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Nonviral Vehicles for Gene Delivery

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Nonviral gene delivery (NVGD) is an appealing alternative to viral gene delivery for clinical applications due to its lower cost and increased safety. A variety of promising nonviral vectors are under development, including cationic polymers, lipids, lipid-polymer hybrids (LPHs) and inorganic nanoparticles. However, some NVGD strategies have disadvantages that have limited their adoption, including high toxicity and low efficiency. This review focuses on the most common NVGD vehicles with an emphasis on recent developments in the field.

Keywords: Nonviral gene delivery; cationic polymers; cationic lipids; cell-penetrating peptides; inorganic nanoparticles.

1. Introduction

Gene therapy continues to grow in popularity as an effective method for treating a growing number of diseases and conditions.[1] According to the Alliance of Regenerative Medicine (ARM), the number of clinical trials involving gene, cell, and tissue therapies is steadily increasing worldwide, with 1052 in 2019 and over 1220 in 2021.[2] Twenty two different gene and cell therapies have already been approved for a variety of genetic disorders, such as Leber's Congenital Amaurosis (LCA), spinal muscular atrophy (SMA), Duchenne's Muscular Dystrophy (DMD) and multiple B-cell lymphomas.[1,3–8] Most of the ongoing clinical trials and approved treatments employ viral vectors that can efficiently deliver a variety of nucleic acid cargoes, but a few disadvantages to viral gene therapy have emerged.[9] For example, transduction efficiencies can be low in some patients and some viruses can cause undesirable side effects.[1,3,10,11] Indeed, early viral vectors suffered major setbacks, including a fatal immune response to an adenoviral vector in a patient during a clinical trial in 1999 and the induction of leukemia from a lentiviral vector in two patients in 2003.[12] It is important to mention that the modern viral vectors (e.g., AAV and γ-retroviruses) used in CAR-T therapy and other treatments have been specifically engineered to avoid these side effects. However, viruses still have some significant disadvantages, including high manufacturing costs and a limited payload capacity.

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In contrast, nonviral gene delivery (NVGD) methods are highly appealing because they are less expensive and generally safer, since they are non-immunogenic, do not randomly integrate into the genome, and could potentially deliver larger genetic payloads.[10] These benefits have motivated efforts to develop a nonviral vector that can provide the same level of gene transfer efficiency as a viral vector.[13] A growing fraction of clinical trials are now using nonviral methods, including injection of naked plasmid DNA,[8] transfection with cationic lipids or polymers and electroporation.[14,15] For example, Bioray Laboratories is currently conducting a clinical trial in which electroporation is used to deliver an anti-CD19 chimeric antigen receptor (CAR) gene that is integrated into the *PD1* gene with Cas9 to simultaneously induce CAR expression while knocking out PD1 expression to prevent T cell inactivation by target cancer cells. Preliminary data from clinical trials have already shown that these electroporated CAR-T cells can achieve levels of cytotoxicity and remission that are comparable to CAR-T cells produced with lentivirus (NCT03229876).[16] Similar studies are also being conducted by Servier and Cellectis that use electroporation to deliver a CAR gene with TALENs (TAL effector nucleases) that integrate the CAR into the TCR (T cell receptor) or CD52 loci (NCT02808442, NCT02735083, NCT02746952, NCT03203369).[17] Overall, these studies and others have shown that electroporation is a promising alternative to viral gene delivery.[18] However, this review will focus on the cationic polymers, lipids and nanoparticles that have been developed as NVGD vehicles.[19]

2. Polymeric Vehicles

Many cationic polymers (see Fig. 1 for commonly used examples) have been developed as gene delivery vehicles, due to their relatively low cost and high affinity for negatively charged DNA.[20] In addition, some cationic polymers also exhibit a "proton sponge" effect that allows them to escape the endosome before it acidifies into a mature lysosome that would otherwise degrade the transgene. Specifically, these polymers buffer the pH inside the endosome by protonating their amine/amide groups as the endosome begins to acidify, which induces a massive influx of chloride ions that increases osmotic pressure and ruptures the endosome, thereby releasing its contents into the cytosol.[21–23]

2.1. *Poly(L-lysine)*

Studies on cationic polymers began in the 1980s using poly(L-lysine) (PLL) as a gene delivery vehicle, since it is biodegradable. PLL was later improved upon by the addition of the hydrophilic polymer PEG, which increases its stability by increasing its hydrophilicity and decreasing systemic clearance *in vivo*.[9,24,25] However, while PEGylated PLL was shown to successfully treat cystic fibrosis during a clinical trial in 2004,[26] use of PLL as a gene delivery vehicle is ultimately hindered by poor transfection rates and high cytotoxicity compared to other cationic polymers.[9]

2.2. *Polyethyleneimine*

Polyethyleneimine (PEI) was first used as a gene delivery vehicle in 1995 and became one of the most widely used NVGD vehicles due to its high transfection efficiency.[27] The high efficiency of PEI is due to its high charge density, which facilitates complexation with DNA and increases endosomal escape by the proton sponge effect.[28,29] This high charge density may also lead to an increase in cytotoxicity, but toxicity can be decreased somewhat by adding neutralizing ions (e.g., NaCl).[29] Alternatively, linear PEI polymers (e.g., jetPEI) have also been shown to have lower cytotoxicity than branched PEI, along with higher transfection efficiencies.[29–31] This is likely due to the lower

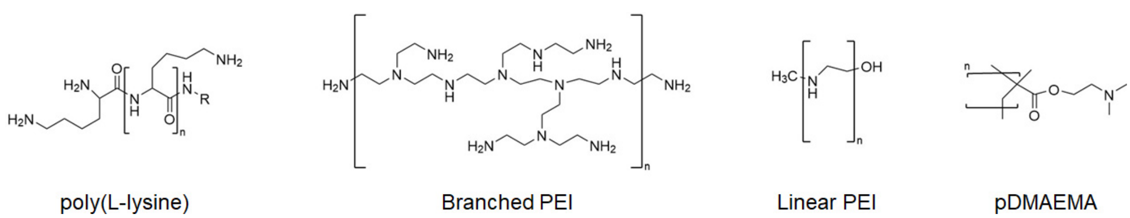

Fig. 1. Chemical structures of four cationic polymers commonly used for gene delivery.

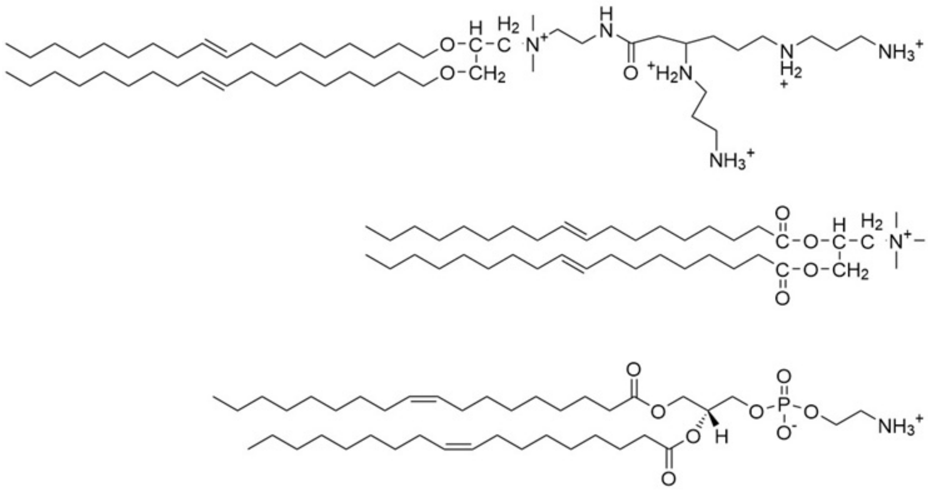

Fig. 2. Chemical structures of three cationic lipids commonly used for gene delivery.

molecular weight of linear PEI (typically 22 kDa for linear versus 25–50 kDa for branched), as the molecular weight of PEI is widely considered to affect its cytotoxicity.[32,33] Nonetheless, there have only been a few Phase 1 or 2 clinical trials of PEI and PEI derivatives for cancer gene therapy, all of which were hampered by relatively high cytotoxicity.[15]

2.3. *Poly(dimethyl amino ethyl acrylate)*

In addition to PLL and PEI, several other cationic polymers have also been developed. For example, methacrylate-based polymers like poly(dimethyl amino ethyl acrylate) or pDMAEMA, have been shown to be rather effective in multiple studies.[30] pDMAEMA has been used to transfect both mRNA and pDNA into primary human T cells while maintaining greater than 90% viability, although the transfection efficiency of pDMAEMA (10% for mRNA delivery and 20% for pDNA delivery) is still lower than the higher transfection efficiencies achieved with electroporation.[20]

2.4. *Poly(β-amino ester)s*

Some applications have used poly(β-amino ester)s (PBAEs) as gene delivery vehicles. These are synthetic polymers with tertiary amines that provide a high charge density and high affinity for DNA. PBAEs also have a high buffering capacity and biodegrade quickly under bodily conditions, which is ideal for limiting any potential immune response. However, this quick degradation often makes PBAE complexes unstable before the transgene is delivered, which is a disadvantage of this vehicle type. Despite this, the ease with which the surface of PBAEs can be chemically customized makes them worth considering as gene delivery vectors.[34]

2.5. *Poly(glycoamidoamine)s*

Reineke *et al.* showed that poly(glycoamidoamine)s (PGAAs) were efficient biodegradable gene carriers *in vivo*. PGAAs are a stable combination of monosaccharides and ethyleneimines that functionally work the same way as PEGylated cationic polymers, known as glycopolymers.[37] The biodegradability of these polymers is an attractive feature, as some of the toxicity associated with polymers such as PEI comes from their lack of biodegradability and subsequent accumulation in tissue.[38] These highlighted examples have been benchmarks for the field of gene delivery polymers, but the field is still rapidly evolving and many new polymers are currently being developed and studied.[35,36]

3. Cationic Liposomes

Cationic lipids are another commonly used type of NVGD vehicle.[13,19,39,40] Figure 2 displays some examples of lipids frequently seen in gene delivery. While several studies have shown cationic polymers can be highly cytotoxic, the relatively lower toxicity of cationic lipids makes them an attractive alternative to polymers.[41,42] These lipids function similarly to any other cationic vector in that they shield the naked DNA from degradation and facilitate its cellular uptake, but their unique characteristics are their high stability and alternative method of endosomal escape. Indeed, the lipids/liposomes used for gene delivery can easily fuse with the lipids in the endosomal membrane, leading to highly efficient release of DNA into the cytosol.[43,44] Second, these lipids have a high structural stability formed from linking a charged lipid with an uncharged helper lipid to form a complex.[19] One such combination is between 2,3-Dioleyloxy-*N*-[2(sperminecarboxamido)-ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and 1,2-Dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE). This reagent, commonly known as lipofectamine, is at the forefront of NVGD vehicles for both high transfection efficiency and low cytotoxicity in most cell lines.[45] Following a similar recipe for lipid formulation, lipofectin (DOTMA/DOPE) has also been successfully used for NVGD.[46,47]

However, the transfection efficiency of some lipid formulations has been observed to be significantly lower than viral vectors in some studies.[13] For example, a study directly comparing lipofectin to a lentivirus (LV) vector in rat mesenchymal cells showed that the lentivirus provided a 95% transduction efficiency whereas lipofectin showed a transfection efficiency of only 25% and a significantly higher cytotoxicity than the LV.[48] The cytotoxicity of these lipids may be due to activation of the innate immune response via the TLR4 pathway. TLR4 is typically activated by lipopolysaccharides, but Lipofectamine has also been shown to trigger TLR4 and upregulate interferons, cytokines, and other inflammatory host cell genes.[49–51] These factors illustrate the current limits of cationic lipids as gene delivery vehicles, which must be addressed before their widespread adoption can be achieved.

Nonetheless, approximately 4.5% of gene therapy clinical trials have utilized cationic lipids as a delivery vehicle.[14] For example, cationic lipids demonstrated some limited success in the delivery of the CFTR gene to treat cystic fibrosis patients, but the investigators expressed a desire for more efficient gene delivery vehicles to further improve patient outcomes.[52] Alternatively, a Phase I study using DOPC lipids to deliver an siRNA to transiently knockdown EphA2 and treat solid tumors was recently completed in 2020, but results for that study are not yet available (NCT01591356). Nonetheless, the most promising clinical development for cationic lipids has come with the recent approval of the Pfizer and Moderna COVID-19 vaccines in 2020, both of which use lipids to deliver mRNA encoding the viral spike protein. Despite the aforementioned reports of low transfection efficiency for cationic lipids, these vaccines have been able to induce a sufficient amount of spike protein expression to activate the immune system and achieve greater than 94% efficacy.[53] Therefore, cationic lipids may be especially useful in the rapid development of future mRNA vaccines.

4. Lipopolymer Hybrids

Some groups have also created lipid-polymer (lipopolymer) hybrid (LPH) vehicles that combine the benefits of lipids and polymers while omitting some of their weaknesses. In general, LPHs provide the low cytotoxicity and efficient endosomal escape of lipids while providing the structural stability, controlled gene release and customizable structural features of polymers.[54] LPHs can be structured in a few different ways, but they typically have a polymeric core that binds the transgene cargo and an outer shell consisting of lipids that interact with the cell membrane.[54]

In one study, an LPH consisting of the polymer PLGA (poly lactic-co-glycolic acid) and the lipid DOTAP was used to deliver a luciferase gene to both HEK-293 and PC-3 cells. Interestingly, the initial transfection efficiencies of Lipofectamine and the LPH were similar, but cells treated with the LPH sustained transgene expression for up to 28 days, while the cells transfected with Lipofectamine only expressed the transgene for 9 days.[55] Another study showed combining triolein, PEI, EPC and PEG-DSPE to form an LPH enabled efficient transfection of MDA-MB-231 and HEK293 cells at a higher rate than Lipofectamine.[56] Likewise, a study by Baghdan *et al.* showed that a hybrid carrier of lipids (DPPC/Cholesterol) and chitosan (a cationic polymer) delivered DNA twice as efficiently as PEI.[57] *In vivo* data for LPHs as gene delivery vehicles is relatively limited, but at least one LPH (EGEN-001, a PEG-PEI-cholesterol conjugate) has progressed to Phase 1 and 2 clinical trials as a delivery vehicle for an IL-12 gene to treat ovarian, fallopian and peritoneal cancers (NCT01489371 and NCT01118052). The disease state was stabilized in seven of the 16 patients in these trials, but none of the patients displayed a partial or complete response to the treatment.[58]

5. Cell Penetrating Peptides

Cell penetrating peptides (CPPs) are relatively short amino acid sequences (20–30 amino acids) that facilitate cellular uptake. The first CPPs to be discovered were TAT (derived from HIV-1) and Penetratin (derived from the Antennapedia homeodomain).[59] The seminal work with these two CPPs also fueled the discovery and design of additional peptides and CPPs with similar effects (see Table 1). There are currently over 1,800 CPPs that have been investigated, 55% of which are synthetic.[60]

CPPs are generally split into three categories: cationic, hydrophobic and amphipathic. Cationic CPPs are typically derivatives of the TAT or Penetratin sequences that contain multiple arginine amino acids and/or at least five positive amino acids. Amphipathic CPPs contain both hydrophobic and hydrophilic moieties. Penetratin is also considered an amphipathic CPP because of its combined polar and nonpolar properties. Some other amphipathic CPPs are VP22 (derived from herpes simplex virus), MPG, and Transportan 10. Finally, hydrophobic CPPs contain mostly nonpolar amino acids (e.g., C105Y and Pep-7). The sequences for these peptides can be found in Table 1.

The cellular uptake mechanism of CPPs is not well understood, but endocytosis is thought to be the primary mechanism.[59,61] The initial interaction between the peptide and cellular membrane is believed to be through electrostatic interactions between positively charged residues in the CPP and the negatively charged cell membrane, especially between arginine and glycosaminoglycans (GAGs) like heparan sulfate. This interaction allows for nonspecific aggregation of CPPs on the cell surface and subsequent uptake via endocytosis. Cationic CPPs may then escape the endosome through the proton sponge effect to release their cargo into the cytosol.[59]

The ability of these peptides to successfully penetrate cells with no cytotoxic effects has led to their use as vehicles for nucleic acid delivery. For example, MPG has been used to deliver siRNA to tumor cells in mouse models to target cyclin B1 and stop tumor proliferation.[62] Transportan10 was also able to silence the SASH1 gene in cancer cell lines HCT116 and HT29 by delivering siRNA.[63]

TAT has been shown to deliver DNA to cells and has been conjugated to polymers (e.g., PEI) and liposomes to increase their transfection efficiencies.[64–68] Alternatively, Zhao *et al.* also showed that conjugating TAT to RGD and PEI enabled targeted delivery to B16 cancer cells with $\alpha v \beta 3$ receptors *in vitro*.[66] However, while CPPs have been used to successfully deliver proteins and drugs *in vivo*, preclinical and clinical studies investigating the use of CPPs for gene delivery have not been conducted.[69]

6. Inorganic Nanoparticles

Interest in inorganic nanoparticles (NPs) as NVGD vehicles has been steadily growing, with over 800 papers published between 1999–2020.[70] These nanoparticles tout several benefits, including customizability, small size and an inorganic composition that limits inflammation.[19,71]

6.1. *Gold nanoparticles*

A variety of diverse inorganic NPs have been developed for gene delivery, but the most common designs have been gold nanoparticles (AuNPs), with

Table 1. Amino acid sequences of several CPPs.

CPP	Sequence	Type
TAT	GRKKRRQRRRPQ	Cationic
Penetratin	RQIKIWFQNRRMKWKK	Cationic/Amphipathic
VP22	DAATATRGRSAASRPTQRPRAPARSASRPRRPVE	Amphipathic
MPG	GLAFLGFLGAAGSTMGAWSQPKKKRKV	Amphipathic
Transportan10	AGYLLGKINLKALAALAKKIL	Amphipathic
C105Y	CSIPPEVKFNKPFVYLI	Hydrophobic
Pep-7	SDLWEMMMVSLACQY	Hydrophobic

the occasional use of carbon nanotubes (CNTs).[9,26,72–75] Xiao *et al.* showed that gold nanoparticles synthesized from folic acid-conjugated poly(amidoamine) dendrimers could transfect HeLa cells at levels comparable to Lipofectamine with lower levels of cytotoxicity.[76] Another study by Rosi *et al.* used gold nanoparticles to deliver antisense RNAs for gene knockdown and showed an improvement relative to Lipofectamine in terms of higher percent knockdown and lower cytotoxicity.[77]

6.2. *Carbon nanotubes*

A few studies have successfully used functionalized CNTs as gene delivery vehicles. These vectors are appealing because their hollow shape allows DNA or small molecules inside to be protected and then systemically released into cells.[78] Although most CNTs are inherently hydrophobic, which would render them incompatible with biological systems, conjugation of positively charged groups like ammonia or PEI to their surface allows them to dissolve in water and significantly reduces their cytotoxicity. Additionally, conjugation of CNTs to PEI is thought to prevent the cytotoxic effects associated with PEI, since these effects are thought to be mostly associated with the freely dissolved polymer.[79] However, functionalized CNTs are not without their own immunostimulatory effects, as a few studies in mice have directly linked inflammation to CNTs, most likely through the activation of TLR4 by the oxidative stress indicator 4-hydroxy-2-nonenal (4-HNE). 4-HNE was shown to be upregulated in mice as early as one day after treatment with CNTs in a study by Shvedova, *et al.*, showing that CNTs may cause potentially harmful effects *in vivo*.[80–82]

6.3. *Iron oxide nanoparticles*

Iron oxide (Fe_3O_4) nanoparticles have also shown promise as gene carriers. A unique advantage of these types of nanoparticles is that they can be manipulated in a magnetic field to enhance transfection.[83] This application of a physical force to enhance transfection is called "magnetofection" and it has been shown to significantly improve gene delivery.[84,85] As described for functionalized CNTs, iron nanoparticles are also typically conjugated with PEG or PEI to facilitate DNA binding, though techniques involving gold coating or conjugation with lipids have also been used.[86] Magnetic nanoparticles have been successfully used as gene delivery vehicles in several studies that have shown magnetofection can provide up to four-fold higher transfection efficiencies than PEI in a variety of cell lines *in vitro*.[83,85] Furthermore, an *in vivo* study by Xie *et al.* used magnetic nanoparticles to achieve a five-fold increase in transfection efficiency over PEI.[87] These studies have also reported relatively low cytotoxicities for these magnetic nanoparticles, which has motivated their further development.[88]

7. Targeting Strategies

One effective strategy to improve NVGD vehicles is to conjugate the vehicle to an antibody or ligand that can bind receptors or markers on specific cell or tissue types.[89] It is important to note that most receptors and cell-surface markers are present in multiple cell types, but targeted gene delivery can still be achieved by selecting a receptor that is expressed at a much higher level in the target site than other tissues. For example, several nonviral delivery vehicles have been conjugated to folate, which enables them to bind to the folate receptor that is significantly over-expressed in different types of tumor cells. Indeed, DOPE liposomes conjugated with folate were able to target and deliver the p53 gene to tumors more effectively than liposomes without folate in a mouse xenograft model.[90] Likewise, hybrid lipopolyplexes conjugated with folate were also shown to successfully deliver siEG5 in leukemic mice.[91] Finally, a PEI-folate conjugate also successfully delivered genes for GFP and luciferase to tumor bearing mice, while no transgene expression was observed with unmodified PEI.[92]

Another commonly used targeting ligand is transferrin, which binds to a transferrin receptor that is abundantly expressed on tumors. This transferrin receptor was successfully targeted with a PEI-transferrin conjugate, which delivered siRNA to silence luciferase in a mouse model when injected intratumorally and intraperitoneally (but not intravenously).[93] Liposomes conjugated to transferrin were also previously shown to enhance transfection efficiency in JSQ-3 cells compared to nonconjugated liposomes.[94] In addition, liposome-transferrin conjugates also specifically delivered p53 *in vivo* to nude mouse tumor models when administered intravenously.[95]

8. Conclusion

Many nonviral vehicles have been developed for gene delivery, all of which employ different strategies to transport DNA into the cell. Some are highly efficient but all nonviral vehicles still face limitations (e.g., low transfection efficiency or high cytotoxicity). Therefore, these nonviral vehicles will require further optimization before they can be considered a viable alternative to viral vectors for *in vivo* gene delivery, but they may still be useful for *ex vivo* gene therapy or in the development of mRNA vaccines.

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