



Polymeric vehicles for nucleic acid delivery

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ABSTRACT

Polymeric vehicles are versatile tools for therapeutic gene delivery. Many polymers—when assembled with nucleic acids into vehicles—can protect the cargo from degradation and clearance *in vivo*, and facilitate its transport into intracellular compartments. Design options in polymer synthesis yield a comprehensive range of molecules and resulting vehicle formulations. These properties can be manipulated to achieve stronger association with nucleic acid cargo and cells, improved endosomal escape, or sustained delivery depending on the application. Here, we describe current approaches for polymer use and related strategies for gene delivery in preclinical and clinical applications. Polymer vehicles delivering genetic material have already achieved significant therapeutic endpoints *in vitro* and in animal models. From our perspective, with preclinical assays that better mimic the *in vivo* environment, improved strategies for target specificity, and scalable techniques for polymer synthesis, the impact of this therapeutic approach will continue to expand.

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Abbreviations: ATRP, atom transfer radical polymerization; bp, base pairs; Cas, CRISPR-associated protein; CD, cyclodextrin; CDI, 1,1'-carbonyldiimidazole; CRISPR, clustered regularly interspaced short palindromic repeats; DEAE, diethylaminomethyl; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; EPR, enhanced permeability and retention; EVNP, *ex vivo* normothermic organ perfusion; Flow-IEG, flow synthesis and iterative exponential growth; GMP, Good Manufacturing Practice; GSH, glutathione; HCA, high content analysis; HFNP, highly functionalized nucleic acid polymer; HPG, hyperbranched polyglycerol; HTS, high-throughput screening; iPSC, induced pluripotent stem cell; mRNA, messenger RNA; miRNA, micro RNA; N, amine group; NIR, near-infrared; NP, nanoparticle; OAA, oligo(aminoamide); P, phosphate group; PACE, poly(amine-co-ester); PAMAM, polyamidoamine; PBAE, poly(beta amino) ester; pDMAEMA, poly(2-dimethylaminoethyl methacrylate); pDNA, plasmid DNA; PDSA, pyridyl disulfide; PEG, poly(ethylene glycol); PEI, polyethylenimine; PGA, poly(glycolic acid); PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PNA, peptide nucleic acid; RAFT, reversible addition-fragmentation chain transfer; RGD, Arg-Gly-Asp; RNAi, RNA interference; ROS, reactive oxygen species; RRM2, M2 subunit of ribonucleotide reductase; sgrRNA, guide RNA; siRNA, small interfering RNA; TALEN, TALE nuclease; TF, human transferrin protein; TRAP, tartrate-resistant acid phosphatase; tRNA, transfer RNA; ZFN, zinc finger nuclease.

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1. Introduction

1.1. Introduction to nucleic acid delivery

Nucleic acids are powerful tools to exogenously regulate cellular processes that can have an impact on the whole organism. Strategic delivery of these molecules can make changes to the expression of genes in an organism in either permanent or transient ways. For therapeutic purposes, nucleic acids can be used to correct a disease-related inherited gene by adding, removing, or replacing genetic material, or to make cells and tissues behave in a certain way by activating, suppressing, or supplementing gene expression [1,2]. Applications of this therapeutic strategy are growing as our understanding of human gene regulation and the links between genetic defects and the molecular basis of disease expands.

Nucleic acid-based therapeutics must act intracellularly, but most nucleic acids are rapidly cleared and degraded after systemic administration and do not readily cross the plasma membrane. Therefore, vectors that facilitate their transport inside cells can enhance the efficacy of these therapeutic strategies [3]. Successful intracellular delivery of nucleic acids has been achieved using a wide variety of tools and techniques, including physical methods, viral vectors, and non-viral vehicles [4]. Within the category of non-viral vehicles are polymeric delivery systems (Fig. 1), which facilitate the protected transport of nucleic acids through the extracellular space and the plasma membrane. There are several types of delivery vehicles that can be formulated, including polyplexes, nanoconjugates, micelles, nanocapsules, dendrimers, and nanoparticles (NPs). Polyplexes are common formulations used for the delivery of nucleic acids, particularly with cationic polymers. These vehicles are spontaneously formed by entropically driven electrostatic interactions between positively charged polymers, often containing ionizable amine (N) groups, and negatively charged nucleic acids containing phosphate (P) groups. For gene delivery purposes, an excess of polymer is typically used (N/P ratio > 1) to condense nucleic acid cargo, generating vehicles with a positive surface charge [5].

There are various design criteria that must be met to ensure that polymeric vehicles reach their final destination and deliver their cargo effectively. These criteria depend on target cell populations, the duration over which the genetic material needs to be present/expressed, and the properties (ex. size/molecular weight, sensitivity to degradation, etc.) of the nucleic acid material to be delivered. The major advantages of this class of vehicles stem from versatile polymer chemistries and physical characteristics which impart an element of control to the delivery strategy; polymers can be manipulated to provide controlled release of cargo, support transfection, and affect biodistribution. This review provides an overview of polymer materials used to deliver nucleic acids along with the current status of clinical trials. We also outline the major challenges faced by polymeric vehicles for gene delivery as well as strategies to overcome them.

1.2. Types of nucleic acid cargo

Therapeutic nucleic acids encompass a wide range of sizes, stabilities, as well as various modes and intracellular sites of

activity. The diversity of these molecules is important to consider when designing delivery strategies (Fig. 1), as their properties will affect encapsulation efficiency as well as delivery vehicle characteristics.

There are several varieties of therapeutic RNA molecules, most of which are small and intended to act in the cytosol without entry into the nucleus. Since they are not integrated into the genome and are eventually degraded, their activity is temporary. They are often chemically modified in order to improve their stability and bioavailability [6]. Such modifications can include 2' hydroxyl modifications that introduce fluorine or methoxy groups or replacement of the phosphodiester backbone to phosphorothioates, phosphorodiamidates, or polyamides to reduce susceptibility to nucleases [7].

The smallest RNA molecules are small interfering RNAs (siRNA) and microRNAs (miRNAs), which are both about 20 base pairs (bp) in length. siRNAs are short duplex, non-coding RNA molecules ~20 bp in length. They act to silence specific genes after they are expressed through the RNA interference (RNAi) pathway. Specifically, siRNA molecules silence a single targeted messenger RNA (mRNA) sequence [8]. Similarly, miRNAs are non-coding RNAs that regulate translation, but act through partial recognition of mRNA targets. Transfer RNAs (tRNAs, ~70–90 nucleotides) interact with ribosomes to incorporate a desired amino acid based on the native mRNA sequence. The largest RNA molecules are single-stranded mRNAs, which direct the synthesis of an encoded protein in the cytoplasm. The size of these molecules is dependent on the length of the gene encoded, which can be up to thousands of nucleotides.

With sizes in the kilobase range, the largest therapeutic nucleic acids by far are DNA molecules. The most extensively studied form of DNA is plasmid DNA (pDNA). pDNAs are circular double-stranded molecules that are capable of replicating autonomously in a cellular host. They consist of an anionic phosphodiester backbone and a stable deoxyribose structure. These large nucleic acids are capable of strong electrostatic interactions and condensation, particularly with cationic carriers [9]. As with all DNA molecules, successful transfection of pDNA requires transport to the nucleus to be effective. These effects can be long-lasting, especially if components of the plasmid are integrated into the host genome.

Therapeutic nucleic acids also include synthetic DNA or RNA analogs such as peptide nucleic acids (PNAs). PNAs have a charge-neutral peptide-like polyamide backbone with nucleobases capable of hybridization with DNA or RNA with high affinity. They are resistant to degradation by nucleases and have increased serum stability compared to other nucleic acids [10], but are also hydrophobic and not easily taken up by cells. However, these properties can be advantageous for loading into hydrophobic polymer NPs [11]. PNA monomers are composed of N-(2-aminoethyl)-glycine units with nucleobases attached *via* methylene carbonyl linkages [12]. Therapeutically, PNAs can be utilized in multiple ways to manipulate gene expression, including antisense, anti-miR, and gene editing [11]. These nucleic acids range in size, with larger therapeutic molecules up to ~40 nucleotides in length. As PNAs have a different




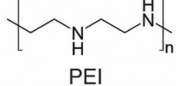
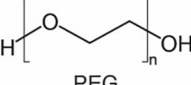
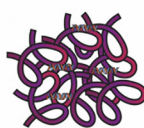

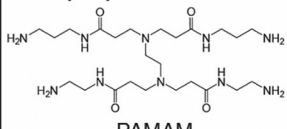
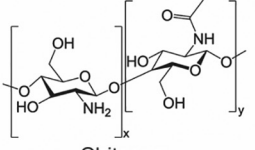
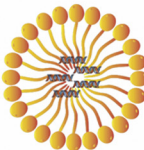
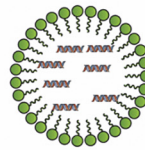


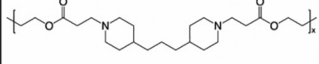
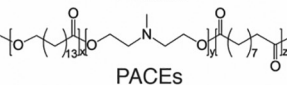

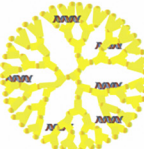
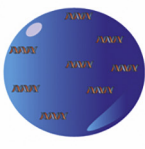


Cargo	Polymers	Modifications	Vehicle Designs	
DNA  pDNA ~1-200K base pairs  dsDNA ~5-100 base pairs  ssDNA ~5-100 bases	Linear Polycations  PEI	Immune System Stealth  PEG	Polyplex 	Nanoconjugate 
	Amphiphilic/Nonlinear  PAMAM			
	Natural Biodegradable  Chitosan	Micelle 	Nanocapsule 	
RNA  mRNA ~1-10K bases  siRNA/miRNA ~20-25 base pairs	Synthetic Biodegradable  PBAEs  PACEs	Targetability  Antibody	Dendrimer 	Nanoparticle 
PNA  Synthetic DNA or RNA Analog ~20-40 bases	Sequence/Mw Defined 			

Fig. 1. Polymeric nucleic acid delivery vehicle formulation "menu" including example choices of cargo, polymers, modifications, and end vehicle design. Vehicle designs include polyplexes - short-lifetime electrostatic complexes that require an excess of polymer to nucleic acids to be formed, nanoconjugates - very short-lifetime linear polycations with limited structure and random organization, micelles - MW-dependent lifetime core-shell complex composed of dynamic amphiphilic polymers, nanocapsules - natural polymer shell complex that encapsulates cargo, dendrimer - branched polymer complex with dendritic encapsulation of nucleic acids that can have peripheral functionalized for improve delivery, and nanoparticles - solid particles with homogeneous loading of nucleic acids that require degradation to assist cargo release.

backbone compared to their natural nucleic acid counterparts, they cannot be incorporated into the host genome.

Gene editing agents can also be delivered in polymeric vehicles for genetic manipulation. In addition to synthetic PNAs mentioned above, there are several families of nuclease-based gene editing agents including meganucleases, zinc finger nucleases (ZFNs), TALE nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated proteins (Cas). Each of these nucleases can be programmed to introduce single or double-strand breaks in the genome [13,14]. The CRISPR/Cas9 system has been the most widely used gene editing approach since its discovery in 2013, in part due to the relative ease of design for various applications [15]. CRISPR/Cas9 gene editing platforms use a single guide RNA (sgRNA) to direct the bacterial Cas9 enzyme to a target locus in the genome [16,17]. In nucleic acid form, Cas9 can be delivered as a plasmid (~8–10 kilobases) or mRNA (~4000 nucleotides) alongside a sgRNA (~100 nucleotides) which can also be delivered in plasmid form; a donor DNA molecule (up to ~100 nucleotides) is also delivered to serve as a template if a site specific genome modification is desired [18]. Protein and mRNA forms of Cas9 are often preferred as they limit the duration nuclease activity, reducing the potential for off-target modification of the genome. Incorporating these gene editing components together results in large and complex nucleic acid payloads within any delivery vehicle.

1.3. Extracellular and intracellular barriers to nucleic acid delivery *in vivo*

Upon *in vivo* administration, polymeric delivery vehicles encounter a complex set of extracellular and intracellular barriers which remain a key challenge in clinical translation (Fig. 2). For each application, accessibility of target tissues and cell types needs to be carefully considered when choosing a route of administration. If administered intravenously, delivery vehicles can interact with molecules present in the blood, such as serum proteins which can accumulate on carrier surfaces. The formation of a "protein corona" around polymeric carriers can change the surface properties and hinder the effectiveness of the delivery vehicle by altering the carrier's interactions with cells and tissues [19,20]. Most intravenously injected carriers, particularly those that are larger than 100 nm, rapidly accumulate in the liver due to phagocytosis by Kupffer cells. Avoiding this rapid liver accumulation is essential to achieve the long carrier circulation times that are needed to increase accumulation in other tissues. The ability of non-viral vectors to evade liver clearance and accumulate in target tissues depends on their size and surface properties. Small vectors (<100 nm) and vectors with certain coatings that confer stealth properties by reducing protein corona formation remain in circulation longer and may be better able to accumulate in organs other than the liver [21]. Of course, the carrier must be large enough (>10 nm) to avoid efficient filtration by the kidney. Aside from elimination from the blood, once the polymer vehicle reaches the organ of

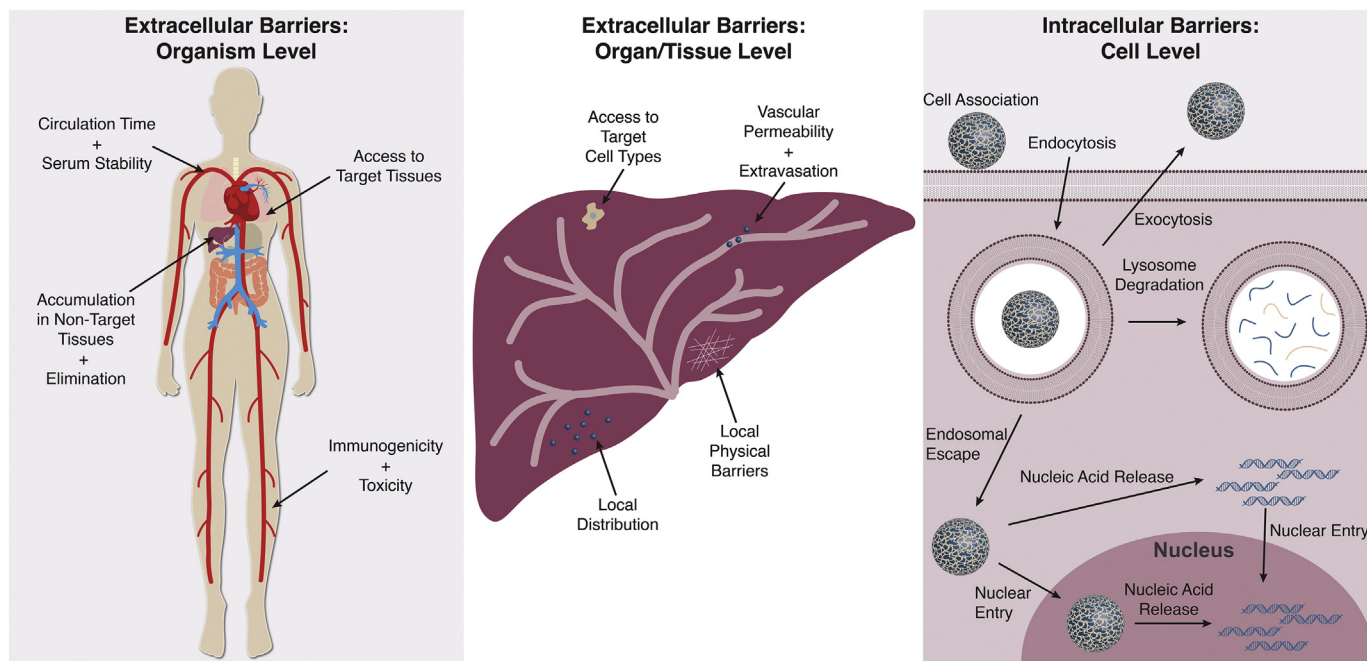


Fig. 2. Extracellular and intracellular barriers to nucleic acid delivery *in vivo*. A schematic illustrating physiological barriers relevant to polymeric nucleic acid carriers at the organism level, organ/tissue level, and cell level. Systemically administered vehicles must adequately circulate in the blood, accumulate in and penetrate target tissues, be internalized by target cells, and achieve intracellular cargo release.

interest, there may be additional physical barriers that prevent access to certain cell types including vascular impermeability and hydrostatic pressure preventing extravasation, poor local distribution, as well as difficulty traversing through local mucus or matrix barriers. If the target tissue of interest is accessible by local administration, such as direct injection into the eye, many of the challenges associated with systemic delivery can be avoided.

Once delivery vehicles reach their target cells, they face additional barriers [22]. The first challenge is cellular association and entry, which can occur in a non-targeted or targeted manner *via* specific receptors on the cell surface. Cell association and uptake of delivery vehicles will depend on the interactions between the delivery vehicle and cell membrane, and generally occurs by endocytosis [23]. Endosomal escape of the carrier is essential to avoid lysosomal degradation of the cargo [24]. Delivery vehicles must facilitate transport of cargo to the cytosol or the nucleus for the genetic manipulation to take place, after which remnants of the delivery vehicle must be eliminated without disrupting normal cellular processes.

In sum, to be successful as nucleic acid carriers, polymeric vehicles must perform multiple functions. They must efficiently load and protect nucleic acid cargo, evade the immune system and premature clearance mechanisms, achieve cellular uptake and endosomal escape, and disappear without toxicity. Designing vehicles to fulfill all of these criteria has been challenging, but recent approaches aim to modify or combine traditional polymers into materials that both capitalize on proven strengths and balance pitfalls to create well-rounded vehicles for various applications.

2. Polymeric vehicles for nucleic acid delivery

Polymer physicochemical properties (ex. composition, molecular weight, and polydispersity) can be modified to achieve specialized formulations for nucleic acid delivery. One common feature in most polymers designed for nucleic acid delivery is the incorporation of cationic groups, with two purposes: first, to aid with the loading of negatively charged nucleic acid cargo, and second, to facilitate the

interaction with negatively charged glycoproteins on the cell membrane [25]. Intended applications may dictate stability, size, and cargo requirements, which can be achieved with various formulations. Some commonly used formulations are illustrated in Fig. 1. Both naturally derived and synthetic polymers have been utilized for non-viral delivery systems, although an advantage of synthetic polymers is the ability to incorporate versatile chemistries in a controlled manner, providing more options for the final formulation.

2.1. Early linear polycations

One of the first linear polycations investigated for nucleic acid delivery was diethylaminomethyl (DEAE) dextran. This polycationic derivative of the carbohydrate dextran was shown to enhance poliovirus RNA transfection in 1965 and simian virus 40 viral DNA three years later [26,27]. The advantages of DEAE-dextran include its chemical simplicity, reproducibility, and low cost; disadvantages consisting of low transfection efficiency, cytotoxicity in primary cells, and inhibition of cell growth *in vitro* have limited its use *in vivo* [28].

Polylysines and polyamines have also been used as gene carriers. Polyamines and nucleic acids spontaneously condense into compact structures that closely resemble viruses [29,30]. Polylysines were the first polymers used in targeted gene transfection strategies by incorporating specific ligands for cell surface receptors [31,32]. Vehicles formulated from these polycations can be toxic (with low molecular weight polymers tending to be more toxic), and endosomal escape can be inefficient due to complete protonation at neutral pH, which limits their endosomal buffering ability. To overcome these limitations, copolymers which incorporate these low molecular weight polycations with other polymer blocks, such as polyesters, can be synthesized [33].

2.2. Proton sponges and amphiphilic polycations

Proton sponges and amphiphilic polymers are more efficient at endosomal escape than early linear polycations. Often, proton sponges are composed of multiple blocked linear and/or non-linear polycations

that enable a larger buffering capacity and enhanced physicochemical properties (such as increased molecular weight for stability). These materials generally contain many proton-accepting groups including primary, secondary, and tertiary amines, and can achieve endosomal escape by causing an influx of chloride and water into the endosome upon increased protonation in an acidified environment. This influx leads to subsequent bursting of the endosome from the osmotic pressure and membrane destabilization [34]. One example is polyamid-amine (PAMAM), which was developed in the early 1990s and demonstrated effective pDNA transfer [35]. Dendrimers, like PAMAM, inherently contain hundreds of coupling sites with primary amine termini due to their multivalent structure, and as such have been extensively explored as drug and gene carriers [36]. Indeed, the abundance of charged coupling sites enables efficient nucleic acid loading of even large structures. On the other hand, the high cation density leads to non-specific cellular uptake and systemic toxicity *in vivo* [37–40]. A strategy to improve specificity is to utilize the amine groups to chemically attach cell-specific ligands that enhance binding to delivery targets. Using this technique, PAMAM-based formulations have been used to deliver gene therapy to injured microglia [41] and tumor cells [42]; another similar dendrimer (polypropylenimine) was used to treat prostate cancer cells [43]. These strategies report no secondary effects to healthy tissues. Conjugation of poly(ethylene glycol) (PEG) to PAMAM also reduces toxicity and improves efficacy [44].

Another well-known proton sponge is polyethylenimine (PEI), which has become a “gold standard” for gene delivery. The PEI polymer family consists of linear and branched polymers with varying molecular weights and structures, adding to their versatility. These materials have been used to deliver a wide variety of nucleic acids including pDNA, miRNA, and siRNA both *in vitro* and *in vivo* [45–48]. While transfection efficiency is high, the mechanism of action for PEI and similar polymers remains unclear [34,49–53]. Beyond the osmotic effect of a proton sponge, it is possible that the higher charge density of PEI molecules results in direct interaction with the endosomal membrane and subsequent permeabilization. Another possible mechanism for endosomal lysis by PEI is thermomechanical disruption, driven by swelling of PEI vehicles to more than four times their original size with a temperature shift [54,55]. While membrane disruptions are helpful for transfection, the high charge density of this polymer can cause extensive damage to cellular membranes, leading to apoptosis, necrosis, and inhibition of ATP synthesis. Additionally, polyplexes with rapid release of nucleic acid cargo may raise concerns over the stability of the vehicle for delivery. Strategies that address this “polyplex dilemma” are described in detail in a recent review by Wagner et al. [56]. Other limitations of PEI are that it is not degradable, it exhibits molecular weight-dependent cytotoxicity, and can stimulate a systemic immune response [57–59]. To address some of these limitations, PEI has been used in combination with other, more biocompatible polymers. For example, blending or grafting PEI with PLGA produces stable, cationic carriers [60] that have been complexed simultaneously with large nucleic acids (pDNA) as well as peptides and have shown improved transfection over branched PEI alone [61,62]. Along these lines, other groups have developed derivatives of PEI, by forming hybrids of PEI with other polymers or lipids [63]. Another way to reduce the cytotoxicity of polymers with high charge density is through rational architectural changes. For example, one hypothesis suggests that increased branch density of dendrimers reduces toxicity [64]. This was demonstrated in studies of linear branched and cyclic branched poly(2-dimethylaminoethyl methacrylate) (pDMAEMA) [65]. Alternatively, the high surface charge of PEI was shielded when heparin was conjugated to the surface, increasing hemocompatibility and transfection [66].

Some amphiphilic polymers, such as poly-alkyl-carboxylic acids and polyvinyl ethers, appear to enhance endosomal escape due to their hydrophobic domains. This process can be further enhanced by incorporating redox-sensitive and pH-dependent chemical groups into the polymer. For example, pyridyl disulfide (PDSA) [67] is pH-sensitive

and, under acidic conditions, can enhance endosomal membrane disruption due to increased hydrophobicity [68–72]. Many amphiphilic polymers lack cationic groups and therefore exhibit reduced nucleic acid binding, though cationic groups can sometimes be incorporated into the polymer design. For example, cationic polyvinyl ethers contain amine groups and achieve both efficient nucleic acid loading and transfection activity [73,74].

2.3. Biodegradable polymers

Many non-biodegradable polymers have no established way to leave cells and tissues after administration. The prolonged exposure to foreign materials and the potential accumulation of materials can lead to toxicity. Biocompatibility is enhanced with the use of biodegradable polymers as carriers, particularly if the polymer degrades to non-toxic and natural metabolites. Both naturally-derived and synthetic polymers can be biodegradable, making them potentially safer options for systemic gene delivery.

2.3.1. Natural polymers

Among naturally-derived polymers, structural proteins and polysaccharides, such as cationic collagen derivatives and chitosan, have been investigated as gene delivery vehicles. Cationic collagenous proteins have been used for nucleic acid delivery to articular cartilage and bone for regenerative medicine and metastatic tumor treatment [75–80]. Chitosan, a linear cationic polysaccharide, is produced by the deacetylation of chitin (poly-d-glucosamine). It is non-toxic, even at high concentrations [81,82], and can be formulated into polyplexes for effective protection and transfection of small and large nucleic acid cargos [83,84]. For example, chitosan has been recently used to deliver miR-124 to rat microglia *ex vivo* [85], osteopathic tumors [86], tumors in multiple myeloma [87], and for *in vivo* bone regeneration [88]. An advantage of chitosan compared to other cationic polymers with higher charge densities (e.g. PEI) is the ready dissociation between nucleic acid cargo and polymer upon internalization, leading to more efficient gene transfection [89]. However, incorporating chitosan and PEI into a combined vehicle can take advantage of the stronger nucleic acid association with PEI while reducing negative consequences from charge density [90–94].

Cyclodextrins (CDs) are another family of naturally-derived carbohydrate-based polymers with favorable physicochemical properties for gene delivery. CDs in α -, β -, or γ - forms can be used either as stand-alone vectors or in combination with other cationic polymers (including PEI) [95–103]. β -CD can form host-guest interactions with adamantane which has supported the formulation of β -CD, adamantane-PEG, and PEI polyplex blends [104]. These formulations achieved high transfection efficiency in the liver, metastatic tumors, and *via* oral administration [105–108].

2.3.2. Synthetic polymers

Synthetic biodegradable polymers are generated by assembling low molecular weight monomers into polymers *via* bioreversible linkages such as sulfide or ester bonds. Examples of widely used biodegradable synthetic polymers are poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and random or block copolymers such as poly(lactic-co-glycolic acid) (PLGA) [109]. These materials degrade slowly *via* bulk hydrolysis, a process which can facilitate sustained release of cargo. PLGA-based delivery systems have been used for a wide variety of therapeutic agents [110], including nucleic acids such as siRNA, miRNA, and PNAs [111–113]. Importantly, PLGA has been approved by the FDA for certain delivery applications. Practically, these materials have been difficult to use for nucleic acid delivery, as formulations usually have low nucleic acid loading, reducing their effectiveness [114,115]. Precomplexation of nucleic acids with spermidine improves loading of siRNA [111]. Alternatively, PLGA can be blended or copolymerized with cationic polymers such as PEI [62], poly(beta amino) esters (PBAEs) [116], poly-L-arginine [117], and chitosan [118] for enhanced gene

transfection. Interestingly, a number of these studies capitalized on the chemical properties of both polymers to deliver both nucleic acids and peptides [62].

PBAEs are a family of degradable cationic polymers with the ability to effectively encapsulate nucleic acids, promote cellular uptake, and act as a proton sponge to achieve endosomal escape [119]. Like polylysine, PEI, and PAMAM, PBAEs contain amine groups which interact with negatively charged nucleic acids, but they also have hydrolyzable moieties which render them degradable. PBAEs are relatively easy to synthesize compared to other cationic degradable polyesters and they are amendable to custom modifications [120], enabling the generation of large libraries of polymers, which can be screened to identify structures that are optimized in different applications [121,122]. Strategic modifications to the polymer structure have been reported to optimize delivery to pediatric brain cancer cells [123], hepatocellular carcinoma [124], and retinal pigment epithelial cells [125]. While PBAEs are biodegradable and exhibit high transfection efficiencies, the high density of amine groups in these polymers makes them susceptible to aggregation with anionic serum molecules and increases toxicity [126,127]. In blended formulations of PBAE and PLGA, cytotoxicity was reduced, which decreased as the ratio of PBAE to PLGA decreased [128].

Poly(amine-co-esters) (PACEs) are another family of biodegradable cationic polymers designed to have lower charge densities to reduce toxicity. These polymers are synthesized via copolymerization of hydrophobic monomers and amine-containing monomers that promote nucleic acid association and endosomal escape [129,130]. PACE synthesis is often accomplished with an enzyme (lipase) as the catalyst, making the polymer easy to purify and free from potentially toxic metal catalysts used to synthesize many other degradable polymers. The physical properties of PACE can be controlled by composition and molecular weight, and these materials can be formulated into various delivery vehicles including polyplexes and solid NPs [131]. The lower charge density of PACE, compared to PEI and PBAE, improves biocompatibility, while maintaining enough charge to promote transfection. Like other cationic polymers, PACE polyplexes have the tendency to aggregate in serum, although this can be mediated through the use of coatings with synthetic peptides, apolipids, or surface modifications [131–133].

Various synthetic strategies now exist to control polymerization reactions and, therefore, the properties of the resulting polymer. Until recently, the evolution of sequence-defined synthetic polymers in the laboratory was limited to analogs of nucleic acids and polypeptides [134–136]. With new knowledge and technical capabilities, sequence-defined synthetic polymers are possible. This synthesis approach provides high chemical precision and flexible design of the resulting polymer, and is therefore a powerful tool to systematically evaluate the effect of minor chemical changes on nucleic acid delivery. Examples include controlled free-radical polymerizations such as reversible addition-fragmentation chain transfer (RAFT) polymerization and atom transfer radical polymerization (ATRP) [137]. RAFT polymerization, in particular, can result in high end-group fidelity and has been used to synthesize amine-based polymers for nucleic acid delivery [138]. Another example is highly functionalized nucleic acid polymer (HFNAP) libraries which are the first in-lab evolution of sequence-defined synthetic polymers that are not limited to the constraints of polymerases or ribosomes [139]. A ligase-based polymerization method provides great flexibility for this polymer system to produce libraries of different side-chain compositions for parallel experimentation to gain structure-function relationships between the resulting polymers and the genetic code to find the best candidates for the application of interest. Sequence control can also be achieved with approaches that are similar to peptide synthesis: in sequence-defined cationic oligo (aminoamide) (OAA) polymers, for example, monomer and functional group placement in the polymer chain is controlled by using stepwise solid phase synthesis [140]. By generating libraries of polymers built with minor chemical changes, OAAs with different architectures and structural motifs were identified for improved stability as a cationic

carrier for formation of siRNA [141], for Cas9/sgRNA lipo-OAA complexes [142] and for combinations of pDNA and siRNA [143]. While techniques to control polymer sequence and structure are time-consuming, they enable control over the properties of the polymer, including hydrophilicity/hydrophobicity, charge density, functional domains, and architecture.

2.4. Vehicle characteristics and surface modifications

Strategic modifications to polymer vehicles can manipulate their behavior *in vivo*. Chemical or physical changes to the vehicle profile can capitalize on natural transport phenomena (transport-based) or can include molecules to aid in specific binding (molecular binding-based). Perhaps most simply, the size of polymer vehicle can significantly alter transport capabilities in the blood. A recent study comparing PAMAM dendrimers with minor size differences (4.3 nm vs 6.5 nm) found profound changes to circulation time and enhanced brain accumulation with the larger polymers [144]; another study found that the size of PLGA NPs determined the fraction that were rapidly accumulating in the liver, and the fraction that were able to target the bone marrow and lung [145]. Similarly, reducing the size of PEI/pDNA complexes resulted in the evasion of normal clearance mechanisms for these polyplexes [146]. In addition to evading clearance, it has long been appreciated that the size of the carrier is an important consideration in capitalizing on enhanced permeability and retention (EPR) effect seen in tumors and other instances of leaky vasculature [147]. However, this strategy does not apply to many tissue types and can result in accumulation in off-target regions [148,149].

Another tool to accomplish increased circulation time is through the incorporation of stealth coatings, such as PEG, as described above. Originating in the 1960s [150], PEGylation is a strategy to reduce aggregation, improve stability, reduce clearance, and increase systemic circulation time *in vivo* [151]. Hydrophilic PEG moieties sterically hinder interactions with the neighboring NPs or serum proteins and blood components by creating a hydrated cloud around the NP [152,153]. Unfortunately, the use of PEG to enhance circulation time has other consequences. The steric hindrance and stealth properties can prevent intended cellular uptake and reduce transfection efficiency [154]. Further, there is still a fraction of serum proteins that can adhere to the vehicle even after PEGylation [153]. In addition, there have been reports of several adverse events including anti-PEG antibody production [155] and complement activation [156]. Despite these potential limitations, the addition of PEG to polymer vehicles remains a popular technique, and is included in many of *in vivo* applications [122,157].

An alternative to PEGylation is the addition of other hydrophilic moieties, such as hyperbranched polyglycerol (HPG). HPG has a dendrimer-like structure which increases the availability of surface-accessible hydroxyl groups [158]. The structure of HPG can be beneficial for further chemical modifications. For example, the vicinal diols can be chemically modified to aldehyde groups, conferring bioadhesive properties [159]. Like PEG modifications, HPG also increases blood circulation time when added to NP surface, and in some cases may outperform PEG in extended circulation half-life and reduced liver accumulation [160]. Advantages of HPG are low toxicity and reduced immunogenicity *in vivo* [161]. Other molecules added to the surface can also affect biodistribution of injected NPs based on natural transport mechanisms. Different types of cholesterol, for example, can direct NPs and oligonucleotides to accumulate in either the liver exclusively (low density lipoprotein) or spread throughout the liver, gut, kidney, and steroidogenic organs (high density lipoprotein) [162,163].

To enhance vehicle accumulation within a certain cell type or tissue, molecules can be incorporated into the vehicle that will increase the likelihood of vehicle binding. Although this strategy will not help the polymer vehicle “home” to a particular site, in many cases it can increase the concentration delivered to a target site if the vehicle encounters this target naturally [164]. Using this strategy to deliver nucleic

acids to tumors, many recent reports describe the use of sugars, proteins or drugs attached to the surface of the vehicle that will bind to uniquely upregulated receptors on cells [42,43,84,117,165]. This effort is summarized in Fig. 3, which illustrates *in vivo* gene delivery applications, which have primarily been targeted to tumors. Enhanced accumulation at the tumor site was observed when hyaluronic acid (which binds to the CD44 receptor) [42,84,117], lactoferrin [43], folate [166–168], or epidermal growth factor [169] were used. Similarly, lactose, which binds to hepatocytes through asialoglycoprotein receptors, improved transfection efficiency of poly(lysine)-PEG vehicles in hepatocellular carcinomas [165]. More details on nanoparticle designs made to enhance delivery in the tumor microenvironment can be found in the recent review by Mukalel et al. [170]. Another study demonstrated enhanced pDNA delivery using the drug eprosartan to bind to upregulated ATR1 in myocardial ischemic regions [171].

In addition to the molecules described above, a number of smaller peptides have also resulted in enhanced tumor accumulation. Certain peptides such as Arg-Gly-Asp (RGD) [172,173] and RGERPPR [174] are frequently used. New peptides, discovered through phage display libraries, can enhance binding to specific, hard-to-access sites. Some examples of novel peptides identified in this way include the tartrate-resistant acid phosphatase (TRAP)-targeted peptide for bone regeneration [175] and an epithelial targeted peptide for lung delivery [176]. Although not yet applied to polymer vehicles, a recent study using peptides discovered with phage display increased nanoligand siRNA carriers in the brain from ~2% (random peptide) to 6% using the targeted peptide [177]. Traditional binding molecules such as antibodies and aptamers can be used as well for specific binding if highly expressed antigens are identified [70,178–181]. Furthermore, coating NPs with a complete cell membrane is a biomimetic strategy that can prolong

circulation and can be used for immune cell targeting [182]. Ultimately, any of these strategies can be used to enhance the accumulation of polymer vehicles at a target site, which can increase the therapeutic efficacy from a single administered dose.

2.5. Clinical trials

Worldwide clinical trials for nucleic acid therapy encompass different approaches with approximately 70% using viral vectors and 30% using non-viral vectors. While there have not yet been any polymeric NPs for gene delivery approved by the FDA, there are several recent and ongoing clinical trials (Table 1). The development of safe, efficient, and controllable delivery vehicles remains a bottleneck to clinical translation, highlighting the need for further vehicle optimization.

In 2010, Davis et al. reported on the first polymeric NP-based gene delivery system (CALAA-01) tested in a Phase I trial for solid cancers [183]. CALAA-01 is a NP system in which an siRNA against the M2 subunit of ribonucleotide reductase (RRM2) is encapsulated. The nucleic acid is loaded into a NP composed of a CD-based polymer and PEG with a human transferrin protein (TF) targeting ligand on the surface. Results from the clinical trial indicated that these NPs could deliver siRNA to melanoma cells after systemic administration and that an anti-proliferative response was achieved. Though apparently successful in delivery of the siRNA to several types of cancer cells, this trial was terminated after Phase I when patients experienced dose-limiting toxicity. Preclinical studies indicated that primary toxicities were in the liver and kidneys as elevations in liver enzymes, creatinine, and blood urea nitrogen were observed [184]. However, in humans, most adverse events consisted of hypersensitivity and acute immune responses. Subsequently, several cationic PEI-based systems (ex. BC-819/PEI, BC-819,

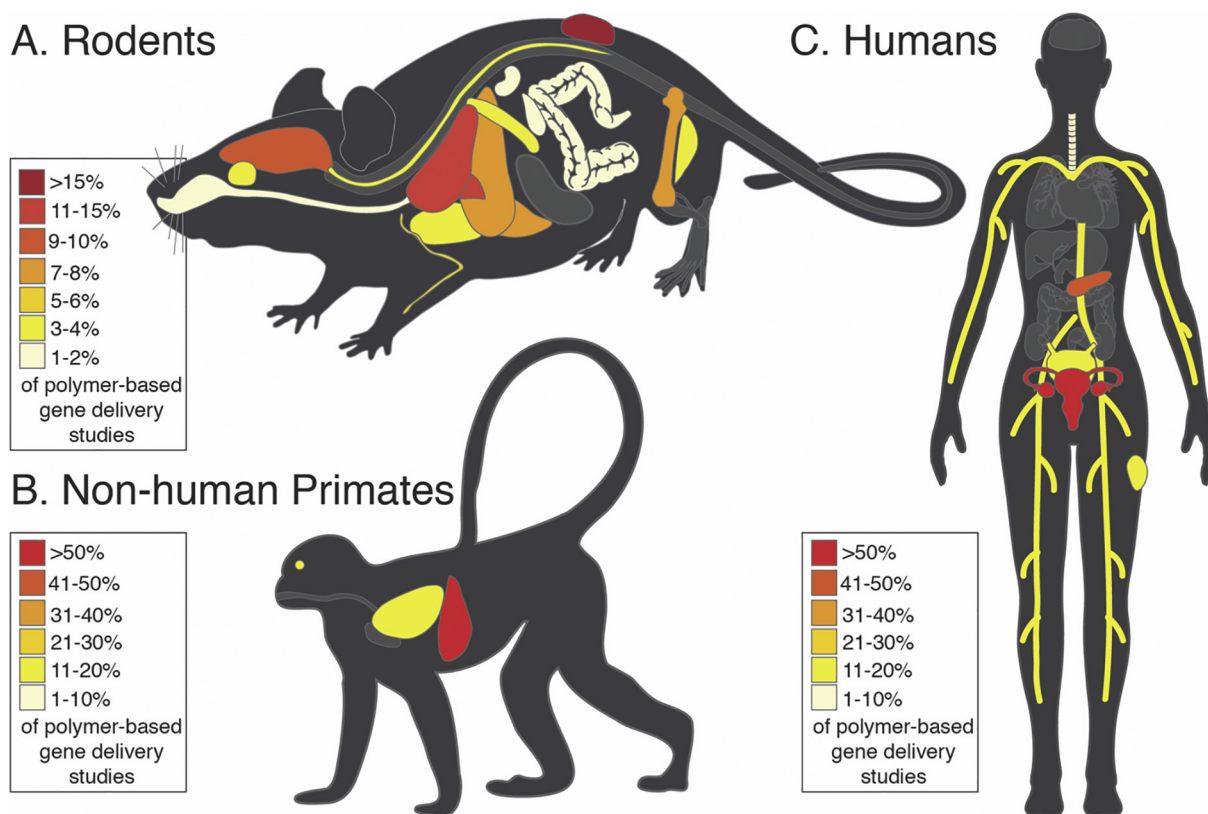


Fig. 3. Nucleic acid delivery in polymeric vehicles grouped by target organ. Heat maps in this figure demonstrate the relative number of publications that have described polymer delivery vehicles for targeted gene delivery per organ or tissue type. The rodent representation is compiled from approximately 100 studies between 2015 and 2020. The non-human primate is compiled from approximately 8 studies between 2005 and 2020, and the human representation is compiled from 11 clinical trials presented in Table 1. These studies were identified using key words such as “gene delivery”, “*in vivo*” and “polymer” in the Web of Science database. In order to be included, investigations had to report activity-based assays demonstrating effective gene delivery.

Table 1
Clinical trials evaluating polymeric vehicles to deliver therapeutic nucleic acids.

Product name	Delivery system	Cargo	Disease/target organ	Administration route	Phase	Status	Identifier
CALAA-01	CD NP with PEG	siRNA (RRM2)	Solid tumor	Systemic (IV)	I	Terminated	NCT00689065
BC-819/PEI	PEI NP	pDNA (diphtheria toxin A)	Superficial bladder cancer	Local	II	Completed	NCT00595088
BC-819/PEI	PEI NP	pDNA (diphtheria toxin A)	Superficial bladder cancer	Local	I	Completed	NCT01878188
BC-819	PEI NP	pDNA (diphtheria toxin A)	Ovarian cancer	Systemic (IP)	I/II	Completed	NCT00826150
BC-819	PEI NP	pDNA (diphtheria toxin A)	Pancreatic cancer	Local	II	Terminated	NCT01413087
BC-819	PEI NP	pDNA (diphtheria toxin A)	Non-muscle invasive bladder cancer	Local	II	Active	NCT03719300
DTA-H19	PEI NP	pDNA (diphtheria toxin A)	Superficial bladder cancer	Local	I/II	Completed	NCT00393809
DTA-H19	PEI NP	pDNA (diphtheria toxin A)	Pancreatic neoplasms	Local	I/II	Completed	NCT00711997
CYL-02	JetPEI	pDNA (SSTR2 + DCK::UMK)	Pancreatic cancer	Local	I	Completed	NCT01274455
CYL-02	JetPEI	pDNA (SSTR2 + DCK::UMK)	Pancreatic cancer	Local	II	Recruiting	NCT02806687
MK 4261/JetPEI	JetPEI	RNA (DDX58 activator)	Solid tumor	Local	I	Active	NCT03739138
BO-112	JetPEI	dsRNA (TLR-3, MDA5, RIG-I agonist)	Solid tumor	Local	I	Active	NCT02828098
EGEN-001	PEG-PEI-cholesterol	pDNA (IL-12)	Ovarian cancer	Systemic (IP)	I	Terminated	NCT00137865
EGEN-001	PEG-PEI-cholesterol	pDNA (IL-12)	Ovarian cancer	Systemic (IP)	I	Completed	NCT00473954
EGEN-001	PEG-PEI-cholesterol	pDNA (IL-12)	Fallopian tube/peritoneal/ovarian cancer	Systemic (IP)	II	Completed	NCT01118052
EGEN-001	PEG-PEI-cholesterol	pDNA (IL-12)	Fallopian tube/peritoneal/ovarian cancer	Systemic (IP)	I	Completed	NCT01489371
GEN-1	PEG-PEI-cholesterol	pDNA (IL-12)	Fallopian tube/peritoneal/ovarian cancer	Systemic (IP)	I	Active	NCT02480374
siG12D LODER	PLGA NP	siRNA (KRASG12D)	Pancreatic cancer	Local	I	Completed	NCT01188785
siG12D LODER	PLGA NPs	siRNA (KRASG12D)	Pancreatic cancer	Local	II	Recruiting	NCT01676259
SNS01-T	PEI carrier	siRNA (eIF5A) pDNA (eIF5AK ^{50R})	Myeloma/lymphoma/leukemia	Systemic (IV)	I/II	Unknown	NCT01435720

DTA-H19) were developed for clinical trials for the treatment of various cancer types after local administration [185]. However, the substantial cytotoxicity of PEI polymers at the preclinical level has limited their clinical application and consequently several modifications have been investigated. For example, glycosylated JetPEI was used in CYL-02 to deliver SSTR2 and DCK::UMK in pDNA form to inhibit tumor cell proliferation and increase chemosensitivity of tumor cells to gemcitabine, respectively [186]. In this case, treatment-related toxicities were mild. Further, EGEN-001, a PEG-PEI-cholesterol polymer-lipid hybrid vehicle was developed for the delivery of an IL-12-expressing plasmid for immunotherapy of epithelial ovarian cancer. This clinical trial progressed to Phase II but resulted in patients exhibiting adverse side effects, including fever, chills, nausea, vomiting, anemia, thrombocytopenia, and leukopenia [187]. Another trial using PLGA-based NPs, a polymer system that has been used safely in many FDA-approved drug delivery and medical device applications, delivering siRNA targeting the oncogene KRAS for pancreatic cancer treatment is currently recruiting for Phase II. In summary, polymeric vehicle-based gene therapy at the clinical level is still in its infancy, and the polymeric NPs that have progressed to clinical trials with published results have mostly failed to meet their rigorous endpoints. Further, the adverse events reported thus far, though mild in some cases, suggest that the therapeutics that have been investigated at the clinical level exhibit off-target activity. However, it is difficult to determine whether these adverse effects are due to the polymeric vehicles themselves, their nucleic acid cargo, or administered combination therapies where applicable. Nonetheless, it is clear that therapeutics involving polymeric carriers must still overcome challenges associated with safety and efficacy before translation to humans. In particular, advances that reduce cytotoxicity while increasing transfection efficiency and target specificity are needed. Strategies to improve vehicle design are outlined below. However, it is important to note that increasing the complexity of polymeric carriers increases the difficulty of maintaining the high quality standards needed for clinical trials.

3. Strategies to mitigate current challenges facing polymeric nucleic acid delivery

While innovation to produce novel polymeric materials and delivery vehicles continues at a rapid pace, significant challenges remain. The

clinical viability of any nucleic acid delivery system requires proper dissemination without interfering with normal bodily functions, eliciting an immune response, or producing adverse effects in non-target organs [188]. We have discussed strategies and examples for balancing biocompatibility and transfection efficiency in our review of polymeric delivery systems above. In broad terms, new innovations that focus on *in vivo* efficacy and target specificity as well as translation to commercial production will lead to increased clinical usage of this class of therapeutics. Here, we describe these challenges and identify forward-looking strategies to address the issues.

3.1. *In vivo* efficacy and target specificity

Many nucleic acid delivery systems have been successful *in vitro* but are not effective *in vivo*. This discrepancy is likely due, at least in part, to the lack of physiologically relevant screening tools and challenges controlling tissue tropism. It is well understood that traditional 2D monolayer culture systems, which are widely used for initial screens, are not a realistic representation of cell behavior in the human body. Fluid flow, 3D cell and tissue organization, and vehicle clearance are critical aspects of gene delivery that are not adequately addressed using conventional *in vitro* experimental designs [189]. Further, animal models of disease can be inadequate, costly, and not truly predictive of human results [190,191]. As shown in Fig. 3, a number of studies deliver genetic material to specific human cancer cell lines (hepatocellular, prostate, lung, breast) which are grown in rodents as tumors. The physical constraints of this type of delivery may not mimic organ-specific cellular organization and limit the predictive results of these experiments.

3.1.1. Strategy: improve preclinical screening models to more accurately predict *in vivo* activity

Advancements in technologies that create platforms to better mimic human disease and physiological barriers can enhance or replace typical *in vitro* and *in vivo* preclinical studies. 3D cell culture systems have emerged over the past few decades as physiologically relevant cellular environments. Using technologies such as 3D printing and microfluidic devices, cell culture environments can mimic the tortuous and complex tissue environments found in tumors, the liver, and other tissue types [192–194]. In particular, micropatterning of cell culture devices allows

researchers to incorporate sophisticated gradients and patterns of signaling molecules, growth factors, or environmental confinement [192,193]. Evaluating polymeric vehicle efficiency in these systems can help to elucidate the effect that physical constraints have on vehicle stability and cell association.

In addition to replicating the physical environment, cells with genetically accurate human disease can be produced from human induced pluripotent stem cells (iPSCs) with the advent of CRISPR/Cas9 and other gene editing technologies. The ability to generate iPSCs from somatic cells provides tremendous promise for gene delivery screening as it is possible to produce many different cell types from patient cells [195]. Candidate therapies can be tested directly in cells modified to include defects responsible for hematological, neuronal, muscular, and other disorders [196]. iPSCs are also more physiologically relevant cells with which to conduct preclinical safety studies than immortalized cell lines.

Lastly, new assays are being developed to rapidly screen many gene delivery vehicles. In one example, Yonamine and colleagues modified an ELISA (enzyme-linked immunosorbent assay) assay into a high throughput screening (HTS) method to select NPs with high affinity to target proteins [197]. The screen identified polymeric NPs with distinctive functional group compositions that exhibited high affinity to either histone or fibrinogen which allowed for identification of synthetic polymers with affinity for the target. These types of assays are often used to screen only one aspect of the delivery process, but they can be a powerful addition to the more complex models described above. *In vivo*, other factors may be relevant and vehicle specific, such as time in circulation and biodistribution. For high-throughput *in vivo* screening, we recently described an accessible approach to measure the circulation half-life of fluorescently labeled agents after intravenous injection [198]. Further, Dahlman and colleagues have designed a way to directly compare the biodistribution of many formulations of nanoparticles at once by labeling the encapsulated DNA with unique barcodes [199]. Evaluating the activity of many polymer vehicles side-by-side in the intended setting can provide valuable insights to behavior in the physiological environment.

3.1.2. Strategy: strategic administration and new vehicle designs for improved target specificity

As described, using molecular targeting strategies for systemic delivery can enhance accumulation at a specific site, but often will still result in unwanted accumulation elsewhere [172,200,201] and cannot provide access to every organ [41]. One strategy to overcome this challenge is site-specific administration, either by using an implant in response to an injury that anchors polymer delivery vehicles at the site [202,203] or through an injection into an accessible organ or tissue (e.g. eye [125], spinal cord [61], brain [123]). Similarly, localized genetic manipulation has been achieved by pre-treating cells with polymeric vehicles *ex vivo*, and then transplanting the treated cells back into the patient [62,112]. This strategy takes advantage of sustained delivery available with some polymer vehicles to maintain the nucleic acid activity throughout cellular development or differentiation. Finally, in instances where organs or tissues are being transplanted out of the body, *ex vivo* normothermic organ perfusion (EVNP) could provide a period of privileged access to deliver therapeutics without off-target possibilities. EVNP is being investigated for many organ transplant practices, and both polymeric NPs and naked siRNA have been successfully delivered during this process [204,205]. *Ex vivo* treatment or direct injections can be invasive and are not suited for all applications. Another strategy for systemic delivery is to temporarily blockade the reticuloendothelial system with decoy materials, allowing the critical cargo to circulate without liver interference [206–208]. Recent work in the field of lipid NP-mediated gene delivery has also shown that modulating the internal charge of delivery vehicles can enable tuning of biodistribution and tissue-specific gene delivery [209].

Alternatively, stimuli responsive polymers offer a tool to constrain nucleic acid availability to only intended regions [210]. In this strategy, polymers react to environmental or applied stimuli, and a conformational

or chemical change triggers the release of the nucleic acid cargo. Externally applied stimuli such as a magnetic field, an electric field, ultrasonic waves, or light can be spatially applied for treatment of tumors or organ specific pathologies [211–213]. For example, near-infrared light was used to trigger PEI-derived NP decomposition-driven release of siRNA to tumors *in vivo* [214–216]. Environmental factors that are unique to the cell, tissue, or pathology (inflammation, cancer, infection) can also be used, such as pH [217–222], temperature [223–225], and the presence of ATP (reviewed in [63]). Polymers incorporating disulfide bonds can be transformed into thiols via reduction in the presence of glutathione (GSH) or reactive oxygen species (ROS), triggering a release of nucleic acid cargo [226–228]. ROS-sensitive polymers are particularly useful tools for cancer cells that exhibit higher levels of ROS radicals and GSH [229].

3.2. Translation to commercial production

Polymeric materials must be inexpensive to produce, easy to synthesize, facile to purify and characterized completely to accomplish commercial-scale production. Control over polymerization mechanisms and chain polydispersity are key factors that can lead to variable performance [230,231]. Since polymers are generally not a specified molecular entity, their characterization for manufacturing can prove difficult with regard to achieving Good Manufacturing Practice (GMP) certification. In terms of vehicle production, the size, morphology, and functionality of polymeric carriers need to be reproducible and scalable. For example, on a research scale, formulation processes such as nanoprecipitation and emulsion evaporation begin with milligrams of a dissolved polymer and end with the formation of precipitates that require the removal of solvents. On an industrial scale, grams to kilograms of materials would be required, with accompanying changes to techniques which may change formulation properties [232]. Many formulations, especially polyplexes, are unstable and cannot be stored long term, and as such, stability and shelf-life need to be considered as well.

3.2.1. Strategy: improved synthesis, purification, and formulation methods

Precise and inexpensive synthesis of polymers is a challenge, especially due to the cost of reactor systems, materials (especially catalysts and initiators), and purification equipment. Enzymatic catalysts can provide a cost-effective and environmentally friendly solution for biodegradable polymers compared to conventional metal or salt catalysts [233]. For example, bacterial-derived lipase can perform essential synthesis steps while maintaining high selectivity, high efficiency, the ability to operate under mild conditions, and potential catalyst recyclability [129,234,235–239]. In a non-aqueous environment, more efficient catalysis can occur due to the increased solubility of the substrates in solvents, a reduction in the number of side-reactions, and a shift in the reaction equilibrium to favor synthesis over hydrolysis [240,241]. Enzymes can also be separated from the reaction mixture as they are often immobilized to an inert bead which can be collected via filtration [242,243]. Lastly, their thermal stability can be enhanced, allowing for synthesis reactions to occur more efficiently at higher temperatures [244].

As cationic charge in polymers has continued to be essential for nucleic acid encapsulation and delivery, appropriate synthesis schemes incorporating specific amine and other end groups are crucial. For example, specified crosslinking with 1,1'-carbonyldiimidazole (CDI) is a useful technique to obtain user-defined end groups. Activation and cross-linking with CDI results in a variety of gene delivery polymers with precise end group chemistries for efficient delivery of nucleic acid cargos [245–248]. Subtle differences in the structure and properties of polymeric materials used for gene delivery can lead to dramatic differences in transfection efficiency and CDI chemistry is an efficient way to develop a library of materials [132].

As described above, it is also desirable to have control over the entire polymer sequence and not just the end group composition. Multistep

flow synthesis and iterative exponential growth (Flow-IEG) is a technique has been utilized for macromolecules such as PNAs and synthetic RNAs, and has been adapted for polymers with semi-automatic processes with molecular weight distribution control [249,250]. The development of the next generation of polymeric materials could utilize an iterative approach to find the appropriate chain length, charge density, and end group chemistry to form the most efficient non-viral vectors in a scalable manner [249,251–255].

Alongside advanced apparatus systems and novel catalysts, new polymer purification equipment and techniques have been employed with the same scalability. Enzyme catalyst reactions often require solvents for appropriate viscosity for mixing during synthesis [237,239,241]. This could be seen as a disadvantage as purification requires the complete removal of the solvent, but it can be advantageous as removal of the polymer and separation of the catalyst can be completed in a series of wash steps that utilize solvent miscibility principles [131]. Other methods of advanced purification, including the use of supercritical fluids and innovative membrane filtration, have aided in large scale polymer syntheses [256–260].

Scale-up of formulation methods requires added consideration of the fragility of nucleic acid cargo, where techniques such as membrane extrusion and supercritical fluid technology are not applicable. Nonetheless, a few methods have been developed in order to produce NPs at larger scales with desired characteristics, such as microfluidic mixing technologies for nanoprecipitation processes [261]. Such processes still require the removal of organic solvent, achievable with tangential fluid flow systems. However, these methods are not amenable to the production of surface functionalized or targeted polymeric carriers, which may require additional processing steps.

4. Conclusions

The future of nucleic acid-based therapies will depend on innovations in delivery systems. For polymeric vehicles, this will involve the development of materials that excel at nucleic acid association, are less toxic/immunogenic, and are efficient at nucleic acid delivery both *in vitro* and *in vivo*. It will also be essential to define the mechanisms by which these materials achieve delivery to maximize effectiveness and minimize unexpected side effects. Studies aimed at understanding the structure-function relationships of polymeric vectors and the mechanisms by which they interact with cells in physiological environments are likely to produce promising candidates. Determining the requirements for optimal timing and longevity of genetic manipulation for each application will also help to guide vehicle design. Given the rapid progress in the field, nanoscale polymeric delivery systems will likely continue to serve an important role in the clinical translation of gene therapies.

Declaration of Competing Interest

Amy C. Kauffman was a Postdoctoral Fellow at Yale University and transitioned to an employee of Corning Life Sciences during the time this review was completed. During the time of writing this review, ASP and WMS were consultants to Trucode Gene Repair Inc.

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