cancer cells and suppressed tumor development.

CAR T therapy

In recent years, there has been a notable rise and swift advancement in CAR technology. CAR-T immunotherapy has demonstrated remarkable clinical achievements for refractory and relapsed hematopoietic malignancies [169]. Motivated by these advancements and achievements, researchers have expanded the application of CAR technology beyond CAR-T to encompass CAR-NK, CAR-CIK, and CAR-M therapies [170]. Conventionally, the process of manufacturing and administering autologous CAR-T therapy follows a relatively standardized procedure involving several key steps: (1) isolating and enriching T cells from cancer patients through leukapheresis; (2) activating and expanding the extracted T cells; (3) introducing a CAR gene vector into the T cells using either viral or non-viral systems; (4) expanding the genetically modified CAR-T cells in vitro; (5) formulating the cell product and preserving it via cryopreservation; and (6) administering lymphodepleting treatment followed by reintroducing the CAR-T cells into the patient (Scheme 4). Other CAR-modified immune cells, such as CAR-NK, CAR-M, and CAR-CIK, are also developed following the similar protocol. Throughout these procedures, stringent quality control measures and release criteria are essential to ensure the integrity of the final CAR products. These measures include monitoring production materials (particularly cell sources and gene modification vectors), in-process controls and testing, release testing, validation of the production process, and stability assessments. Key factors evaluated during these tests include CAR expression levels, lymphocyte subpopulations, cell purity, the count and ratio of viable cells, in vitro potency, and microbiological safety (such as sterility testing, mycoplasma screening, detection of replication-competent viruses, rapid microbial detection, and endotoxin levels).

CAR T cell therapy, approved by the FDA and EMA, represents a groundbreaking approach for the management of B cell malignancies and multiple myeloma [171]. Although CAR-T therapy demonstrated huge possiblity for the treatment of cancers with high efficiency, compelling challenges still exist regarding the feasibility of this therapy for severe immune deficiency and other cytotoxicity caused by its "on-target, off-tumor" effect [172, 173]. Recently, facts have demonstrated the possibility of applying NPs as vesicles for the delivery of CAR instead of virus vectors for the higher transfection efficiency, lower cost and off-target drawbacks it presents [174]. Various strategies were designed for the optimization of NPs to achieve the maximal and specifically targeted delivery of CAR therapy. In this section, we will discuss non-viral gene delivery methods utilized for engineering CAR T cells, with a focus on lipid and polymer-based nanosystems as a leading nanotechnology.

NPs as CAR carriers

In 2017, Smith et al. [174] developed the first off-the-shelf CAR-T through NPs instead of lentivirals. The DNA cargo encoding leukemia-specific 194-1BBz CAR was loaded on the biodegradable polymer-based NPs, which absorbed the conjugate formed by the coupling of polyglutamic acid and αCD3e f(ab′)2 via electrostatic interactions to achieve the selective targeting towards T cells. Peptides that incorporate microtubule-associated sequences and nuclear localization signals were designed to functionalize the coating polymer to ensure fast-track nuclear importation. And with the help of mobile piggyBac inverted terminal repeats serving as transposons, the vectors were integrated into chromosomes via a cut-and-paste mechanism [174]. This in vivo expansion strategy largely reduced the cost and provided a novel platform for active immunotherapy.

In addition to the above transposon-based integration, cationic lipid- and polymer-based engineered to serve as vesicular carriers for CAR delivery via transfection. Their tunable structure and shelf stability make them not limited to the dimensions or category of the cargo, thus could be regarded as an excellent substitute to electroporation because this method would probably lead to genome alteration and significant cytotoxicity, while also failing to ensure uniform membrane disruption across all cells [175]. Studies have demonstrated that polymers are able to deliver pDNA and mRNA into primary human T cells with moderate efficiencies, achieving rates of up to 18% and 25%, respectively, while preserving high cellular viability [176]. Interestingly, the shape of NPs is associated with the efficiency of CAR-T transfection. Comb- and sunflower-shaped polymers are more effective in safeguarding the cargo, making them more attractive for ex vivo gene delivery applications [176]. Recently, ionizable LNPs were designed for delivering mRNA to the primary human T cell ex vivo, which have been demonstrated to induce equivalent expression levels of CAR but reduced cytotoxicity compared with electroporation [177]. In this study, the platform utilized ionizable lipids along with three distinct excipients in ethanol.: (1) cholesterol for NPs stability and membrane fusion capability.

(2) DOPE for better endosomal escape. (3) C14-PEG for reduction of aggregation and endocytosis. In the orthogonal experiments designed to optimize LNPs, the findings also highlighted the influence of excipients on LNP performance, which in turn affected the reprogramming efficiency of CAR-T cells [178].

Traditional CAR-T engineering requires a series of cumbersome processes. However, the novel program uses nanocarrier to deliver in vitro-transcribed antigen receptor mRNA, which bypasses the process of lymphocytes extraction and culture from patients [179]. The injectable nanocarrier with a transposon/transposase system encoding the CAR for transiently reprogramming T cells for specific recognition of tumor-associated antigen. This innovative approach eliminates the complexity and high expenses associated with traditional methods of generating disease-specific T cells in vitro. However, several clinical challenges remain for its widespread application. A key consideration is the requirement for an adequate number of functional T cells in patients. Additionally, the effectiveness of mRNA may be diminished by induced immune responses, necessitating multiple administrations.

NPs combined with CAR

Currently, studies have found the vast majority of solid tumors failed to effectively respond to the CAR-T cell therapy. The obstacles that must be crossed are the thorough penetration of the tumor and the overcoming of the immunosuppressive environment. Tightly interconnected dense tumor tissues and a compact extracellular matrix are closely associated, the produced tissue pressure by physical barrier limits the complete permeation and hinders the infiltration of CAR-T cells in internal tumors [180]. Additionally, TME poses challenges for the survival of CAR-T cells due to its characteristics of hypoxia, low nutrition and pH, and high permeability. Additionally, various immunosuppressive cells such as Treg cells and several immune checkpoints would suppress the cytotoxic activity of CAR-T cells in different manners [181]. Therefore, to effectively suppress the solid tumor, CAR-T would probably be applied in combination with other nano-based approaches.

Microenvironmental modulation plays a crucial role in the treatment of solid tumors and represents an emerging breakthrough for enhanced CAR-T immunotherapy. A variaty of strategies based on nanosystems have been used for remodeling the TME. One of the feasible combinations with CAR-T therapy is the rational delivery of immunomodulatory cytokines into the tumor microenvironment using nanocarriers to enhance antitumor immunity. A novel approach has been suggested for the treatment of solid tumors, which involves chemically linking adjuvant drug delivery with T cell activation in a synergistic manner. By using T cell receptor (TCR)-signaling-responsive drug-loaded lipid NPs (referred to as "backpacks"), interleukin (IL)-15 super-agonist could be focused release to the tumor microenvironment, enabling substantially improved tumor clearance (Fig. 10a) [182]. This research also demonstrated that this controllable NP delivery of cytokines might enhance the CAR-T therapy due to the higher proportion of tumor eradication in those NG-backpacked mice. Similarly, Fang et al. [183] created a lipid-based nano delivery system that includes potent drug cocktails that could reshape the tumor microenvironments. Data from the mice model of glioma demonstrated that iRGD peptide-decorated liposomes enhanced tumor localization of systemically administered liposomes. And co-delivery of two immunomodulatory agents called PI-3065 (an inhibitor of the PI3K kinase) and 7DW8-5 (an agonist of NK cell) swayed the TME from suppressive to permissive using the "releasing immune brakes" while "stepping on the gas" strategy, thus triggered tumor-specific regression and undergo robust expansions. While showing potential, the continuous secretion of cytokines by CAR-T cells may raise safety issues in clinical applications [184]. These challenges can potentially be mitigated by engineering CAR -T cells to secrete cytokines only upon activation or by altering cytokine receptors or their downstream signaling pathways instead of modifying the cytokines themselves. Although CAR-T cells demonstrate potential through their continuous secretion of cytokines, this characteristic might pose safety concerns in clinical settings.

Several combination therapy strategies are currently in clinical development, integrating CAR-T cells with checkpoint inhibitors, cancer vaccines, or radiotherapy. These approaches aim to alter TME in solid tumors and enhance the effectiveness of CAR-T cell therapy [185]. Preclinical studies have also investigated various combination therapies that hold promise for clinical application. For example, researchers have explored using CAR-T cells in conjunction with stimulator of interferon genes (STING) agonists. For instance, Zhu et al. [186] developed a nanosystem that expresses anti-PD-L1 and loaded with the STING agonist. This innovative approach aims to reprogram the TME, consequently boosting the efficacy of CAR-T cell therapy (Fig. 10b).

Then, like most gene therapy approaches, the next issue that should be addressed is tissue-selective release. Numerous environment-responsive strategies combined with nanocarrier have been designed for drug delivery to achieve tissue-selective release [187]. Mild photothermal therapy has recently emerged as a promising approach for remodeling TME. For instance, nano-photosensitizer served as a microenvironment modulator, has been wildly applied to solid tumor immunotherapy via biohybrid with CAR-T [188]. Chen et al. [188] developed

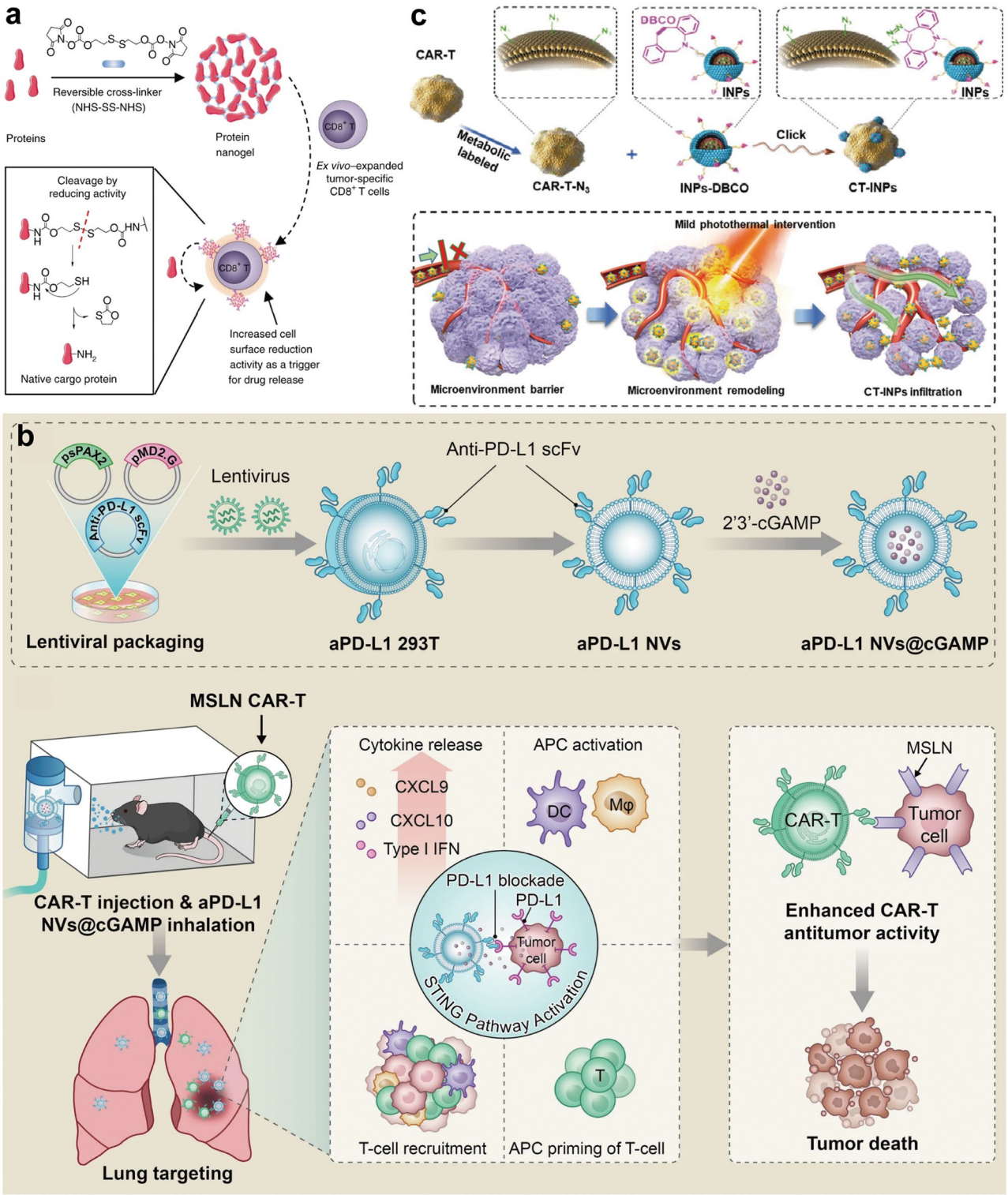


Fig. 10 Schematic illustration of strategies for designing NPs combined with CAR therapies. **a**, Schematic illustration of "backpack" large amounts of protein-based therapeutics on T cells. Reprinted with permission [182]. Copyright© 2018, Springer Nature America, Inc. **b**, Schematic diagram of application of aPD-L1 NPs@STING agonist in antitumor efficacy. Reprinted with permission [186]. Copyright© 2025, The Author(s). **c**, Schematic diagram of gentle photothermal remodeling to dismantle the physical barriers and reconfigure the microenvironment to achieve improved infiltration of CT-INPs within tumors. Reproduced with permission [188]. Copyright© 2021 Wiley-VCH GmbH

indocyanine green NPs engineered CAR-T biohybrids effectively collapsed the physical barriers by dilating vascular structures, relaxing dense tissue, and activating antitumor elements secretion, further robustly boosting the CAR-T antitumor immunotherapy (Fig. 10c). Combining the above phototherapy with chemotherapy seems to be a more promising platform.

Recently, nanoparticle-sensitized photoporation has been utilized in adoptive cell therapy. Effector molecules including CRISPR/Cas9 RNP and siRNA could be successfully delivered to hard-to-transfect T cells without influencing cell growth or characteristics. With the help of photothermal electrospun nanofibers, siRNA targeting PD-1 was transfected to the CAR-T, leading to the reduced expression level of PD-1 receptor, further strengthening the tumor-eradication capability [189]. Based on the dual response of tumor-antigen and light, the light-switchable CAR-T cells could remotely regulate the elimination of antigen-specific masses with substantially attenuated side effects.

Application and challenges of NP-gene therapeutics in clinical trials

Over the past twenty years, many nano-drugs have been investigated in clinical and preclinical studies for enhanced gene delivery against cancers (Table 1). While lipid-based nanoparticles dominate clinical applications, other systems including MSC, cell membrane-coated systems, and RNA-LNPs, are emerging through preclinical studies [190]. The main takeaways emphasize the essential requirement for improved targeting, reduced immunogenicity, and manufacturing processes that can be scaled up efficiently. Nanoparticles in clinical trials are predominantly reformulations of approved drugs using polymers, liposomes, micelles, and dendrimers [191]. While passive targeting *via* the EPR effect remains common, active targeting strategies are increasingly explored. Recent advancements involve multicomponent delivery systems [192]. Nanoparticles are increasingly utilized in cancer gene therapy to enhance the delivery and efficacy of genetic materials such as siRNA, mRNA, CRISPR-Cas9, and miRNA mimics. These therapies aim to silence oncogenes, restore tumor suppressor function, or induce immune responses against tumors. Several clinical trials have examined the feasibility of encapsulating RNA within LNPs. These studies focus on utilizing LNPs both as standalone treatments and in conjunction with immune checkpoint inhibitors, such as pembrolizumab and navuliumab, to improve treatment efficacy and address resistance issues (Table 1) [193, 194]. Additionally, research has been conducted on the administration of mRNA-2752 via LNPs in combination with durvalumab, aiming to elicit immune responses against tumor-associated antigens [195]. A further significant application involves the use of LNPs for delivering siRNA (drug product NBF-006), which targets glutathione S-transferase Pi, a regulator of the KRAS and JNK pathways [196]. Liposomes have been employed in clinical trials for delivering siRNA targeting protein kinase N3, siRNA targeting EphA2, and miR34a to regulate oncogenic pathways and genes associated with tumor immune evasion [197–199]. Additionally, polymer nanoparticles and exosome have been utilized for the targeted delivery of siRNA targeting the M2 subunit of ribonucleotide reductase RRM2 and siRNA against KrasG12D, aiming to decrease tumor burden [200, 201]. The adaptability of polymer-based delivery systems has also enabled the incorporation of a human transferrin protein-targeting ligand to specifically target cancer cells and enhance overall therapeutic effectiveness.

However, the high production and commercialization costs associated with nano-drugs have become the main challenge for their successful development. These products are considerably expensive due to the high costs associated with both the manufacturing process and raw materials. Manufacturing nanomedicines under Good Manufacturing Practice conditions presents a unique challenge, as even minor changes in the process can significantly affect properties such as size, shape, composition, drug loading and release, biocompatibility, toxicity, and in vivo performance [202]. Another critical challenge in nanomedicine development involves determination of an appropriate sterilization method without compromising the stability or physicochemical attributes of the therapeutic agents [203]. Biological molecules, such as proteins, are highly sensitive to deactivation during

Table 1 Clinical trials involving nanoparticles for cancer gene therapy

Type	Drug Delivered	Target Cancer	Trail Phase	Target gene	Ref.
LNP	mRNA-5671/V941	Solid tumors	I	KRAS	[193]
LNP	siRNA	Non-small cell lung cancer; pancreatic cancer; colorectal cancer	I	Glutathione S-transferase Pi	[196]
LNP	mRNA-2752	Solid tumor malignancies or lymphoma	I	Human OX40L, IL-23, and IL-36γ	[195]
Liposome	siRNA	Advanced solid tumors	I	Protein kinase N3	[197]
Liposome	siRNA	Advanced solid tumors	I	EphA2	[198]
Liposome	miR-RX34	Primary liver cancer; advanced or metastatic cancer	I	-	[199]
PNP	siRNA(C05C)	Solid tumors	I	anti-RRM2	[200]
Exosome	siRNA	Pancreatic cancer	I	KrasG12D	[201]

sterilization, necessitating additional care for nanomedicines based on these substances [204]. Endotoxin contamination poses significant health risks and accounts for over 30% of nanoformulation failures in early preclinical studies [205]. Thus, the endotoxin level in nanomedicines must be meticulously evaluated using suitable techniques. Currently, assessing the stability and storage characteristics (shelf life) of nanomedicines remains a complex task [206]. The properties of nanomedicines may also alter under storage conditions, whether in aqueous solutions or in lyophilized forms [207]. Moreover, evaluating the toxicological impacts of nanomaterials is essential yet challenging. While the toxicological impacts of nanomaterials require evaluation, certain effects remain ambiguous. There is a need to develop frameworks for standardizing preclinical nanomedicine studies. Such frameworks can enhance quantitative analysis, ensure reproducibility, and support modeling efforts, ultimately improving the cost-efficiency, safety, and suitability of nanoformulations and accelerating the transition from fundamental research to clinical application. In addition, regulatory considerations play a critical role in advancing technologies for the characterization and quality assurance of nanopharmaceuticals.

Safety screening of NP types

It is crucial to evaluate the safety of various NP types, as well as any documented long-term effects. Here, the screening safety aspects of LNP, PNP and inorganic NPs are summarized below.

LNPs

Cytotoxicity assessment: Cationic lipids in LNPs are a concern due to their potential cytotoxicity. For example, reducing the positive charge of cationic lipids normally decreases toxicity while leading to decreased genetic material encapsulation and transfection efficiency. To evaluate the NP safety, in vitro cytotoxicity tests are commonly used. These tests examine the impact of LNPs on cell viability, often using cell lines relevant to the target tissue or organ. For instance, when developing LNPs for liver-targeted gene delivery, hepatocyte cell lines are used to assess how LNPs affect cell behaviors.

Immunogenicity evaluation: Exogenous mRNA encapsulated in LNPs can trigger an immune response as it may be recognized by TLRs. To screen immunogenicity, preclinical studies involve administering mRNA-LNPs to animal models and monitoring immune-related responses such as cytokine production, antibody formation, and immune cell activation. Chemical modifications to mRNA and LNPs are also explored to balance stability and immunogenicity. For example, rationally designed ionizable lipids can potentially reduce immunogenicity while maintaining delivery efficiency [208].

PNPs

Biocompatibility and biodegradability: PNPs made from polymers such as polyethylenimine, PEG, and PLGA are biocompatible and biodegradable. Safety screening involves assessing how these polymers interact with biological systems. In vitro studies evaluate their impact on cell growth, proliferation, and function. In vivo studies in animal models are used to monitor tissue distribution, clearance, and potential long-term accumulation. For example, the degradable PLGA-based PNPs are analyzed to ensure that they do not cause adverse effects [209].

Off-target effects: Since PNPs can be engineered to target specific cells or tissues, screening the off-target effects are essential [210]. This is often done by tracking the distribution of PNPs in the body using imaging techniques such as fluorescence microscopy or radiolabeling. If PNPs accumulate in non-target tissues, some unwanted side effects may be caused, so researchers strive to optimize their targeting ligands and surface properties to minimize such off-target effects.

Inorganic NPs

Physicochemical stability and cytotoxicity: Inorganic NPs normally have stable physicochemical characteristics. However, their safety screening is still required about how they interact with biological systems. For example, AuNPs' surface properties, such as PEGylation and surface charge, can affect their toxicity [211]. In vitro and in vivo studies are conducted to assess their cytotoxicity, genotoxicity, and potential to cause oxidative stress. MSNs' pore-size and surface modifications were also studied to ensure they do not cause adverse effects on cells or tissues [212].

Long-term accumulation: Inorganic NPs may have long-term accumulation in the body. To screen the safety, animal studies with long-term follow-up were carried out. The accumulation of NPs in organs (liver, spleen, and kidney) was monitored over time, and any associated histological or biochemical changes were evaluated. For example, if AgNPs accumulate in the liver, it could potentially lead to liver dysfunction, so continuous monitoring of liver function markers is part of the safety screening process [213].

Distinct advantages and limitations of different NP types

The development of diverse nanoparticles (NPs) for tumor gene therapy has been driven by their unique physicochemical properties, yet each class of NPs presents distinct advantages and limitations that influence their applicability in translational research [214]. Below is a synthesis of the strengths and challenges associated with major NP types (Table 2).

The choice of NP type hinges on the therapeutic cargo (e.g., mRNA, siRNA, CRISPR RNPs), target tissue (e.g.,

Table 2 Advantages and disadvantages of different NP types

Nanoparticle Type	Advantages	Disadvantages
Organic Nanoparticles	PNPs - Tunable biodegradability and biocompatibility - Flexible surface functionalization - Versatile cargo encapsulation	- Degradation kinetics complexity - Lower transfection efficiency - Limited stability in biological fluids
	LNPs - Superior mRNA delivery - High transfection efficiency - Tailored tissue tropism	- Cationic lipid toxicity - Short circulation half-life - Limited DNA delivery
Inorganic Nanoparticles	AuNPs - Exceptional stability & biocompatibility - Easy surface modification - Multi-modality applications	- Heavy metal toxicity - Limited degradability - Immune activation
	MSNs - Large pore volume & High loading capacity - Chemical stability - Biocompatibility	- Poor degradability in vivo - Size-dependent clearance - Surface charge challenges
Extracellular Vesicles	EVs - Natural biocompatibility and low immunogenicity - Inherent tissue tropism, engineerable with ligands - Capable of crossing biological barriers	- Low cargo loading efficiency - Scalable production challenges - Short circulation half-life in unmodified form
DNA Nanostructures	- Precision engineering - High biocompatibility - Nuclear targeting potential	- Mechanical instability - Limited cargo diversity - Scale-up challenges

solid tumors, metastatic sites), and desired mechanism (e.g., transient gene silencing vs. permanent genomic editing). For example, LNPs are optimal for mRNA vaccines due to their transfection efficiency, while EVs excel in delivering siRNA across barriers [215, 216]. Inorganic NPs like AuNPs offer dual functionality for imaging and therapy, but their safety profiles require rigorous long-term toxicity studies [217]. Future advancements must address shared challenges, including off-target accumulation, immunogenicity, and cargo release kinetics, through interdisciplinary approaches-such as AI-driven NP design, biomimetic surface engineering, and multi-responsive materials-to balance efficacy and safety. Ultimately, no single NP type is universally ideal; instead, rational design tailored to the biological context of each cancer type will drive progress in clinical translation.

Nanoparticle-mediated gene therapy for precise tumor treatment

While significant progress has been made in NP-mediated tumor gene therapy, several critical open questions remain, particularly regarding dosing frequency control for repeated therapy and strategies to minimize off-target effects, especially in CRISPR-based approaches. Addressing these challenges will be pivotal for advancing translational research and clinical implementation.

Dosing frequency control for repeated gene therapy

In order to achieve a suitable dose control, we first need to understand the major issues that currently hinder dosing frequency control. The first thing is NPs' pharmacokinetics and biodistribution. The optimal dosing frequency for NP-based gene therapies requires further investigation to determine its efficacy and safety, as it depends on NP clearance kinetics, target tissue accumulation, and therapeutic payload persistence [218]. For example, LNPs delivering mRNA or CRISPR components may have short half-lives due to immune-mediated clearance or renal filtration, necessitating repeated administrations. However, repeated dosing can trigger adaptive immune responses, reducing efficacy or causing adverse events. The second challenge is the narrowed therapeutic window. Frequent dosing may lead to saturation of cellular uptake mechanisms or overload of intracellular processing machinery, potentially diminishing transfection efficiency or increasing off-target toxicity. Moreover, TME dynamics will also influence cellular uptake. Solid tumors exhibit heterogeneous blood flow and extracellular matrix barriers, which may alter NP accumulation kinetics over repeated doses.

To solve this issue, it is a promising strategy to develop predictive models integrating NP physicochemical properties, biological clearance pathways, and target tissue characteristics [219]. Machine learning could optimize dosing schedules by analyzing preclinical data on NP distribution and therapeutic response [220]. Moreover, researchers should also explore strategies to mitigate immune responses to repeated NP administration, such as transient immunosuppression during dosing, stealth NP designs, or tolerogenic NP formulations that dampen adaptive immunity. Beyond that, we may engineer NPs with stimulus-responsive release mechanisms to enable

on-demand payload release, reducing the need for frequent dosing. For example, NIR-responsive inorganic NPs could release drugs only upon tumor irradiation, minimizing systemic exposure [221].

Minimizing off-target editing

How to reduce the off-target effect is the most important to gene editing (e.g. the CRISPR gene editing system). Off-target DNA cleavage by CRISPR-Cas9 nucleases remains a critical safety concern, particularly with viral or non-viral delivery systems that exhibit prolonged Cas9 expression [222]. Even RNP-based delivery, which offers transient Cas9 activity, may still induce off-target effects due to incomplete RNP clearance or unintended nuclear localization [223]. Thus it is of great importance to precise the targeted delivery system. Traditional NPs may lack spatiotemporal control over CRISPR component release, leading to non-specific accumulation in off-target tissues. This is exacerbated in solid tumors, where NP penetration is limited, forcing higher doses that increase off-tumor exposure [224]. While base editors and prime editors reduce double-strand break (DSB) risks, they introduce new challenges, such as cytosine or adenine misediting and off-target RNA interactions, which are poorly understood in NP-delivery contexts.

Tumor targeting

Targeting solid tumors: overcoming heterogeneity and microenvironmental barriers

Solid tumors, characterized by dense extracellular matrices (ECM), abnormal vasculature, and immunosuppressive milieus, pose unique targeting challenges. Nanosystems leverage passive and active targeting strategies to enhance accumulation and penetration. Passive targeting is achieved via EPR effect. Most nanosystems (e.g., liposomes, polymeric NPs) rely on the enhanced permeability and retention (EPR) effect, exploiting leaky tumor vasculature to accumulate in solid tumors [225]. For example, PEGylated liposomes loaded with doxorubicin (Doxil®) improved tumor uptake in breast and ovarian cancers. However, EPR efficiency varies widely. Poorly vascularized tumors or that with high interstitial fluid pressure exhibit limited passive accumulation [226]. Active targeting can be achieved with ligand modification. Surface conjugation of tumor-specific ligands (antibodies, peptides, aptamers) enhances tumor specificity [166]. In addition, microenvironment-responsive release, Acidic pH (~6.5 in tumor interstitial fluid) or overexpressed enzymes (e.g., matrix metalloproteinases, MMPs) trigger payload release [227], can also help achieve active targeting. pH-sensitive polymers or MMP-cleavable linkers ensure cargo release primarily within tumor microenvironment, minimizing off-target toxicity.

Targeting hematological malignancies

Blood-borne tumors and their metastatic niches require nanosystems to navigate systemic circulation and engage circulating or disseminated tumor cells. Thus leukemia targeting could be achieved by conjugating nanosystems with cell surface markers such as CD19 (in B-cell leukemia) or CD33 (in acute myeloid leukemia) [228]. On the other hand, lymph node targeting can be completed via modifying lipid NPs with lymphotropic ligands (e.g., mannose for dendritic cell uptake), which is critical for treating lymphoma or priming anti-tumor immunity [229]. Similarly, to achieve bone marrow penetration, engineered EVs or cationic polymers functionalized with integrin ligands enhance uptake in bone marrow niches, enabling targeted delivery to metastatic breast or prostate cancer cells [43].

Targeting brain tumors: overcoming BBB

Brain tumors (e.g., glioblastoma) are notoriously difficult to target due to the BBB, which restricts passive diffusion of NPs (>200 Da). Strategies to enhance brain delivery include receptor-mediated transcytosis, BBB disruption and biomimetic NPs (such as EVs) and exosomes that naturally cross the BBB. NPs were engineered with tumor-targeting ligands (e.g., aptamers for glioma-specific antigens), which enhances delivery of CRISPR-Cas9 or anti-angiogenic siRNA to glioblastoma in brain tumors [105, 230].

Targeting metastatic sites: Organ-specific tropism

Metastases often occur in specific organs with distinct microenvironments, requiring nanosystems with the tailored tropism. For example, LNPs were formulated with galactosylated lipids to target asialoglycoprotein receptors on hepatocytes, enabling efficient delivery of siRNA to liver metastases [231]. As for Lung metastases, inhalable NPs were deposited in pulmonary metastatic nodules through surface modifications enhancing adhesion to lung epithelial cells [17]. Moreover, designing NPs with tissue-specific targeting ligands and responsive release mechanisms restricts CRISPR component delivery to tumor cells. For example, integrin-targeted LNPs for metastatic tumors or pH-sensitive polymers released Cas9 only in the acidic TME [232, 233]. Combined with AI-driven lipid design, this could optimize NP tropism and minimize off-tissue accumulation.

Addressing these open questions will require interdisciplinary efforts spanning nanotechnology, genomics, immunology, and bioinformatics. By prioritizing dosing frequency optimization and off-target minimization, especially for CRISPR-based approaches, researchers can bridge the gap between preclinical promise and clinical safety, paving the way for next-generation NP-mediated gene therapies with precise, predictable, and durable outcomes.

Perspectives and conclusion

The key factor that influences the therapeutic effectiveness of nanocarriers is derived from the off-target effects resulting from the nonspecific accumulation in non-target tissues. To enhance the cellular uptake of NPs for the intracellular delivery of diagnostic agents, many optimized surface modifications incorporating specific targeting ligands have been designed for specific interactions between delivery vectors and receptors on the cell surface [234]. The parameters including ligand length, density, hydrophobicity and avidity should all be taken into account for perfect adjustment. Studies have demonstrated that the benefits achieved through the conjugation of targeting ligands to particles stem from improved target binding affinity, optimized biodistribution, and enhanced cellular uptake, which can potentially reduce the dosages needed to achieve therapeutic outcomes [235]. In addition to active targeting strategies, various novel strategies based on environment-specific targeting release and improvement towards the diffusion ability seem to be more feasible, especially for the on-demand CRISPR-Cas9 delivery system for specific remote spatiotemporal control over genome editing. Affected by specific environmental factors, physical or chemical factors, nanocarriers release active ingredients by undergoing phase transformation or depolymerization, then the formed local high concentration gradient in tumor tissue microcirculation facilitates the cellular uptake with the advantage of selectively high permeability and retention of particles, thus the environment-specific responsive drug delivery system exhibited more promising capacity in the treatment of solid tumors. How to design the intelligent nano-based stimuli-responsive delivery system with good biocompatibility which "switch" itself owns, efficient chemotherapy and integrating tumor imaging has been the major challenge in the research of gene therapy, especially in tumor diagnosis and treatment. The next-generation responsive system might focus on reducing the possible toxicity of by-products, and addressing the obstacles of irreversibility and low selectivity in certain response processes. Additionally, enhancing properties such as particle size, surface charge, and other characteristics of delivery vehicles can facilitate targeted accumulation of therapeutic agents in specific organs or tissues, such as liver, spleen, lung, lymph, and tumor. Recently, the strategy of capturing NPs by the inherent phagocytosis of macrophages provides a new idea for the specific targeting of nanocarriers though the exact mechanism remains unclear [131]. In addition to the therapeutic potential of phagocytizing tumor cells, presenting antigens and secreting cytokines, macrophages also have the drug delivery ability of phagocytosis, drug loading, inherent targeting and deep penetration. But the biggest barrier is clinical transformation and industrial development. On the one hand, the immunogenicity of individual rejection and in vivo safety have yet to be confirmed. On the other hand, the maintenance of specific phenotypes in vivo should be comprehensively evaluated combining with prolonging the cycle half-life of vectors. With the continuous development of cellular drug delivery systems, these above strategies for enhancing target specificity will have better prospects in the future.

Another appealing nanotechnology for delivery system construction to enhance the therapeutic effectiveness is the co-delivery strategy. Though the multi-drug "cocktail therapy" has made great progress in gene therapy, due to the different pharmacokinetics and pharmacological characteristics of different drugs, as well as the personalized distribution after systemic administration, the strategy always exhibits potential toxicity and unsatisfactory efficacy in both preclinical and clinical application. As for those nano-based CRISPR systems combined with chemotherapy, drugs with synergistic effects should be co-encapsulated in nanocarriers in an optimal dose proportion. Besides, the sequential release order of chemotherapeutic drugs and other components in nano-carriers may affect the efficacy. For example, P-gp inhibitors should be released preferentially than drugs in the designed system or the drug would be pumped out of cells before the inhibitor works, which prevent achieving the ultimate effect [236]. In recent years, developing co-delivery strategies tailored to the tumor microenvironment has emerged as a key focus due to its significant influence on tumor development, growth, and metastasis. Enhancing anti-tumor efficacy by mitigating immunosuppressive signals within this environment can be synergistically combined with targeted in vivo delivery to immune cells. This integrated approach offers fresh perspectives for advancing more effective immunotherapies.

The safety of successfully delivered NPs is another key component that clinical applications must take into consideration [177–180]. Enhancing the biocompatibility and biodegradability of delivery mechanisms would significantly expedite the transition of nanomedicines into clinical applications [237]. One example is exosomes, which have served as a new drug delivery system for biological therapeutics including siRNAs, ASOs, antibodies, and small molecules. They exhibited huge potential in gene therapy as natural carriers for their natural material transport characteristics, inherent long-term circulation ability and excellent biocompatibility. Also, the capability of crossing the blood-brain barrier makes it been wild applicated in brain-associated diseases. Due to the different components of exosomes from different cell sources and their potential biological functions, the selection of exosome-derived cells is the premise of whether the best therapeutic effect can be achieved. Additionally, tumor-derived exosomes may have certain potential safety

hazards, which need to be systematically evaluated in the future. The universality of large-scale production is also a problem. Further research at the industrial level is ongoing to fully harness the potential of novel biomaterials, such as exosomes.

Abbreviations

NPs	Nanoparticles
CRISPR	Clustered regularly interspaced short palindromic repeats
CAR	Chimeric antigen receptor
PNPs	Polymeric NPs
PEG	Polyethylene glycol
PLGA	Poly(lactic-co-glycolic acid)
pDNA	Plasmid DNA
LNP	Lipid NPs
AuNPs	Gold NPs
GQDs	Graphene quantum dots
AgNPs	Silver NPs
MSNs	Mesoporous silica NPs
TLRs	Toll-like receptors
LDH	Layered double hydroxide
EVs	Extracellular vesicles
mRNA	Messenger RNAs
siRNAs	Small interfering RNAs
miRNAs	MicroRNAs
ASOs	Antisense oligonucleotides
Cas9	CRISPR-associated protein 9
AAV	Adeno-associated virus
AI	Artificial intelligence
AGILE	AI-Guided Ionizable Lipid Engineering
TME	Tumor microenvironment
CS	Chondroitin sulfate
TNBC	Triple-negative breast cancer
BBB	Blood-brain barrier
RMT	Receptor-mediated transcytosis
Tf	Transferrin
RVG	Glycoprotein
nAchR	Nicotinic acetylcholine receptor
Plofsomes	Polymer-locked fusogenic liposomes
GBM	Glioblastoma multiforme
ApoE	Apolipoprotein E3-reconstituted high-density lipoprotein
siPKM2	Pyruvate kinase M2 siRNA
MDM2	Mouse double minute 2
NIR	Near-infrared radiation
sgRNA	Single-guide RNA
DSB	Double-strand break
NHEJ	Non-homologous end-joining
HDR	Homology-directed repair
ROS	Reactive oxygen species
SPs	Semiconducting polymers
SORT	selective organ targeting
RNP	Cas9/gRNA ribonucleoprotein complex
OMVs	Outer membrane vesicles
TCR	T cell receptor
IL	Interleukin
STING	Stimulator of interferon genes

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Declarations

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The authors declare no competing interests.

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