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Toward Gene Transfer Nanoparticles as Therapeutics

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Genetic medicine has great potential to treat the underlying causes of many human diseases with exquisite precision, but the field has historically been stymied by delivery as the central challenge. Nanoparticles, engineered constructs the size of natural viruses, are being designed to more closely mimic the delivery efficiency of viruses, while enabling the advantages of increased safety, cargo-carrying flexibility, specific targeting, and ease in manufacturing. The speed in which nonviral gene transfer nanoparticles are making progress in the clinic is accelerating, with clinical validation of multiple nonviral nucleic acid delivery nanoparticle formulations recently FDA approved for both expression and for silencing of genes. While much of this progress has been with lipid nanoparticle formulations, significant development is being made with other nanomaterials for gene transfer as well, with favorable attributes such as biodegradability, scalability, and cell targeting. This review highlights the state of the field, current challenges in delivery, and opportunities for engineered nanomaterials to meet these challenges, including enabling long-term therapeutic gene editing. Delivery technology utilizing different kinds of nanomaterials and varying cargos for gene transfer (DNA, mRNA, and ribonucleoproteins) are discussed. Clinical applications are presented, including for the treatment of genetic diseases such as cystic fibrosis.

1. Types of Nanomaterials

1.1. From Viral Vectors to the Revolution of Nanoparticles

Historically, the predominant approach for gene therapy has been the use of viral vectors. Viral vectors are engineered to deliver a therapeutic gene of interest to target cells. Typically, they are also engineered for safety, including to become replication-deficient, so that they cannot amplify to dangerous levels within a patient. As natural biological "nanoparticles" (such viral vectors generally have diameters of 100 nm or less), they have been a frequently investigated biotechnology for gene transfer preclinically and in patients.[1] Approximately 70% of the gene therapy clinical trials to date have used viruses, which include adenoviruses, adeno-associated viruses (AAVs), and retroviruses.[2] Historically, adenoviruses have been the most extensively investigated virus for human gene therapy, although more recently AAVs have been preferred due to their more advantageous safety profile.[3]

In an exciting step forward for the field of gene therapy, the FDA has recently started to approve viral vector gene therapeutics, with AAV approaches shown as safe and effective for certain applications.[4] Although suitable for indications such as intracellular delivery of medium-sized genes, more broadly speaking, AAV vectors have certain manufacturing challenges such as restrictions on nucleic acid cargo size.[5] Although other types of viral vectors can have a high transfection capacity, there have historically been certain safety concerns in terms of oncogenic and immunogenic potential, as well as potential viral recombination. Several clinical trials have been halted due to subjects developing severe complications following treatment. For example, two independent gene therapy trials using retroviral vectors to treat SCID-X1 patients reported events of leukemogenesis arising from insertional mutagenesis.[6] These limitations and potential safety concerns have created growing interest in developing nonviral methods of gene delivery.

While viral vectors have had a long history of investigation for DNA delivery, there has been comparatively little exploration of their utility for mRNA delivery.[7] In contrast, nonviral nanoparticles (NPs) have quickly emerged as leading materials for mRNA delivery as they are viewed as not only safer, but more economical and simpler to produce at scale. Nonviral mRNA delivery can be designed to be less immunogenic and cytotoxic, with little chance of mutagenesis, compared to other types of gene transfer. The largest limitation for nonviral NPs historically has been transfection efficacy as it is difficult to transport large hydrophilic anionic polyelectrolytes (DNA and RNA) through extracellular barriers and into the cytosol of target cells, although recent innovative strategies have drastically boosted nonviral transfection efficiency.[8]

NP chemical composition can vary greatly, from organic materials (lipids,[9] polymers,[10] peptides,[11] and sugars[12]) to inorganic materials (such as silica[13] and gold[14]) (**Figure 1**).

Natural materials such as peptides and sugars are typically the most biocompatible, while lipid-based materials, including

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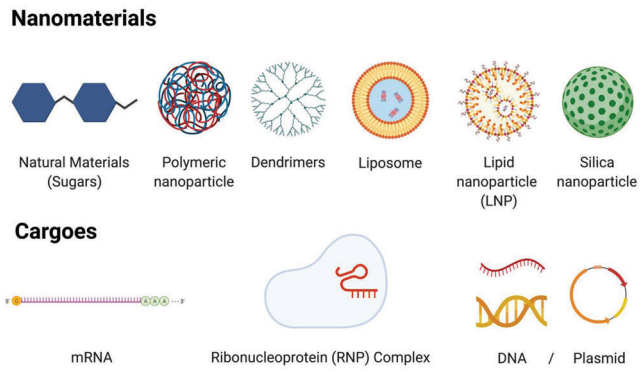


Figure 1. Structure of different nanomaterials and cargoes.

liposomes and lipid nanoparticles (LNPs), have had good success in recent years, including demonstration of utility for gene editing, due to their intracellular delivery properties.[9c,15] Polymeric NPs, such as polyplexes, polymersomes, dendrimers, and polyester-based NPs, can have a diverse array of NP surface properties and functionality, and inorganic materials, like silica and gold nanoparticles, enable smaller sizes and controllable physical properties. These varied nanomaterials share certain features in common, such as their small size, flexibility to encapsulate different types of cargos, and relatively straightforward scale up and manufacture. Key differences include their biodegradability, tissue tropism, and efficiency at delivery. While not all nanomaterials are biodegradable, these materials are generally designed to have high levels of biocompatibility as well as low risk of toxicity. In addition, they can be chemically modified to increase target specificity and cellular uptake.[16] Unlike viral vectors, with limited cargo carrying capabilities, NPs can flexibly encapsulate multiple genetic cargoes such as DNA, RNA, and ribonucleoproteins (RNPs) (**Table 1**), forming nanostructures that can range in size from 10 to 1000 nm. As DNA and RNA both have a strongly negatively charged phosphate backbone, biomaterials that incorporate positively charged amine groups have shown effectiveness at binding and encapsulating these cargoes into NPs. RNPs on the other hand are much larger in size than strands of DNA or RNA and are less negatively charged. This can often necessitate alternative design of biomaterials and NPs to efficiently encapsulate a combination of protein and nucleic acid cargos and facilitate their efficient uptake into cells. [17]

1.2. Natural Materials

Natural materials used to fabricate nanoparticles for nucleic acid delivery include sugars and peptides. Chitosan is a nontoxic polysaccharide polymer that has been demonstrated to be both biodegradable and biocompatible. It binds to and condenses DNA to form a nanoparticle, protecting it from enzyme-mediated degradation.[24] Its ability to condense DNA into small discrete particles and promote cellular uptake enables its utility as a biocompatible non-viral method of gene delivery.[18] Cyclodextrins are cyclic oligosaccharides, typically composed of 6–8 glucose units, that can form molecular inclusion complexes and are useful alone or as conjugates with other biomaterials to enhance gene delivery.[25] These sugars can enable modular incorporation

Table 1. Select gene delivery nanoparticles.

Nanomaterial	Possible cargo	Toxicity	Advantages	Disadvantages	Application	Refs.
Natural material	50–100 nm					
Polysaccharides	DNA, RNA	Low	Complexes well with DNA	Potential off-target effects	Chitosan–DNA nanoparticles improve transfection	[18]
Lipids	30-200 nm					
LNPs	DNA, RNA, RNP	Low	Efficient intracellular delivery, Electrostatic interactions stabilize cargo	Potential immunogenicity	Pfizer/BioNTech, Moderna COVID-19 vaccines	[19]
Liposomes	DNA, RNA	Low	Bilayer forms protective cargo compartment	Subject to disassembly before reaching target	Liposomes enable enhanced gene delivery and targeting to the endoplasmic reticulum	[20]
Polymers	50-1000 nm					
Polyplex	DNA, RNA, RNP	Low to moderate	Easy to chemically modify, target specific cell types	Potential toxicity depending on materials	PBAE-based nanoparticles for treatment of brain cancer	[21]
Polymersomes	DNA, RNA, RNP	Low	High cargo loads possible	Subject to degradation before reaching target	Organelle-specific targeting into the cell nucleus	[10f]
Dendrimer	DNA, RNA, RNP	Low to moderate	Can have a high density of terminal functional groups	Only smaller cargoes possible	Phenylboronic dendrimer enables targeted delivery of Cas9 ribonucleoprotein	[22]
Inorganic	10–200 nm					
Silica	DNA, RNA	Low	Biocompatible, physically robust	Encapsulation lower, often requires modification with positive charge	Mesoporous silica NP-loaded microbubbles enable ultrasound-mediated imaging and gene transfection	[23]

of targeting ligands through the inclusion complexes and also have good biocompatibility. Other natural materials used for gene delivery include peptides, such as those including lysine and arginine to bind nucleic acids and histidine to promote endosomal disruption.[10b] Peptide-based gene delivery materials can also make use of cell penetrating peptides (CPPs) to facilitate crossing of the cell membrane to reach the cytosol.[26]

1.3. Lipids

Based on successful utilization for drug delivery of small molecules, lipid-based nanocarriers have had a long history of investigation for gene delivery and are now quite well-developed. Cationic lipids electrostatically bind anionic nucleic acids to form nanoparticles and other lipids can encapsulate hydrophilic nucleic acids into an aqueous core.

1.3.1. Lipid Nanoparticles (LNPs)

Lipid-based complexes, or lipoplexes formed between cationic lipids and nucleic acids, protect the nucleic acid from degradation and enable intracellular delivery inside mammalian cells.[10d,14] To further optimize potency and safety for intracellular delivery, lipid formulations have been designed consisting of ionizable lipids, helper lipids, cholesterol, and typically a PEG–lipid such as PEG–DMG, that can encapsulate nucleic acids, to form lipid nanoparticles (LNPs). LNPs have demonstrated multiple successes for nucleic acid delivery, including the delivery of siRNA, such as patisiran, an siRNA LNP that is FDA-approved for the treatment of hereditary transthyretin amyloidosis.[27] While LNPs are becoming very well known for their usefulness for vaccination,[19] they are also demonstrating potential for gene editing as well. For example, in an in vivo study to treat alpha-1-antitrypsin deficiency in the liver, a single administration of CRISPR-Cas9 LNPs achieved a >97% reduction in protein levels of its target.[9c] Recently, it was demonstrated in a clinical study with six patients with hereditary transthyretin amyloidosis that treatment with a LNP containing mRNA for Cas9 along with a gRNA targeting transthyretin protein (0.1–0.3 mg dose) had only mild adverse safety events and led to an average of a 52%–87% reduction of serum transthyretin protein at day 28 depending on dose.[28] LNPs compatible with multiple types of nucleic acids also demonstrated a unique ability to target specific tissues, including nonliver targets. Selective organ targeting (SORT) describes LNPs that are engineered to exclusively edit particular cell types such as B cells, T cells, epithelial cells, endothelial cells, and hepatocytes.[29] Clinically, LNPs have met the challenge presented by the global COVID-19 pandemic by demonstrating safety and efficacy for mRNA-based delivery to muscle as next-generation SARS CoV-2 vaccines.[19] With more than 1 billion mRNA nanoparticle doses administered worldwide, these products validate the translational potential of non-viral nanoparticles for gene transfer.

1.3.2. Liposomes

In addition to LNPs, other lipid-based systems, such as liposomes, are also effective for nonviral intracellular nucleic acid delivery. Liposomes are spherical particles made up of a lipid bilayer enclosing an aqueous core and have a history of successful clinical applications for decades.[30] The lipid bilayer provides a layer of protection to the cargo confined to the interior aqueous compartment until the liposome deposits its cargo within the cell. NPs made with liposomes are generally straightforward to manufacture and benefit from a relatively long blood circulation time and reduced systemic toxicity.[31] Liposomes can also be combined with inorganic materials, such as mesoporous silica, to create hybrid non-viral nanoparticles capable of multiplexed RNP delivery and gene editing in the liver.[32]

1.4. Polymers

Early successes with cationic peptide-based and cationic lipid-based nanoparticles pushed the field toward other positively charged non-viral biomaterials such as cationic polymers (**Figure 2**). Cationic polymers are useful biomaterials for gene therapy due to their malleability and control over their physical and chemical properties such as structure and molecular weight.[33] Polymers are easily synthesized as well as modified as single chemical entities with multiple modular functions such as binding to nucleic acids, facilitating cellular internalization, facilitating endosomal disruption, and degrading within a cell to facilitate nucleic acid cargo release.[10e] Further, they can also be designed to be biodegradable and/or bioeliminable, facilitating release of nucleic acids within the cytosol and reducing potential cytotoxicity.

1.4.1. Polyplex

Poly(ethyleneimine) (PEI), branched or linear, is a well-studied polymer for polyplex-mediated gene therapy first described by for this use by Behr and co-workers.[10a] Its favorable properties include high amine content, which aids in both binding and encapsulating nucleic acids (primary and secondary amines) and in endosomal escape to the cytosol (secondary and tertiary amines). While promising for certain applications, one of the potential limitations of regular PEI, a nondegradable polymer, is cytotoxicity, which has been shown by both apoptosis and necrosis.[34] Considerable research has gone into modified PEIs, PEI derivatives, and incorporation of PEI into other biomaterial composites that has shown improved efficacy and reduced cytotoxicity.[35] Other approaches include synthesizing degradable PEI by the inclusion of disulfide linkages between low molecular weight PEI oligomers that are quickly broken down in the reducing environment of the cytosol to improve efficacy and reduce cytotoxicity.[36]

To further reduce potential cytotoxicity, alternative polymers can be synthesized using biodegradable monomer constituents to create biodegradable polymers. Poly(beta-amino esters) (PBAEs) contain amine groups like PEI to promote nucleic acid binding and to facilitate endosomal escape, but also contain hydrolytically degradable ester groups for biodegradability. These functional groups give PBAEs reduced cytotoxicity, aid in DNA release, and enable structural diversity within a library of related polymers.[10e,37] By modifying the end-group of the PBAEs, they can target specific cell types with high transfection efficiency and low toxicity,[38] and in some cases, demonstrate

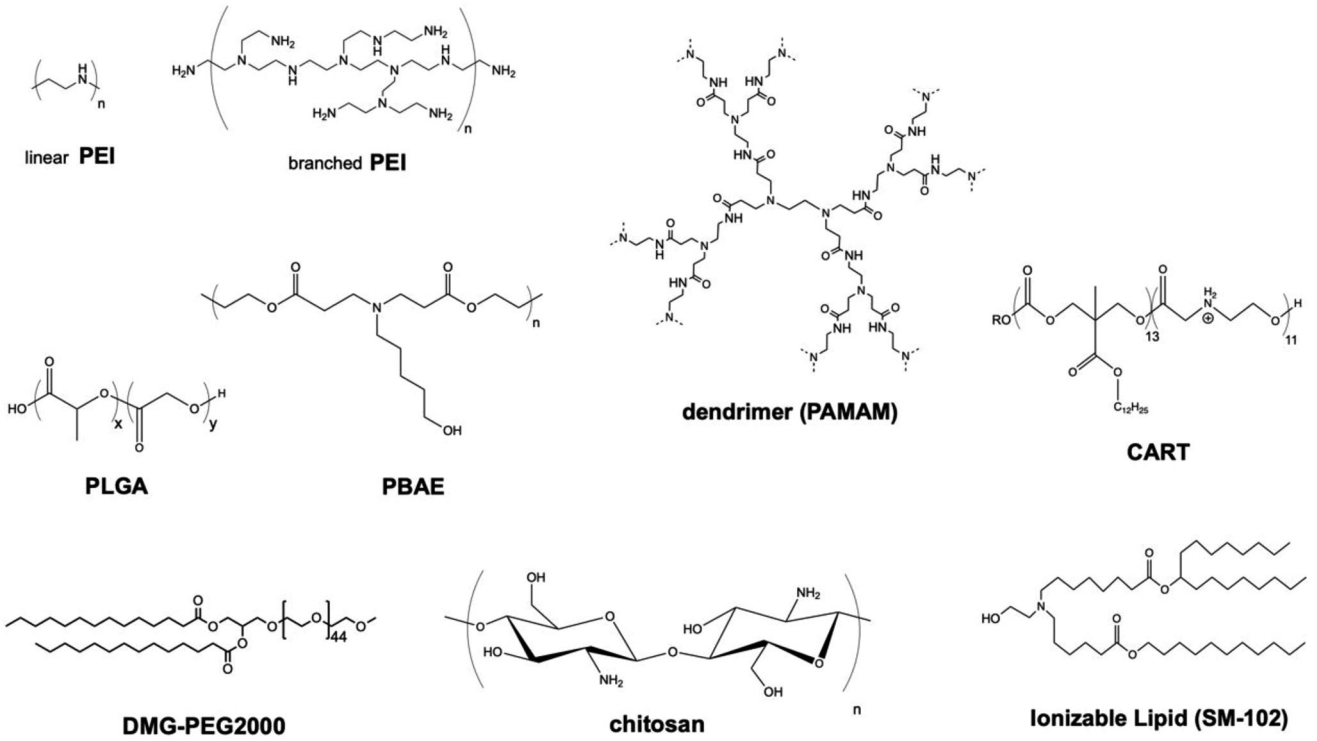


Figure 2. Chemical structures useful for fabrication of gene delivery nanoparticles.

long durability of gene expression.[39] Intriguingly, the cell uptake and transfection are observed to be largely dependent on the structure of the polymer end group, rather than physical or chemical properties of the NPs.[10c] Hybrid PLGA/PBAE particles have also been used for intracellular delivery for genetic material as well as vaccines.[40] While it is advantageous that PBAE NPs are biodegradable and have rapid release of their biological cargo intracellularly, this hydrolytic biodegradability may also cause manufacturing challenges when in aqueous processing conditions.[10c] One approach to increase stability during storage is lyophilization as the polymer-DNA nanoparticles can be stored long-term if freeze-dried with cryoprotectant.[41] Other types of degradable polymers useful for gene delivery include charge-altering releasable transporters (CARTs), which are poly(alpha-amino ester)-based materials that also take advantage of amine groups to aid in nucleic acid binding and intracellular delivery as well as degradable linkages to ensure quick release of the cargo through a self-immolative reaction.[42]

Polyplexes are relatively easily to chemically tune and modify to target certain tissues more selectively. For example, combinatorial PBAE and lipid–PEG nanoparticles have shown systemic delivery of mRNA targeted to the lungs.[43] Recent translational approaches also include inhaled mRNA polyplexes in the form of hyperbranched poly(beta-amino esters) (hPBAEs). This NP formulation allows for protein production in lung epithelium.[44] These technologies are especially beneficial for organs like the lungs where pulmonary delivery has historically been challenging due to mucus and other barriers designed to prevent inhaled agents from reaching or persisting in the lungs.[45] Exciting work has also been demonstrated with CARTs that incorporate lipophilic side chains, showing that mixtures of these materials dramatically boost mRNA delivery, including to the spleen following systemic administration to transfect macrophages, dendritic cells, B cells, and T cells.[46] While cationic polymers have been designed to bind and encapsulate anionic nucleic acids, they can also be modified further to encapsulate a broader set of biological cargos. For example, newer methods of carboxylating branched PBAEs enable the polymers to encapsulate protein cargos and efficiently delivery them intracellularly.[17] This approach in particular enables CRISPR-Cas9 RNP delivery and gene editing with PBAE NPs.[17]

1.4.2. Polymersomes

Polymersomes, which are similar in vesicle structure to liposomes, possess an aqueous core and are composed of polymers rather lipids. They are being actively investigated due to their ease of manufacturing, conjugation, encapsulation, and targeting.[10f] Polymersomes as organelle-specific nanoparticles, are particularly advantageous in gene therapy as they can transport large cargoes not only into the cell, but into the nucleus as well, while being biocompatible and having low cytotoxicity.[8b] In addition, the polymer structure allows for flexibility so that the nanocarrier may change shape and enter organelles without rupturing. Polymersomes are also thought to provide enhanced stability in cellular environments or when binding to cargoes when compared to liposomes.[10f] However, challenges with polymersomes for intracellular delivery include low cellular uptake and inefficient intracellular transport.[47]

1.4.3. Dendrimers

Dendrimers are made up of branched polymer structures. These polymers can include PEI, poly-L-glutamic acid (PGA), polyamidoamine (PAMAM), and other macromolecules that employ step growth polymerization that is either convergent or divergent.[48] The dendrimer itself is hydrophilic, making it an ideal coating agent for delivery of certain biological cargos. In addition, it is easy to make modifications for targeted delivery due to the high density of functional terminal groups on the dendrimers. For example, selective targeting to tumor tissues has been demonstrated.[49] Dendrimers are relatively easy to manufacture and have shown in vivo gene delivery success for a range of cell types. For example, a study using dendrimer gene delivery showed 96% inhibition of tumor growth after delivery of angiostatin gene and tissue inhibitor of metalloproteinases (TIMP)-2 genes.[50] An alternative approach using a phenylboronic dendrimer conjugated with hyaluronic acid demonstrated the ability to deliver Cas9 ribonucleoprotein following systemic delivery, leading to antitumor activity.[22] In the case of gene editing, a particularly innovative approach utilized biodegradable ionizable dendrimer-based lipid nanoparticles (dLNPs) to enable in vivo homology-directed repair (HDR) of ≈20% following a direct injection of the dLNPs formulated with DOPE, cholesterol, and PEG–DMG and carrying Cas9 mRNA, gRNA, and a donor ssDNA template.[15] The convergence of biomaterial technologies (including polymer/lipid hybrid materials) is showing great promise in the design of next-generation nanomaterials for nucleic acid delivery and gene editing.

1.5. Inorganic

Inorganic nanoparticles encompass a broad range of materials with unique physical, chemical, magnetic, and optical properties. For use in medicine, many of these materials may not be biodegradable, but have shown good biocompatibility in a variety of settings. In contrast to many nanostructures formed through self-assembly, such as polyplexes, inorganic NPs generally have much lower polydispersity and enhanced control of physical properties.[51] Inorganic nanomaterials such as silica NPs can also have low toxicity, resistance to physiological conditions, and can be autoclaved. On the other hand, many inorganic NPs such as silica do not natively easily bind nucleic acids and their surfaces require modification with positive charges to be used as vehicles for gene delivery.[31] Gold nanoparticles are also promising for gene transfer, including for gene editing, with similar strengths and limitations. In one recent example, a layered approach was used, with monodisperse gold nanoparticles first conjugated to RNA molecules through thiol-PEG linkers, then nuclease proteins were bound to the RNA, then 2 kDa branched PEI was used to coat the particles with a positive charge, and finally negatively charged ssDNA was added as the last layer.[52] The authors demonstrated efficient ex vivo gene editing of primary human hematopoietic stem cells and progenitor cells with these nanogold formulations.

Spherical nucleic acids, constructed with inorganic nanoparticles like gold as cores, or as purely nucleic acid-based nanostructures, have also demonstrated safe and effective gene therapy.[53] Overall, inorganic nanomaterials, with their well-defined physical properties, can serve as key scaffolds, core NPs, and delivery vehicles that enable multifunctional gene delivery.

1.6. Metal-Organic Frameworks (MOFs)

Metal-organic frameworks (MOFs) are hybrid materials that combine metal ions/clusters such as platinum, zirconium, iron, zinc, copper, or nickel with organic bridging ligands such as polymers. Their advantages include a defined porous structure with high surface area and the ability to precisely tune structural and chemical properties.[54] Nanoscale MOFs are increasingly being designed for varied applications, including in medicine, and can be functionalized with nucleic acids into well-defined nanostructures.[55] While limitations with some of these nanomaterials included variability in manufacture and control of size in comparison to inorganic nanoparticles, the field is rapidly advancing, and sophisticated nanostructures are being generated.[56] Recently, investigators have explored the use of MOFs for gene delivery, including delivery of Cas9 and gRNA for gene editing.[57] Although this field is still nascent, there is significant potential for nanostructured MOFs, porous coordination polymers, to deliver multiple biological cargos in a safe and efficient manner to enable therapeutic gene transfer and gene editing.

2. Delivery Barriers That Nanostructures Must Overcome for Therapeutic Gene Transfer

The biggest challenge for clinical application of nucleic acids is achieving safe and efficient delivery of the genetic cargos to the target tissue, cell type, and intracellular compartment of interest. While nanostructures for gene transfer have different methods of manufacture and varied physical, chemical, and biological properties, they each must be able to cross the same challenging sequential gene delivery barriers to enable successful gene therapy. In the cases of both DNA delivery and gene editing, there are downstream delivery bottlenecks past delivery to the cytosol, making therapeutic delivery more challenging with these cargos than with the siRNA or mRNA. Thus, even though there have been recent clinical successes with siRNA[58] and mRNA delivery,[19] to fully realize the potential for robust therapeutic gene transfer, additional nanoengineering and development is required. Extracellular and intracellular delivery pose independent challenges. Nanoparticle attributes can aid in preventing quick clearance, achieve cell targeting, enhance cellular uptake, facilitate endosomal escape, and enable cargo release to overcome these barriers.

2.1. Extracellular Delivery Barriers

In designing a nanocarrier for extracellular delivery, one must first consider the route of administration. For example, for a clinical application to treat cystic fibrosis, two leading approaches are by inhalation and by intravenous injection. Inhalation has the advantage of locally administering the therapeutic nanoparticle

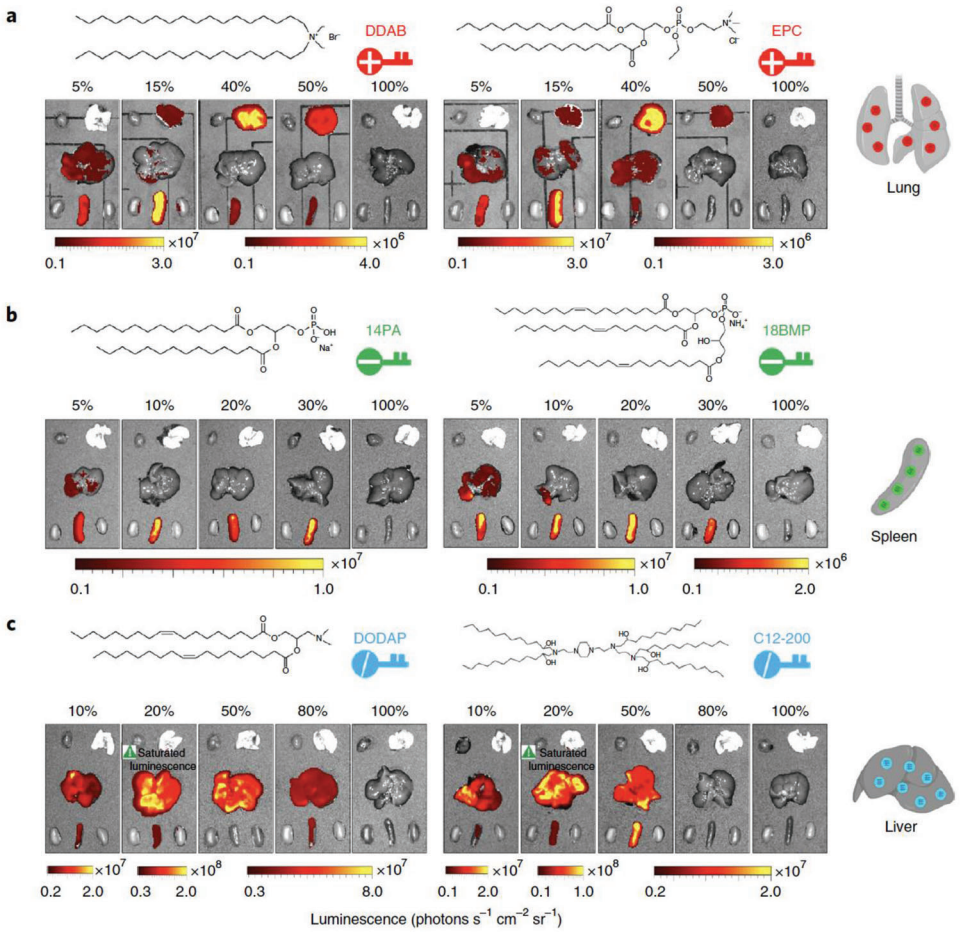


Figure 3. SORT relies on general biophysical properties and not exact chemical structures to deliver mRNAs encoding for therapeutically relevant proteins. a) SORT molecules could be divided into specific groups with defined biophysical properties. Permanently cationic SORT lipids (DDAB, EPC, and DOTAP) all resulted in the same mRNA delivery profile. b) Anionic SORT lipids (14PA, 18BMP, 18PA) all resulted in the same mRNA delivery profile. c) Ionizable cationic SORT lipids with tertiary amino groups (DODAP, C12-200) enhanced liver delivery without luciferase expression in the lungs (0.1 mg kg^{-1}, 6 h). Reproduced with permission.[29] Copyright 2020, Springer Nature.

directly to the lung, the main target organ for cystic fibrosis. Similarly, for the case of treating other genetic diseases, local delivery to the eye, muscle, or brain may have their own unique advantages. Intravenous delivery provides for delivery throughout the body, potentially to many organs. For intravenous delivery, the nanoparticle must be stable in several types of environments, while avoiding systematic clearance from the blood or degradation of the sensitive biological cargos.

Typically, there is a tissue or cell type of interest for the gene transfer, and so there must be a targeting mechanism present for specific expression of the cargo. Two orthogonal ways of achieving targeting can be ligand-directed uptake of the NPs to a target cellular receptor as well as transcriptional targeting that controls the expression of the DNA cargos released from the NPs. Additionally, the biophysical and surface properties of the nanoparticles themselves can help to tune extracellular delivery to tissues such as the lung, liver, or spleen. For example, several classes of LNPs, named SORT nanoparticles, were developed for targeted intravenous delivery without the need for ligands and were found to exclusively edit therapeutically relevant cell types in distinct tissues following intravenous injection (**Figure 3**).[29] How NP surface properties determine interactions with cells and intracellular transport machinery has been a long-term area of active investigation.[59] Targeted gene delivery is important as it can help to maximize high therapeutic efficiency while minimizing off-target side effects. Another concern with extracellular delivery of nanocarriers is potential immunogenicity and toxicity as safety concerns, including after repeat administrations, and these aspects must be fully investigated in preclinical and clinical studies.

2.2. Intracellular Delivery Barriers

2.2.1. Intracellular Gene Delivery Mechanism

Intracellular delivery poses its own challenges (**Figure 4**). Once reaching the extracellular target of interest, the nanoparticles must be taken up by the cell, generally through endocytosis.[60] After entering the cell, the NPs must undergo endosomal escape

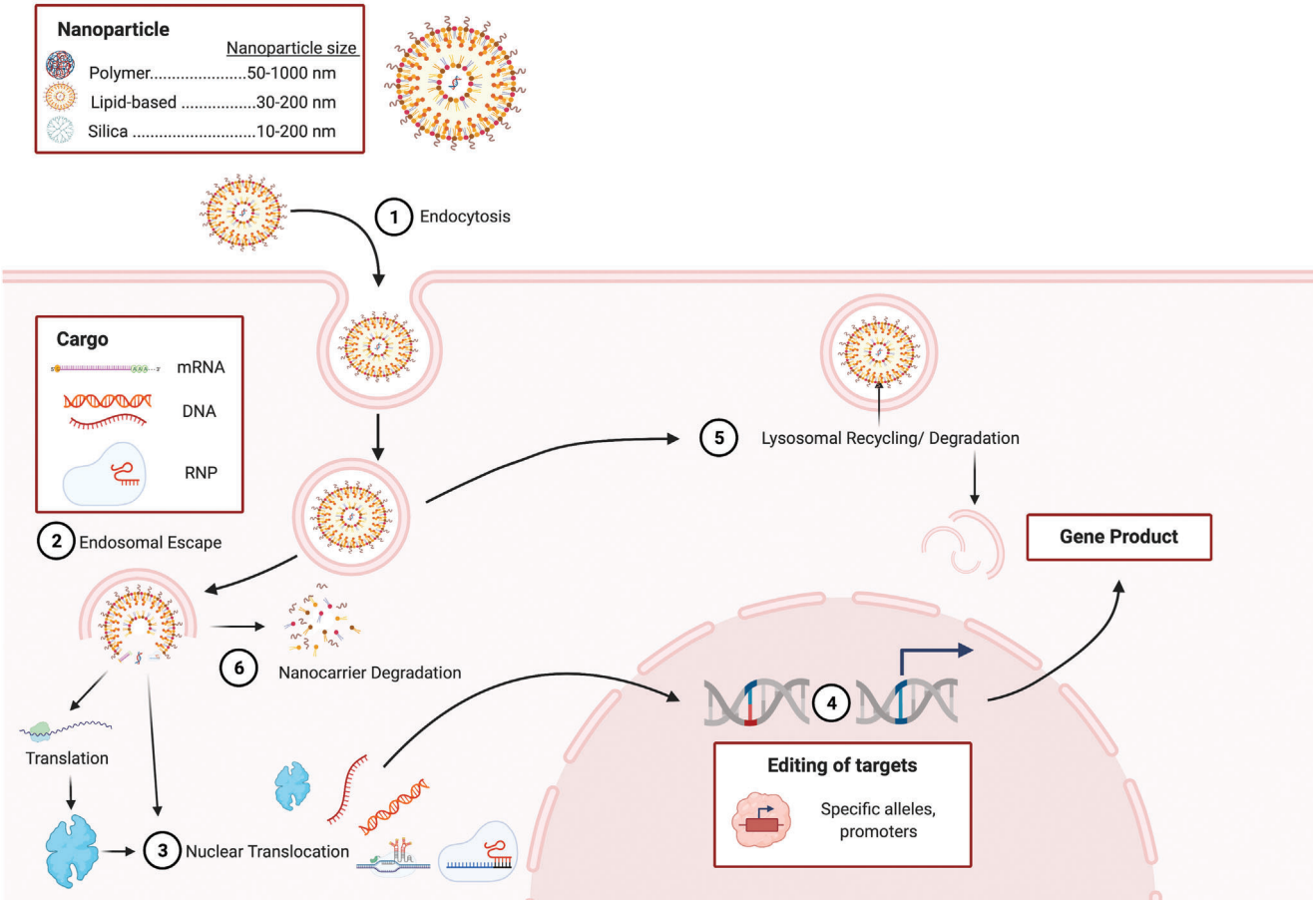


Figure 4. Mechanism of intracellular gene delivery.

to safely reach the cytosol or else the contents of the endosome will be degraded and/or recycled out of the cell.[26] Subsequently, the cargo must be released and trafficked through the cell to reach its area of function. In the case of RNPs and DNA, this means transport to the nucleus via the nuclear pores. Ultimately, gene editing will happen in the nucleus to attempt restoration of wild-type function by repairing a diseased gene. Lastly, delivery being completed, the NP components must be able to safely degrade or risk safety concerns such as apoptosis or necrosis caused by the biomaterial carrier[34] and mutagenesis caused from nucleic acid cargo.[61]

2.2.2. Cellular Uptake

Cellular uptake, biodistribution, and clearance are driven by the biophysical properties of a nanoparticle as well as the chemical functionalization of its surface. For example, in vivo studies have shown that optimizing nanoparticle size and PEG-coating on a nanocarrier increases the half-life during circulation.[62] Regardless of surface modifications, particles >200 nm have been shown to be generally more quickly cleared from the blood circulation, accumulating rapidly in the liver and spleen.[62] For long circulation time in the blood, it is also important that nanoparticles are not smaller than ≈10 nm in size (and/or that molecular agents have a molecular weight of over 5000 kDa) to avoid clearance from the body via the renal system.[10d] To best reach a target tissue, physical and chemical parameters of the system, such as molecular weight and charge, must be precisely tuned.[63]

Chemical modifications can also increase selective targeting, biocompatibility, and circulation times of intravenously injected nanoparticles. PEGylation is an attractive method for protection of nanomedicines, including gene delivery nanocarriers. Nanostructured molecules coated with hydrophilic, noncharged molecules such as PEG and administered systematically have been shown to have reduced cellular uptake through phagocytotic pathways, thus increasing circulation time, although potential immunological responses to PEG remain a subject of active investigation.[64] Benefits of PEGylation of nanocarriers generally outnumber weaknesses and this approach has been demonstrated with many drug delivery systems including liposomes and have led to PEGylated nanomedicine products in widespread use.[19] Modification of NP surface properties is also critical for targeted delivery. These modifications are accomplished through conjugating or coating ligands on the surface of NPs that specifically bind to target receptors and biomolecules on the surfaces of cells. Many types of ligands have been conjugated to the surfaces of NPs for targeting, ranging from small molecules like

folic acid, to peptides such as RGD, to large antibodies, and they have been shown to improve cell specifically and facilitate receptor-mediated endocytosis.[31,65] In one representative example, HER-2 monoclonal antibodies were conjugated to DNA/PEI polyplexes and it was found in vitro in breast cancer cells that these polyplexes had up to 20-fold higher gene delivery compared to non-functionalized PEI polyplexes.[66] In vivo, liposomes conjugated to PEG and an antibody to human insulin receptor has been evaluated for gene delivery to the brains of non-human primates. The authors found that this technology can be successful in crossing the blood-brain barrier via transcytosis and leading to strong exogenous gene expression following intravenous administration.[67] In addition, surface modification via PEGylation can also enable extended release of genetic cargoes from NPs.[10d]

2.2.3. Facilitated Endosomal Escape

A crucial challenge in intracellular delivery of genetic cargoes with biomaterials is the need for a mechanism for endosomal disruption. Many types of gene delivery nanoparticles are successfully internalized by cells into endosomes, but the bigger bottleneck is safe escape from the endosome to reach the cytosol.[68] Through the endolysosomal pathway, internalized NPs and their sensitive biological cargos will be naturally degraded by enzymes in a low pH environment and/or recycled if unable to escape. One method of endosomal escape used by some viral vectors and NPs are peptides on their surfaces that mediate membrane fusion.[69] An alternative approach for endosomal escape relies on endosomal pH, either by using cationic nanoparticles that are pH sensitive or that include chemical groups to buffer and tune the local pH environment. The high transfection activity of certain cationic polymers, such as PEI, has been linked to the "proton sponge" hypothesis.[70] The "proton sponge" hypothesis postulates that polyamines with a high H^+ buffering capacity in the range of endosomal pH (pH ≈ 5–7) create a buildup of positive charge inside of the endosome due to their H^+ buffering activity. To maintain electroneutrality, chloride ion accumulation occurs in the endosome to balance the charge, and this leads to osmosis to balance the tonicity, generating swelling of the endosome and facilitating its rupture, thus allowing the contents within to escape. PEI has been observed to reduce acidification through endosomal buffering with its titratable amine groups, allowing for swelling of the endosome in support the proton sponge hypothesis.[71] Polymeric materials with high titratable amine content such as polyamidoamine (PAM), PEI, and PBAEs have a high rate of transfection, marking the importance of these functional groups. While the definitive mechanism for endosomal escape is still being further investigated, it is clear that pH-responsive biomaterials as constituents of nanoparticles aid in the intracellular delivery of genetic cargos.

2.2.4. Degradability for Cargo Release

After endosomal escape, the next step of delivery a nanoparticle must complete is release of its genetic cargo within the cytosol so that it may be active there and/or be subsequently delivered to the nucleus. In some cases, the nanoparticle degrades itself to facilitate this cargo release and minimize any potential biomaterial-mediated cytotoxicity. Ester linkages, disulfide linkages, and enzymatically degradable linkages have been shown to assist in this process. In a representative example, an in vivo study demonstrated the effectiveness of PEI-ester modified nanoparticles for gene therapy.[72] A quaternary ammonium-modified PEI with a propionic 4-acetoxybenzyl ester group was used for delivery and intracellular esterase created a change in charge from cationic to zwitterionic, triggering efficient intracellular release of DNA.[72] Hybrid materials can also be constructed by coating such polyplexes with DC-chol/DOPE lipids forming lipidic esterase-responsive charge-reversed polymers (LERP). LERPs were observed to effectively accumulate in tumors in vivo and efficiently release DNA into target cells for exogenous expression.[72] Ester linkages in nanoparticles can be easily degraded in water and many types of polyester-based nanomaterials benefit from this mechanism.[10c] Either ester or enzymatic linkages are a simple chemical modification that can ensure the encapsulated cargo is able to be released inside cells. Other approaches have looked at harnessing disulfide linkages for environmentally triggered degradation and release of genetic cargos upon NPs reaching the reducing environment of the cytosol.[73] These bioreducible approaches benefit from quick intracellular release, within just minutes rather than hours or days, and help control release to be in the cytosol, rather than in endosomes or other intracellular compartments.[74]

3. The Need for Gene Editing and Strategies to Achieve It

While many biological cargos can be delivered intracellularly with nanocarriers, including siRNA, mRNA, plasmid DNA, and proteins, robust therapeutic gene transfer, as needed to usher in a new era of genetic medicine, requires the delivery or editing of genes (DNA). Introduction of exogenous DNA into the nucleus as well as genome editing is the most difficult clinical delivery challenges for nanocarriers to accomplish, but they can also be the most impactful. This is because long-term term gene expression of a delivered or edited wild-type gene has the potential to cure an inherited genetic disorder. Further, such an approach has the promise to treat many acquired diseases as well, and in a manner that does not require frequent administrations of a small molecule or biological therapeutic, and that functions at the level of the underlying cause of the disease, rather than at the level of the disease symptoms. Despite the recent clinical successes of nanocarrier-mediated delivery to the cytosol for noncoding gene silencing (siRNA) and transient expression of an antigen (mRNA), "gene" therapy, nonviral delivery of DNA as an exogenous gene and genomic editing of endogenous genes, has not yet reached the same clinical stage. As plasmid DNA delivery is by its nature a nonpermanent change with transient effects, and integrating viral gene therapy runs the risk of insertional mutagenesis, non-viral gene editing approaches have the largest potential to deliver what is needed the most in the clinic: durable efficacy enabled by a precise and safe technology.

Clustered regularly interspaced short palindromic repeats (CRISPR)—CRISPR-Associated protein 9 (CRISPR-Cas9) gene editing, often referred to as simply CRISPR, allows scientists to

precisely cut DNA at any place in the genome across organisms, creating a permanent genetic change. It was adapted from bacteria that have this genome editing system to survive viral assaults. Through CRISPR, bacteria can synthesize and utilize RNA to target viral DNA and then use Cas9 to destroy the viral DNA's activity,[75] Levering this system, scientists around the world are now able to permanently turn off aberrant genes with precise control, under the right experimental conditions.

Although CRISPR-Cas9 has emerged relatively recently, interest from the scientific community on its widespread potential has taken off at an astounding rate. This has allowed for a rapid progression of the technology in just a few years' time, earning the discoverers the Nobel Prize in Chemistry in 2020. CRISPR-Cas9 is an exceptionally enabling technology for genetic editing that could potentially reverse deadly genetic diseases and cure certain types of cancers. However, multiple challenges, particularly delivery and off-target effects, remain before this nascent technology can mature to yield promising treatments for patients. The unique properties associated with intracellular delivery nanoparticles give them great potential to meet the challenges associated with the delivery of the large CRISPR-Cas9 associated cargoes.

While the design of next-generation nanocarriers is critical to enable therapeutic gene transfer, also very important is the design of the needed biological cargos to enable precise gene editing. Multiple strategies are under investigation for the efficient delivery of Cas9 proteins and nucleic acid components. This includes intracellular delivery of Cas9 protein complexed to guide RNA (gRNA, to target the DNA site to be cut), forming a ribonucleoprotein (RNP), Cas9 mRNA to be expressed as Cas9 protein in situ along with delivered guide gRNA, or in situ expression of Cas9 protein and gRNA from delivered plasmid DNA.[76] While there are multiple approaches, the use of plasmid DNA or viral vectors can bring added risks such as potential insertional mutagenesis at the double-stranded break site or at a random site.[77] In contrast, delivery focused on only the transport of RNA and/or protein molecules does not have these risks and also has a shorter time window of activity, further limiting side effects.[78]

3.1. Ribonucleoproteins (RNPs)

RNPs for gene editing are complexes that consist of an endonuclease, such as Cas9 protein, and a guide RNA. Base editors are an example of an RNP that allows for precisely targeted base changes by chemically converting just one nucleotide, performing gene editing without a double-stranded break like traditional CRISPR-Cas9 proteins (**Figure 5**).[2] This allows for lower off-target effects like indel formation and more efficient editing.

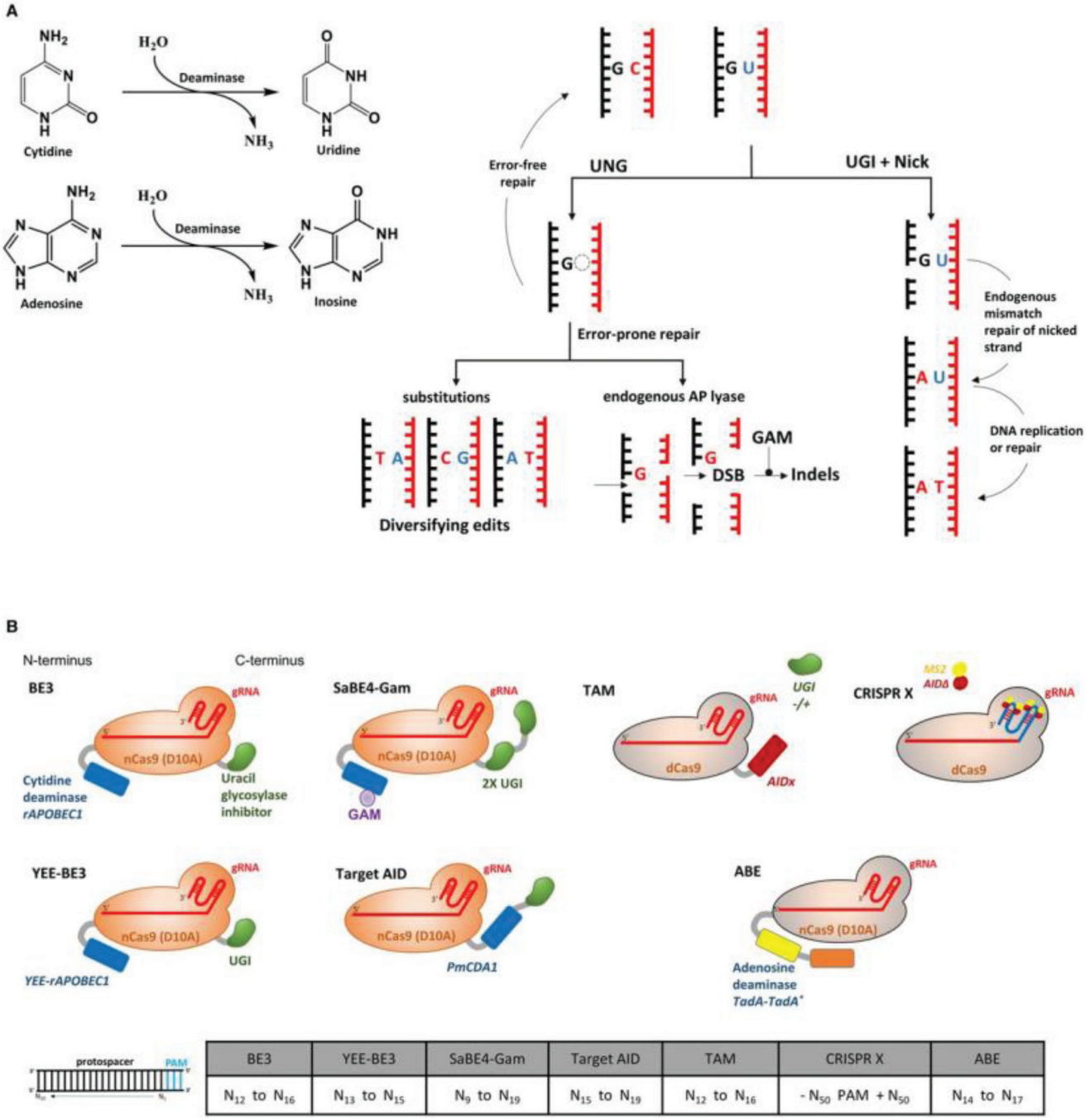
Researchers are using base editors to genetically edit and correct many diseases in animal models such as hemophilia,[76b] DMD,[79] and progeria.[80] Currently, two main classes of base editors exist: adenine base editors (ABEs) and cytosine base editors (CBEs). CBEs are constructed from the APOBEC1 enzyme, a cytidine deaminase that enzymatically converts cytosine (C) to uracil (U), and when fused to Cas9, APOBEC1-Cas9 will convert C to U at a sequence-specific site determined by gRNA. The DNA repair system then corrects this change, leading to a T–A pair as the result of the edit.[80] Studies have shown that CBEs can correct point mutations in mouse and human cells in vitro with high target editing efficiency (35–75%) and a low indel rate (5%) compared with CRISPR–Cas9 (0.1–0.3% efficiency and a 26–40% indel rate).[80] ABEs are one of the newest generations of base editor enzymes that convert A–T base pairs to G–C base pairs. They can correct point mutations that can cause life-threatening disorders such as sickle-cell anemia and hemochromatosis (iron overload).[81] ABEs have also demonstrated success at correcting nonsense mutations in a cystic fibrosis organoid model.[82] Base editor RNP complexes have been successfully used for therapeutic editing in human primary cells and are promising for future therapeutic genetic medicine.[83]

Rather than using gene editing to knock out a gene from a double-strand break (DSB) or a base editor to modify a single nucleotide, gene editing can also be used to knock in genetic sequences to be permanently integrated into the genome at a precise sequence site. This is achieved by also co-delivering a DNA donor template, such as a single-stranded piece of DNA (ssDNA), and a process known as homology-directed repair (HDR). In this process, a DSB is repaired with the ssDNA containing 5' and 3' regions of homology to the endogenous DNA close to the DSB. The ssDNA encoding the new desired sequence then becomes incorporated into the chromosome during the repair. The central challenge is that biomaterials are needed to safely and efficiently deliver these multiple biological components (Cas9 protein, gRNA, donor DNA) to be active within the cell at the same time and at the right dosage. While the types of nanomaterials designed to deliver nucleic acids efficiently generally may not work well for protein delivery as well, research shows that chemical modifications to the nanomaterials that balance their charge can lead to successful intracellular delivery of Cas9 protein, gRNA, and donor DNA simultaneously to enable gene editing.[17]

3.2. Messenger RNA (mRNA)

mRNA delivery is a newer approach to the field due to the relative complexity and expense of mRNA manufacture as a drug cargo. Yet, it is increasingly utilized as it can relay genetic messages to cells while side-stepping several of the safety risks of utilizing DNA as well as the physical challenges of intracellular protein delivery. Once mRNA enters the cytosol of the cell, it can be translated by the ribosome into protein. The exogenous proteins are then shuttled by chaperone proteins to whichever area of the cell they are instructed to go (lipid membrane, nucleus, etc), while the original mRNA is degraded. mRNA is a useful cargo to modulate a wide variety of states of health and disease, including proteins to combat a genetic deficiency, trigger an immune response, or act as a direct therapeutic. However, mRNA is also highly unstable and requires a vehicle that can both stabilize and safely transport it to its target. mRNA delivery NPs are an increasingly preferred approach to achieve gene editing in vivo.[84]

Lipid-based delivery for mRNA in a popular approach and these systems have been explored and found successful at treating multiple genetic disorders in animal models.[2] One representative study in hemophilia used functionalized derivates of lipid-like nanoparticles to deliver human factor VIII mRNA (a large mRNA molecule at ≈4.5 kb), which resulted in restored protein level expression in hemophilia A mice.[76c] These nanoparticles, at a low dose of base editor mRNA (≈5.5 kb) and gRNA, also



BE3	YEE-BE3	SaBE4-Gam	Target AID	TAM	CRISPR X	ABE
N_{12} to N_{16}	N_{13} to N_{15}	N_9 to N_{19}	N_{15} to N_{19}	N_{12} to N_{16}	- N_{50} PAM + N_{50}	N_{14} to N_{17}

Figure 5. The enzymatic activity, subsequent cellular repair events, and molecular modules of base editors. a) Hydrolytic deamination of adenosine (A) and cytidine (C) into inosine (I) and uridine (U) that are read as guanosine (G) and thymine (T), respectively, by polymerase enzymes. The conversion of C into U might result in the onset of base excision repair, where a U from the DNA is excised by uracil DNA N-glycosylase (UNG). This is followed by a repair into C through error-free repair or error-prone repair that results in base substitutions. Blocking the base excision is promoted using uracil DNA glycosylase inhibitor (UGI). A DNA nicked by nCas9 induces the long-patch base excision repair that will use the non-nicked deaminated strand as a template for the repair event. Using nCas9 also might lead to the formation of a basic site removed by AP lyase leaving a DSB. nCas9–GAM fusions reduce this effect by binding to the DSB site, thus reducing the frequency of indels. b) Structural representation of base editors and their activity window corresponding to PAM sites. Base editors (CDAs), Target-AID, CRIPR-X, and ABEs (ADA) are all shown. Fusions of base deaminases with either dCas9 (orange) or nCas9 (gray) showing various activity windows from the PAM site. Reproduced with permission. Copyright 2018, Portland Press, Ltd.

showed effective base editing of PCSK9 cells in vivo.[76c] These lipid-like nanoparticles were shown to be successful for not only mRNA delivery in vivo, but base editing as well. As mentioned above, hybrid dendrimer-based LNP materials have also shown efficiency at co-delivery of mRNA, gRNA, and ssDNA to enable efficient gene editing, including HDR in vivo.[15] While multiple COVID-19 vaccines have been developed, two formulations with high efficacy that have been widely used are both mRNA LNPs. BioNTech/Pfizer and Moderna have both shown that LNP vaccines can be produced at large scale, be satisfactorily stored and distributed, and achieve 94–95% efficacy, leading to the first mRNA vaccines to be authorized for use in the United States by the FDA.[19] Once administered, the LNP systems allow uptake of the nanoparticles by host cells and intracellular delivery of the S, or "spike" protein mRNA (≈3.8 kb)[85] into the cytosol where ribosomes can translate it into the S protein and it can subsequently mediate an immune response. This vaccine approach represents the first successful integration of mRNA into a nanoparticle for widespread clinical use and highlights the translatability of nanoparticle-based genetic medicine.

3.3. DNA

Rather than delivering RNPs directly or codelivery of RNAs (both mRNA encoding an endonuclease and gRNA), gene editing can be accomplished by intracellular delivery of DNA plasmids that encode all the needed editing components. A benefit of delivery of DNA is being able to tune promoter and transcriptional control in cells. This allows for the ability to control genetic editing to be precisely limited to just the target cells that can express the DNA. DNA is also easier and more cost effective to manufacture at scale than mRNA or recombinant proteins. An added challenge with DNA delivery is that this large cargo must be delivered to the nucleus, rather than just to the cytosol, as is the case for mRNA. The approach of delivery of DNA only for gene editing with nanoparticles was recently demonstrated with PBAEs that co-delivered plasmid DNA encoding both Cas9 and gRNA. The authors found that this approach is viable for knockout of expression by CRISPR-mediated cleavage at a single site or for gene deletion by CRISPR-mediated cleavage at two sites flanking the region of interest.[76d] Reducible branched ester-amine polymers have also been validated for co-delivery of DNA plasmids and RNA oligos to enable gene editing as well.[86]

4. Toward Therapeutic Gene Editing with Nanomaterials

4.1. Blood Diseases

Gene therapy clinical trials have often focused on regulating a gene rather than correcting the causative mutation. This is now changing with gene editing. As an example, there was a recent successful clinical trial in 2020 where fetal hemoglobin was upregulated from healthy donor cells by using CRISPR-Cas9 to target the erythroid-specific enhancer of the transcription factor BCL11A.[87] Clinicians were successfully able to perform a transfusion of CRISPR-Cas9-edited $CD34^+$ cells on two patients, one with transfusion-dependent ß-thalassemia and another with sickle cell disease. The RNP was prepared by mixing the gRNA and Cas9 protein, then electroporating the cells with the RNP complex. Patients had high levels of edited bone marrow and blood cells for over a year and the treatment eliminated vaso-occlusive episodes in the sickle cell disease patient.[87] One year later, an in vivo study demonstrated the ability for specially designed base editors to directly edit the sickle allele in $CD34^+$ cells at levels sufficient to help patients.[88] Ex vivo editing of cells for diseases such as SCD shows promise for no off-target effects and the ability to achieve high editing capacity with electroporation. However, many diseases are unable to utilize an ex vivo approach due to multi-organ impact. The recent study on hereditary transthyretin amyloidosis mentioned previously demonstrates the potential for LNPs (in this case containing RNAs) to achieve clinical gene editing that functionally turns off a diseased gene in a durable manner.[28] There is a large opportunity for nanoparticles to build on these early successes and meet the in vivo clinical needs through further engineering of nanostructures for even more efficient and safe delivery.

4.2. Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder that is most common in white individuals of European ancestry, although it has been reported in all races and ethnicities. The disease is characterized by defective function of the anion channel cystic fibrosis transmembrane conductance regulator (CFTR) which leads to altered mucous rheology, primarily affecting the respiratory and digestive systems. Current therapies for CF primarily focus on modulator drug therapies such as Trikafta, a triple drug therapy combination to modulate the protein made by the diseased CFTR gene composed of two correctors (Elexacaftor, Tezacaftor) and one potentiator (Ivacaftor) of the CFTR channel.[89] Correctors work to facilitate the functionality of the CFTR protein at the cell surface, most likely by acting like a scaffolding protein. Potentiators allow for an increase in chloride ion flow by increasing the probability of the channel being in the open configuration.[90] When these drugs are combined, the CFTR protein function improves for those with the F508Del mutation.[89]

While Trikafta has revolutionized care for CF patients, it is only approved for children aged 12 years or older with at least one copy of the most common disease-causing variant, F508Del.[91] This fails the 10% of the CF patient population with nonsense variants who are unable to produce any functional CFTR protein and have no therapeutic alternative.[92] For this reason, individuals bearing these mutations are prime candidates for novel therapies such as CRISPR-Cas9 gene editing. Their mutation serves as a potential target for genome editing technology due to the patient's lack of ability to produce any CFTR protein targetable with drug therapies.

Despite several decades of effort, gene therapy clinical trials for cystic fibrosis have thus far been limited.[93] **Table 2** shows the results of an October 2021 search of the clinicaltrials.gov database[94] to identify cystic fibrosis clinical trials that involved the keywords gene therapy, gene delivery, mRNA, virus, or nanoparticle to find gene transfer studies. As Table 2 indicates,

Table 2. Cystic fibrosis gene therapy clinical trials.

Description of study	Vector type	Date of completion	Results	Limitations	Clinical trial #	Refs.
Repeated application of gene therapy in CF patients	pGM169/GL67a lipid vector	May 2014	Stabilization FEV_1 after 12 doses of gene product	Requires repeated monthly administration	NCT01621867	[95]
Phase I pilot study of gene therapy for CF using cationic liposome mediated gene transfer	pGT-1 gene lipid complex	May 2001	No study results posted	N/A	NCT00004471	[94]
Phase I study of liposome-mediated gene transfer in patients with CF	CFTR liposome	Nov. 2002	No study results posted	N/A	NCT00004806	[94]
Phase I randomized study of adeno-associated virus-CFTR vector in patients with CF	Adeno-associated virus-CFTR vector	August 2002	No study results posted	N/A	NCT00004533	[94]
Single dose of pGM169/GL67A in CF patients	pGM169/GL67A	December 2010	Allowed determination of optimal dose, safety to support multidose trial	Adverse effects from higher dosage levels in 20 mL dose	NCT00789867N	[96]
Safety and efficacy of recombinant adeno-associated virus containing the CFTR gene in the treatment of CF	tgAAVCF	October 2005	No study results posted	Terminated	NCT00073463	[94]
Phase I study of the third generation adenovirus H5.001CBCFTR in patients with CF	H5.001CBCFTR adenovirus	Results published December 1999	Gene transfer to epithelial cells in lower respiratory tract can be achieved	Production of neutralizing antibodies limit reapplication efficiency	NCT00004287	[97]
Phase I pilot study of Ad5-CB-CFTR, an adenovirus vector containing the CFTR gene, in patients with CF	Ad5-CB-CFTR	Start date January 1993 end date unknown	No study results posted	N/A	NCT00004779	[94]
Study to evaluate the safety & tolerability of MRT5005 administered by nebulization in adults with cystic fibrosis (RESTORE-CF)	Nebulized mRNA encoding CFTR in LNPs	Start date May 2018 estimated end date December 2021	No study results posted	N/A	NCT03375047	[94]

there have been few completed studies and the result thus far have been disappointing. Beyond the intracellular delivery challenges previously discussed, another major limiting factor for CF delivery is the ability of the nanocarrier to cross extracellular barriers, such as penetration of mucus and navigation of the inflammatory environment in the lung. After many clinical trials, the strongest clinical benefit observed thus far is with cationic liposomes delivering CFTR cDNA where there was the stabilization of lung function (measured by FEV_1) in patients who received 12 doses monthly over the one year compared to the control group, who saw a reduction in FEV_1 values over the following year.[95]

Gene therapy approaches to potential cure cystic fibrosis have been attempted in recent years. In vivo editing with a super-exon 2-27 donor could correct virtually all CF-causing variants. However, large cargos like this will not work with AAV vectors, making non-viral biotechnologies, such as nanoparticles, a necessity.[98] A further challenge is that this approach relies on HDR to correctly integrate a large piece of genomic DNA, which also favors a nanoparticle approach.

The first clinical trials of a drug that attempts to address the underlying cause of the disease are currently ongoing. The drug MRT5005 delivers mRNA of a fully functional CFTR protein via a lipid nanoparticle (LNP) to the lung epithelium through nebulization. It entered Phase I/II human clinical trials and initially proved to increase lung function,[99] but a second interim analysis showed the drug did not significantly improve lung function in patients.[100] Repeated doses were well-tolerated and considered generally safe, however it is yet to be seen whether continuing trials will prove it to be efficacious.

Organoids have become a useful tool for evaluating ex vivo potential of next-generation gene therapeutics. Studies have demonstrated ex vivo functional correction of the CFTR gene in patient-derived cells.[101] Intestinal epithelial organoids electroporated with gRNA and ABE mediated CFTR rescue, resulting in cellular editing in a 3D model that restored CFTR function. Organoid studies have shown functional repair of the CFTR gene, with editing efficiency up to a clinically relevant 9.3%, and without off-target side effects.[102]

Recently, nanoparticle formulations for gene transfer have demonstrated the capability for direct delivery to the lung in preclinical animal models, a discovery that could allow for targeted gene therapy in the future. Polymer-lipid nanoparticles have

achieved delivery of mRNA to the lungs following intravenous injection[43] and inhaled mRNA polyplexes have demonstrated protein production specifically in lung epithelium.[44] With further improvements to targeted, efficient nanoparticle-based delivery, similar nanocarriers could potentially advance the field from the current *ex vivo* proof-of-principle gene editing studies to in vivo therapeutics that could change the lives of CF patients.

5. Conclusions

Viral vectors, while efficient for delivery on a per particle basis, and promising for gene therapy for particular applications, such as local delivery of a small gene, have multiple limitations for use as a general-purpose gene delivery vehicle. Non-viral nanoparticles for gene transfer are becoming an exciting alternative due to numerous advantages that are being validated in animal models and in the clinic. These advantages include: 1) safety, with low toxicity, low risk of insertional mutagenesis, and low immunogenicity; 2) flexible cargo carrying capacity, facilitating the incorporation of large nucleic acid constructs, combinations of nucleic acids, and the opportunity to deliver ribonucleoproteins as well; 3) targeting, including tissue-specific and cell-specific targeting driven by targeting ligands and/or by intrinsic nanomaterial properties; 4) ease in manufacturing, including scalable technologies that are driven by the cost of nucleic acid production rather than the cost of virus production. Lipid nanoparticles have demonstrated strong success in the clinic for RNA delivery and are promising materials for the delivery of DNA as well. In addition, new nanomaterials in development, such as biodegradable polymeric nanoparticles, have the potential to further improve nonviral gene transfer through reduced toxicity and a wider therapeutic window for dosage, ability to design systemic delivery to focus on nonliver targets, and simplicity of production, scale up, and storage.

To move from transient mRNA expression to long-term therapeutic genetic medicine, gene editing technologies hold the key. In important proof-of-principle studies, nanoparticle base editor RNPs have demonstrated successful therapeutic editing in human primary cells in vitro. Nanoparticle systems encapsulating mRNA molecules have also shown therapeutic potential for gene editing in vivo in multiple systems. Yet, gene editing therapeutics still must overcome several barriers for in vivo editing in patients with severe diseases such as cystic fibrosis. Bottlenecks to treat diseases like cystic fibrosis include anatomical and extracellular barriers that exist whether the administration of the nanoparticles is systemic (intravenous) or local (inhalation). In the case of cystic fibrosis as an example, while efficacy for in the phase I/II clinical trial for MRT5005 is yet to be seen, it is encouraging that base editors show rates of editing that can restore CFTR function to clinically meaningful levels with patient-derived organoids and that repeated doses were well-tolerated in patients. For this application and many others, the field is moving step-by-step toward gene transfer nanoparticles as therapeutics.

In related nucleic acid delivery applications, NPs are increasingly showing great success, especially for infectious diseases. COVID-19 vaccines have demonstrated the clinical realization of lipid nanoparticle-based mRNA delivery as a gene therapy strategy on a large scale. Local delivery to the muscle and transient transfection works very well for a vaccination application and ongoing research is investigating how related nanocarrier technology can be extended to efficient, systemic delivery and long-term expression and correction of a genetic disease. To overcome the remaining limitations, several nanocarrier properties need to be further improved. Critically, on a per particle basis or per nucleic acid molecule basis, delivery using NPs is far less efficient than that of the best viral vectors. This limits the efficacy attainable in clinical trials and narrows the therapeutic window. To further improve the potency, several parameters must be considered. These include engineering the nanomaterial, with newer engineered materials showing great promise at improving performance through extracellular delivery bottlenecks and intracellular delivery bottlenecks, such as endosomal escape efficiency. In the case of DNA delivery, nuclear uptake is also a critical limitation that must be overcome through a combination of nanocarrier engineering and DNA engineering to facilitate transport through the cytosol and nuclear import to match the performance capability of many viruses. To best enable systemic delivery, it is critical that the delivered nanoparticles avoid off-target tissues, such as the liver, and off-target cells, such as macrophages, that can quickly reduce the potency of an injected dose. In addition, the durability of delivered mRNA, and even episomal DNA, is too short-lived to treat many chronic diseases and genetic disorders. To overcome this limitation, nanocarriers are being designed to deliver combinations of payloads that can enable precise genetic editing for long-term function, and even potential cures. Finally, many nonviral NPs are composed of sensitive biomolecules that do not tolerate heat and may be challenging for certain manufacturing processes including terminal sterilization. Alternative manufacturing methods for scale up and sterilization are necessary to ensure high quality, reproducibility, and safety. Taken together, these engineering strategies can further improve nanocarriers for gene transfer and enable their broad clinical application as therapeutics.

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Conflict of Interest

Patents related to polymer technology discussed in the manuscript have been filed by Johns Hopkins University with J.J.G. as a co-inventor. J.J.G. is a co-founder, manager and CTO of Dome Therapeutics, cofounder, board member and CTO of Cove Therapeutics, and board member of VasoRx. Any potential conflicts of interest are managed by the Johns Hopkins University Committee on Outside Interests.

Keywords

CRISPR, cystic fibrosis, DNA, gene editing, gene therapy, mRNA, nanoparticles

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