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Review

Polymeric Delivery of Therapeutic Nucleic Acids

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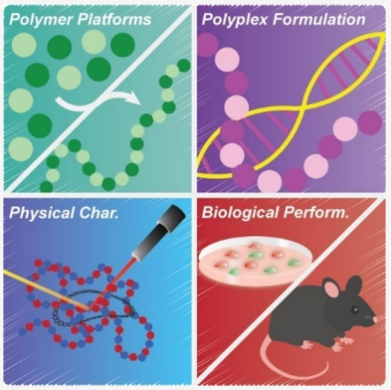
ABSTRACT: The advent of genome editing has transformed the therapeutic landscape for several debilitating diseases, and the clinical outlook for gene therapeutics has never been more promising. The therapeutic potential of nucleic acids has been limited by a reliance on engineered viral vectors for delivery. Chemically defined polymers can remediate technological, regulatory, and clinical challenges associated with viral modes of gene delivery. Because of their scalability, versatility, and exquisite tunability, polymers are ideal biomaterial platforms for delivering nucleic acid payloads efficiently while minimizing immune response and cellular toxicity. While polymeric gene delivery has progressed significantly in the past four decades, clinical translation of polymeric vehicles faces several formidable challenges. The aim of our Account is to illustrate diverse concepts in designing polymeric vectors towards meeting therapeutic goals of in vivo and ex vivo gene therapy. Here, we highlight several classes of polymers employed in gene delivery and summarize the recent work on understanding the contributions of chemical and architectural design parameters. We touch upon characterization methods used to visualize and understand events transpiring at the interfaces between polymer, nucleic acids, and the physiological environment. We conclude that interdisciplinary approaches and methodologies motivated by fundamental questions are key to designing high-performing polymeric vehicles for gene therapy.



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1. INTRODUCTION

Molecular biology tools that remediate genetic defects have steadily grown in their capabilities, with the evolution of nucleic acid therapy tools such as meganucleases,[1] transposons,[2] transcriptor activator-like nucleases (TALENS),[3] ribonucleic acid (RNA) silencing,[4] clustered regularly interspersed palindromic repeats (CRISPR) gene editing,[5] base or prime editing,[6,7] and other innovative editing platforms.[8] In addition to the ability to treat many genetic diseases such as Leber's congenital amaurosis, Duchenne's muscular dystrophy, beta thalassemia, or cystic fibrosis, researchers are slowly uncovering the genetic basis of many acquired afflictions such as cancer, type 2 diabetes, Alzheimer's, and age-related macular degeneration. Vaccine development is also increasingly relying upon delivery of deoxyribonucleic acid (DNA), RNA, or antigens. Many of the aforementioned systems involve systemic infusion or direct tissue administration; however, cellular therapies involving induced pluripotent stem cells and chimeric antigen receptor T-cells[9] have also come to fruition and require ex vivo genome editing, further expanding the therapeutic scope of gene therapy. Indeed, several gene therapy clinical trials have been progressing rapidly with landmark successes being reported in therapies focused on CRISPR/Cas9[10−12] and in the development of mRNA-based vaccines for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[13]

Despite the enormous promise held by gene therapy to solve pressing problems in human health, we must contend with economic and engineering barriers to their clinical translation. To deliver therapeutic nucleic acids during in vivo administration as well as ex vivo applications, engineered viral vectors, especially adeno-associated viruses (AAVs), are employed by default.[14] Over the years, clinicians have perfected approaches to optimize viral capsids to deliver payloads efficiently while minimizing toxicity and preventing adverse events associated with the innate immune system. Despite these efforts to reduce the mutagenic and immunogenic risks originating from viral vehicles, fatal responses to virus administration have been recorded in patients undergoing experimental treatments for Duchenne's muscular dystrophy and X-linked myotubular myopathy.[15,16] The treatment regimens for these diseases require extremely high (and sometimes repeated) doses of viral vehicles, increasing the risk of adverse events. Beyond their non-ideal safety profiles, engineered viral vehicles pose financial and logistical challenges during scale-up and mass-manufacturing, resulting in both exorbitant product costs and long wait-times for production.[17]

The need for nonviral delivery methods is widely acknowledged by both clinicians as well as biotechnologists in the nascent gene therapeutics industry.[18,19] Chemically defined materials can be easily scaled up, made available off-the-shelf, readily formulated, and stored without the need for technical

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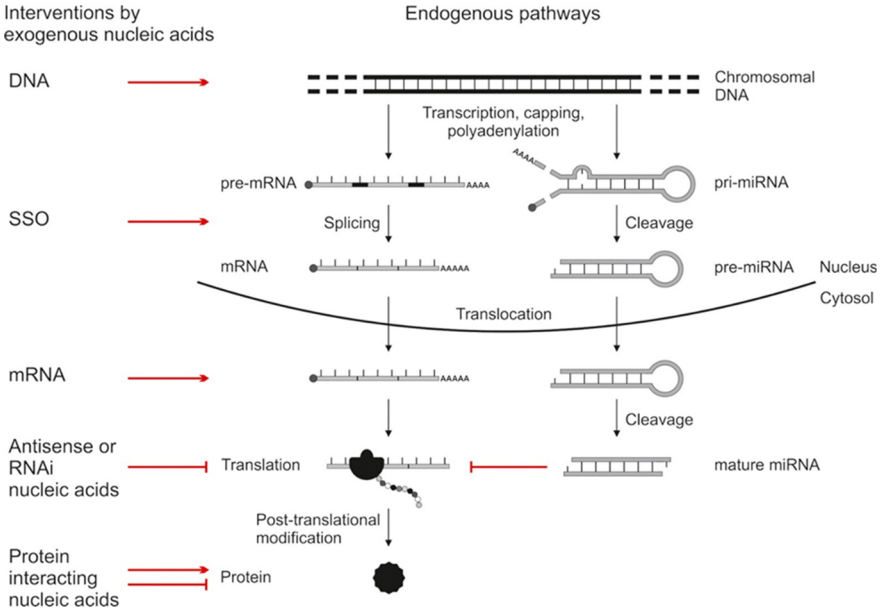

Figure 1. Schematic illustrating the endogenous pathways through which various nucleic acid payloads such as DNA, splice-switching oligonucleotides (SSOs), mRNAs, and ASOs are processed. Reprinted with permission from ref 45. Copyright 2015 American Chemical Society.

expertise or access to refrigeration. The recent approval of two lipid-based mRNA vaccines for the novel coronavirus, SARS-CoV-2, have sparked renewed interest in non-viral gene delivery platforms. While adenoviral and lipid-based delivery approaches have both yielded successful vaccine candidates, there is a dire need for nanomaterial platforms that can address challenges in affordability and rapid world-wide distribution, especially in the developing world where infrastructural deficiencies exist in the cold chain.[20]

Because of their versatility and multifunctionality, polymeric biomaterials have emerged as viable gene carriers.[21,22] Advances in synthetic methodologies, particularly controlled radical polymerization, have allowed researchers to impart desired properties to polymeric carriers by investigating diverse monomer functionalities and polymer architectures. Although our knowledge of intracellular mechanisms involved in polymeric gene delivery remains incomplete, researchers have developed creative ways to characterize and understand interactions between polymers, nucleic acid cargoes, and cellular targets. The field has gradually been making progress towards clinical translation, and the next decade promises to be an exciting one for polymeric vectors.

We note that synthetic methodologies along with architectural and chemical design aspects for polymeric vehicles form the focus of our Account. Hence, we redirect readers to excellent reviews focusing on related classes of biomaterials such as polypeptides,[23] dendrimers,[24,25] nanogels,[26,27] graphene-based materials,[28] poly(ethylene imine) (PEI), chitosan, poly(L-lysine) (PLL), and hydrogels for sustained delivery.[29] Since lipid nanoparticles are outside the scope of our Account we point out some reviews focusing on lipid-based delivery approaches.[30−32] We would also like to highlight payload-specific reviews focused on short interfering RNA (siRNA),[33,34] messenger RNA (mRNA),[35,36] and a slate of recent reviews highlighting delivery challenges involved in CRISPR/Cas9 editing.[19,29,37−44] Since *Chemical Reviews* has published two in-depth reviews on polymeric gene delivery[45,46] in the past two decades, we will only briefly discuss inorganic nanoparticles, PEI, PLL, chitosan, dendrimers, and polypeptides, with references restricted to the most recent literature covering the subject. Given the rich, decades-long history of this field, our Account has been preceded by a wealth of review articles that have also offered critical insights on polymer-mediated gene delivery.[47−52] In this contribution, our goal was to capture the most recent developments in the field, to survey a broad variety of polymer design approaches along with clinical successes in a balanced manner, and to offer conceptual overviews that are of interest to seasoned investigators and novice researchers alike.

Through this Account, we aim to offer the reader a holistic view of significant developments and essential material design concepts in the polymer-mediated transfer of nucleic acids. Our effort encompasses several disciplinary perspectives, including organic synthesis, macromolecular chemistry, and materials engineering; it covers diverse classes of polymeric materials, from free polymer chains to cross-linked hydrogels and polymer coatings. We begin the Account by outlining physiological barriers to delivery that must be traversed by polymeric vehicles to deliver their payload. The second section will present a detailed overview of key design motifs used in polymeric vehicles, paying special attention to chemical and architectural design features. We will discuss how precise design, chemical innovation, and controlled synthesis of polymeric vehicles have come together to impart powerful features such as stimuli-responsiveness and resistance to protein fouling. Subsequently, we will take a deep dive into the synthetic toolkit commonly deployed by polymer chemists to access interesting polyplex properties, with a focus on click-chemistry approaches and post-polymerization modifications. The Account will then transition to discussing the physical aspects of gene delivery and focus on how engineering interventions can resolve kinetic limitations in polyplex assembly. We will briefly describe alternative polymer platforms that address gene delivery challenges from a polymer processing rather than a polymer chemistry perspective. Our Account will conclude by examining clinical success and future research directions for polymer-mediated gene delivery and by

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suggesting profitable avenues of research for aspiring investigators.

2. BIOLOGICAL CHALLENGES RELEVANT TO POLYMER-MEDIATED NUCLEIC ACID DELIVERY

A key driving force for the design of polymers for gene delivery is the incorporation of material properties that aid nucleic acids in overcoming specific biological barriers to gene delivery. In this section we will first describe various therapeutic nucleic acids and the molecular biology principles underlying their functioning, highlighting their unique properties, challenges for delivery, and uses. We briefly describe commonly employed physical strategies to introduce nucleic acids within cells, noting that these approaches are mostly restricted to ex vivo applications. Then, we review biological barriers that are unique to gene delivery, paying special attention to both extracellular (or systemic) barriers as well as intracellular barriers that are encountered by therapeutic nucleic acids as they travel to targeted cells where gene expression must be achieved. While we do not propose solutions to overcome these barriers in this section, we believe that a basic understanding of the biological basis for polymer-mediated gene delivery is essential to engineer synthetic strategies.

2.1. Types of Nucleic Acid Cargoes and Their Biological Mechanisms

Polymeric vehicles can be assembled with various nucleic acid modalities varying widely in their therapeutic application, the design constraints accommodated by the polymeric vehicle, and the desired time frame for therapeutic effects, that is, whether we require permanent changes to the genome or transient expression or silencing of targeted proteins. While all of the cargoes described in the section vary in their size, topology, and mechanism of action (Figure 1), all of them are amenable to being packaged with synthetic polymers to form therapeutically useful nanoassemblies called polyplexes through polyelectrolyte complexation.

2.1.1. Plasmids (pDNA). Plasmids are the most dominant nucleic acid cargoes explored in the gene delivery literature. These are circular double-stranded DNA molecules that are replicated inside bacteria separate from chromosomal DNA. Along with their utility in cloning DNA fragments and producing large quantities of proteins in culture, plasmids have been widely used as vectors in gene therapy.[53] The two primary portions of plasmids are (1) the bacterial backbone, which contains an antibiotic resistance gene and origin of replication for production in bacteria, and (2) the expression cassette, which is the transcriptional fragment containing the gene of interest and regulatory sequences.[54] The expression cassette can encode therapeutic RNAs or proteins, and if successfully delivered to the nucleus of the target cell, endogenous cellular machinery can produce the therapeutic construct in large quantities.[53] Unlike some other nucleic acid payloads, pDNA requires nuclear entry to be effective, placing additional constraints while designing gene delivery vehicles. Delivery efficiency can also be improved by reducing the plasmid size through the removal of the bacterial backbone, forming minicircles or minivectors.[55] Once they reach the nucleus, plasmids and mini DNA vectors do not integrate into the genome, so expression of the transgene is transient and will diminish over time, especially as the cell divides.[56] Plasmids are still widely used for transient gene delivery applications due to the ability to accommodate large gene payloads, their ease of construction, low production cost, and resistance to degradation.[56]

2.1.2. mRNA. An alternative method to achieving transient gene expression in target cells is through the delivery of synthetic messenger RNA (mRNA).[57] One major advantage of using mRNA as a therapeutic payload is that it is readily translated in the cytoplasm and does not need to translocate through the restrictive nuclear barrier. For this reason, mRNA can be expressed more readily than pDNA in non-dividing cells.[58] The biggest concern with mRNA as a gene delivery vector, however, is its relative instability to RNase degradation.[57] To address this concern, significant progress has been made in producing synthetic mRNAs that are more resistant to degradation.[59] The cap, 5′ and 3′ untranslated regions, coding region, and poly(A)-tail are all elements of natural mRNA that are present in synthetic mRNA, and all have been optimized for increasing stability. For example, synthetic caps, called anti-reverse cap analogues, have been developed that are resistant to decapping enzymes while maintaining translation efficiency.[60] Another concern surrounding mRNA is the innate immune response that foreign mRNA can elicit.[61] Some ways to reduce this immune response include modifications to the structure of the nucleic acid base (such as replacing N1-methylpseudouridine for uridine[62,63]) or 2′-O-methylation.[64] Such improvements in synthetic mRNA stability and immunogenicity have helped increase its popularity as a transgene vector.[65]

2.1.3. Antisense Oligonucleotides (ASOs) and RNA Interference (RNAi). Along with nucleic acids that encode for genes, there is a critical need for delivery vehicles that can deliver synthetic nucleic acid oligomers that can induce gene silencing.[66] These include ASOs and RNAs for RNAi. ASOs are short (~20 bp), single-stranded oligodeoxynucleotides (ODNs) that can bind to a target mRNA to silence its expression. When the ASO binds to the target mRNA via base pairing, the RNA-DNA hybrid acts as a substrate for RNase H leading to the degradation of the target mRNA.[67] ASOs can also bind the targeted RNA and block translation without inducing its degradation (steric-blocking oligonucleotides) or modulate the splicing of the RNA (splice-switching oligonucleotides).[68] Similar to ASOs, several types of therapeutic RNAs utilize RNAi, which is an innate biological process that inhibits gene expression.[69] Endogenously, eukaryotes regulate mRNA translation by producing microRNAs (miRNAs) that bind to cytosolic RNAi enzymes to form an RNA-induced silencing complex (RISC). When bound as an RISC, miRNA can base-pair to mRNAs complementary sequences and either inhibit containing complementary sequences and either inhibits translation or promote degradation of the mRNA.[70] Similarly, small interfering RNAs (siRNAs) are fragments of double-stranded RNA (ranging between 15 and 30 bp) derived from exogenous RNA that can use RISC to bind and cut mRNAs of specific sequences to inhibit translation.[71] Improved siRNA constructs have overcome initial setbacks in toxicity and efficacy and have recently earned approvals from the Food and Drug Administration (FDA), re-invigorating their status as impactful therapeutic drugs.[69]

The nucleotides in ASOs and synthetic RNAs for RNAi are chemically modified to impart resistance to degradation, improve immune system tolerance, and enhance binding selectivity.[69,72] Some common modifications of the phosphodiester backbone include phosphorothioate DNAs, phosphorodiamidate morpholinos, and peptide nucleic acids.

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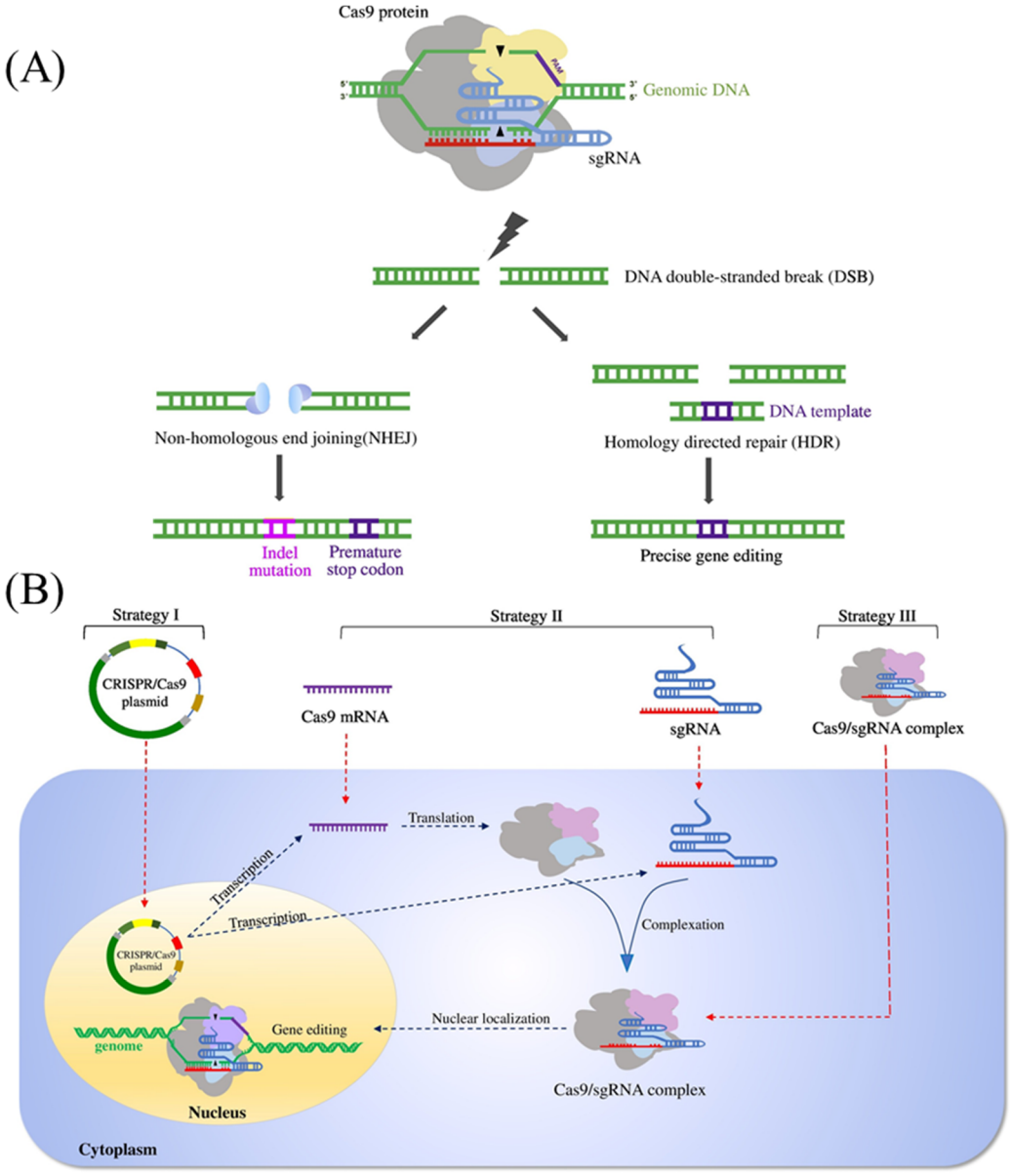

Figure 2. (A) Mechanism of the CRISPR-Cas9 system. Guide RNA recognizes and binds to the target genomic locus, subsequently directing the Cas9 protein to produce a double-strand break in the DNA. The severed DNA can now undergo two types of repair, non-homologous end joining or homology-directed repair. (B) Summary of delivery strategies used for CRISPR/Cas9 editing. Strategy I employs a plasmid to encode both the Cas9 protein and the single guide RNA. Strategy II uses a mixture of Cas9 mRNA and single guide RNA. Finally, the Cas9 protein can be delivered directly after annealing with the single guide RNA to form ribonucleoprotein complexes (Strategy III). Reprinted with permission from ref 79. Copyright 2017 Elsevier.

Some common 2′ substitutions of the ribose sugar include: 2′-O-methyl, 2′-O-methoxyethyl, 2′-F, and 2′4′-locked nucleic acid.[69,72] An additional benefit of ASOs and synthetic RNAs for RNAi is the fact they impart their gene silencing effects in the cytoplasm, so nuclear delivery is not necessary. Similarly to mRNA, though, these constructs still greatly benefit from gene delivery vehicles that stabilize them against degradation, promote cellular internalization, and allow for their entry into the cytoplasm.[66,73] Other therapeutic nucleic acid constructs for gene silencing that can be delivered with polymer-based gene delivery vehicles include ribozymes, DNAzymes, and antagomirs.[74−76]

2.1.4. Genome Editing. The aforementioned nucleic acids impart transient effects on gene expression, and continued modulation of gene expression with these cargoes requires multiple administrations. Many gene therapies are focused on permanently altering the genome of target cells within a patient in a process known as gene editing. These therapeutic strategies utilize nucleic acid and protein-based machinery, the cellular delivery of which can be mediated by polycations. Nonviral genomic insertions of genes can be achieved with the delivery of DNA transposon systems such as *Tol2*, *piggyBac*, and *Sleeping Beauty*.[2] More recently, however, gene therapy strategies have embraced technologies that can achieve genomic manipulations, such as gene insertions and knockouts with greater precision. The most common nonviral gene editing platforms include zinc finger nucleases, transcription activator-like effector nucleases (TALENs), meganucleases, and the CRISPR/Cas9 system.[77] These nuclease systems induce a double-strand break (DSB) in a precise location of

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the genome, which stimulates endogenous cellular repair machinery. Repair of the DSB can occur through non-homologous end-joining (NHEJ) or homology-directed repair (HDR) as depicted in Figure 2A. The NHEJ pathway ligates the broken ends and often introduces insertions and/or deletions that can disrupt genes at the site of the break (knock-out). In contrast, the HDR pathway can repair the break by using a DNA template containing a homologous sequence, and by doing so, the repair can lead to the insertion of an exogenous gene of choice (knock-in).[78] Variations of genome editing with Cas-based derivates are being developed to address other challenges in gene editing at a rapid pace.[8]

The delivery of nucleic acid-based constructs is required for all of these editing strategies to occur. In the case of the canonical HDR-based gene insertion with CRISPR/Cas9, a ribonucleoprotein, consisting of the guide RNA (sgRNA) and Cas9 protein, must be delivered to the nucleus to induce a DSB concurrently with the delivery of a template DNA. The template DNA can be delivered via a plasmid or single-stranded oligonucleotide (ssODN), while the components of a ribonucleoprotein can be delivered directly or expressed from a plasmid or mRNA (Figure 2B).[40] Exemplified by this case, delivery requirements for these sophisticated editing systems are demanding, and there is an urgent need for efficient gene delivery strategies in order to achieve the desired outcomes.[40] Polymer-based delivery platforms are well-suited for the concurrent delivery of these large constructs. A sophisticated polymer-based design is required to help this cargo overcome the extracellular and intracellular barriers to achieve efficient delivery and editing.[80]

We summarized key features of the most widely used nucleic acid cargoes in this section. We also note the emergence of payload systems such as microRNA,[81] self-amplifying or replicon RNA,[82,83] base editor proteins,[6,8] prime editing,[7] and redirect the reader to more detailed articles discussing these molecules.

2.2. Physical and Chemical Methods of Delivery

There are several categories of non-viral gene delivery vectors, each presenting advantages and disadvantages with their application. Physical methods of delivery achieve translocation of hydrophilic macromolecules into the intracellular space by transient permeabilization of the cellular membrane via mechanical means.[84] These processes include microinjection, particle bombardment, electroporation, magnetofection, sonoporation, photoporation, mechanical deformation, and hydroporation.[84] Most of these physical methods are most effective for the transfection of cells in culture (in vitro) or of localized tissue in vivo. In addition, they often require specialized equipment. Alternatively, gene delivery can be achieved using chemical carriers that typically bind the nucleic acid cargo and facilitate its intracellular uptake and delivery. Although the chemical diversity of these systems is vast, chemical carriers can generally be categorized as inorganic, peptide, lipid, or polymer-based systems.[85] Examples of materials used for inorganic gene delivery particles include calcium phosphate, silica, gold, magnetic metals, carbon nanotubes, and quantum dots, among others. These inorganic nanoparticles can vary greatly in size, shape, and surface chemistry, and they are often functionalized with polymeric or bioactive compounds to tune their biological properties.[86]

Alternatively, nucleic acids can be conjugated or electrostatically bound to biologically-derived compounds, such as peptides, to promote nucleic acid delivery. Peptides for gene delivery can be broadly categorized as either cell-penetrating peptides, targeting peptides, endosome disrupting peptides, or nuclear localization signal peptides. While providing effective methods to overcome certain biological barriers, these peptides often suffer from short circulation half-lives, poor stability, and low DNA binding affinity.[87] The most widely utilized non-viral gene delivery vehicle is lipid-based vesicles. Lipids, which consist of a hydrophilic head and hydrophobic tail, can form bilayer vesicles called liposomes, and if lipids with cationic heads are present in the lipid mixture, nucleic acids can electrostatically bind and become encapsulated in the liposome to form a lipoplex. These lipoplexes are often mixtures of charged lipids, uncharged lipids, and cholesterol that can promote fusing and lipid exchange with endogenous cellular membranes. Lipoplexes can also be functionalized with PEG-based coatings or bioactive compounds to improve transfection efficiency, stability, or promote tissue-specific targeting. Each of these non-viral methods has been developed over the last several decades in parallel to polymer-based gene delivery, and each method has its own advantages and disadvantages for any given gene delivery application.

2.3. Extracellular Barriers

Polymer-mediated gene therapy promises to address limitations associated with both viral vectors and physical gene transfer methods, albeit not without its own series of extracellular and systemic biological barriers (Figure 3).[88−90] The vectors must evade the reticuloendothelial system (RES) that would otherwise rapidly eliminate biologically relevant materials from the body. Additionally, there are multiple physiological barriers nonviral vehicles must cross, based on the route of administration (intravenous/mucosal injection, topical application, and oral delivery). Formulation of the polyplexes must also be taken into consideration as, at higher salt concentrations, the electrostatic repulsion between the cationic polyplexes and anionic DNA backbone is screened by electrolytes, leading to a decrease in colloidal stability and a propensity for aggregation.[43] Aggregation of these polyplexes can also occur in the blood (particularly due to plasma proteins and erythrocytes), which can also lead to an unsuccessful localization of the vector to the desired tissue and RES-mediated elimination.[91] Furthermore, upon systemic delivery of these vehicles in vivo, other barriers include phagocytosis of the nanoparticle, enzymatic (DNases, RNases, proteases) and/or hydrolytic degradation, and potential activation of an immune system via a toll-like receptor (TLR)-mediated response or cytokine induction. Each of these barriers will be further discussed below, and circumvention of these barriers will be discussed throughout this Account.

2.3.1. Serum-Induced Aggregation. Like any other biomaterial introduced into a physiological environment, polymeric gene delivery vehicles are susceptible to non-specific protein adsorption (or biofouling), and the formation of an opsonin-enriched protein corona marks them out as a target for clearance by the immune system.[92] Surprisingly, the challenges associated with non-specific protein adsorption are not unique to in vivo delivery, since serum is ubiquitously used in the cultivation of both immortalized cell lines as well as the maintenance of primary cells. Unfortunately, serum contains numerous proteins, which can adsorb onto the polyplex surfaces through electrostatic, hydrophobic, or other inter-

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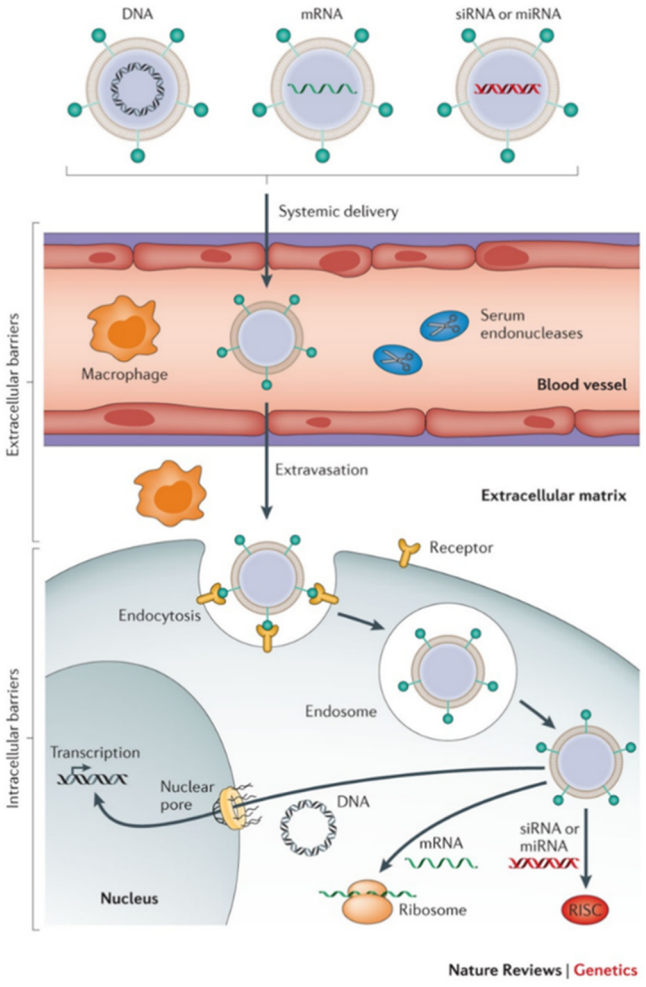

Figure 3. Extracellular and intracellular barriers to nonviral gene delivery vehicles. Reprinted with permission from ref 88. Copyright 2014 Springer Nature.

actions, ultimately causing these colloidal systems to aggregate severely.[93]

Nanoparticles designed to condense the negatively charged backbone of nucleic acids typically consist of cationic lipids or polymers. Although this positive charge allows an efficient complexation with nucleic acids, these polycations tend to be sequestered by proteins present in the serum or by other components of the extracellular matrix.[94] The association with plasma proteins (albumin, lipoproteins, macroglobulin) is the primary mechanism by which the reticuloendothelial system recognizes circulated nanoparticles. Immune recognition initiates a cascade of events that redirect injected polyplexes to the liver or spleen, thereby preventing the vehicle from reaching its target.[95] In addition, the interaction with the serum proteins and nanoparticles can greatly alter their diameter and zeta potential, ultimately influencing its biodistribution profile and compromising organ-specific targeting.[96,97]

Another serious consequence of protein−polyplex interactions is the displacement of the nucleic acid by negatively charged proteins through a competitive binding, causing a premature release and disassembly of formulated polyplexes.[98] Proteoglycans and glycosaminoglycans are also abundant within serum-rich environments and can displace nucleic acids from polyplexes through a competition for cationic binding partners, triggering a polyplex disassembly.[99] Recent work has shown promise in enhancing the transfection efficiency of polymeric gene delivery vehicles even in the presence of high proportions of serum (up to 50%). Among a wide variety of strategies, methods to combat serum instability have included stealth nanoparticle coatings, such as the incorporation of poly(ethylene glycol) (PEG),[100] fluorination,[101,102] phosphonium-containing polymer blocks,[103] and the incorporation of other hydrophilic stealth functionalities such as carbohydrate moieties.[104,105] These chemical design strategies are discussed in detail in Section 3.

2.3.2. Susceptibility to Enzymatic Degradation. An additional deleterious effect of serum exposure is the rapid degradation of nucleic acids through the action of DNAse and RNAse enzymes present in serum. Naked RNA and DNA are known to rapidly degrade via serum nucleases in vivo; thus, nonviral vectors need to prolong the half-life of DNA in circulation. Chemical modifications to the nucleobase furanose sugar or phosphate backbone have been a common method by which researchers have circumvented this barrier (Section 2.1.3).[106] Additionally, delivery via local injections minimizes the time spent in circulation (including contact with serum proteins) and thus can lead to improved gene delivery. Unfortunately, this cannot be applied universally and has only seen utility for the treatment of certain cancers.[90] The propensity for DNA/RNA to be degraded by serum nucleases can not only be attenuated by complexation via cationic delivery vehicles but also modification of these vehicles with cell-specific targeting moieties can direct the cargo to the tissue of interest.[43]

2.3.3. Immune Activation. The immune system is a formidable extracellular barrier that triggers potent non-specific defense mechanisms immediately upon introduction of polyplexes into the organism. While the innate immunity is more pronounced in the case of viral vectors (except some adeno-associated viruses),[95] nonviral vehicles and their macromolecular cargo can trigger an innate immune response as well. Surprisingly, although poly(ethylene glycol) has long held the status of a "biocompatible" material, recent reports suggest that PEG elicits an accelerated blood clearance (ABC) response, as well as a complement activation-related pseudoallergy response (CARPA). We direct readers to Section 3.4.1, which discusses in detail the immunogenic effects of PEG in gene delivery. On the one hand, activation of the innate immune system leads to the recruitment of vascular endothelial cells and platelets, inflammatory cytokine production, and macrophage cell death. On the other hand, previous exposure to exogenous material causes the adaptive immune system to generate an antigen-specific response in the form of neutralizing antibodies, which clears the polyplexes from circulation and prevents successful re-administration.[107] The innate ability for both DNA and RNA to activate the immune system upon a systemic injection in vivo can represent a substantial obstacle during gene delivery.[108] These side effects include toxicity associated with a TLR-mediated inflammatory response and cytokine release. Additionally, changes to the chemical composition of the delivery vehicle and size, aggregation state, and shape and charge of the nanoparticle can provoke varied responses from the immune system.[109] These factors necessitate careful design of nanoparticles in order to side-step anti-polyplex immune responses and ensure the safety and performance of polymeric vehicles. While immune responses to viral vectors comprise various steps (innate immunity, adaptive immunity, and

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humoral and cell-mediated responses), we restrict our discussion to those most relevant to polymers: toll-like receptors and complement activation.

TLRs are a class of membrane-bound receptor proteins that play a key role in the innate immune response. Each type of TLR receptor can recognize specific compounds common to microbial pathogens. TLRs allow immune cells, such as macrophages and dendritic cells, to mount rapid and tailored immunological responses, such as releasing inflammatory cytokines and anti-viral interferons, to attack the microbial invaders.[110] Humans have TLRs that can bind a variety of foreign nucleic acids belonging to viruses or bacteria. For example, TLR3 binds to dsRNA, TLR7/TLR8 binds to ssRNA, and TLR9 binds to ssDNA (especially if it contains unmethylated CpG motifs common to bacterial DNA).[111] This ability of TLRs to sense exogenous nucleic acids, however, can induce immune responses to therapeutic nucleic acids. While in some cases, such as vaccines or cancer therapies, immunostimulation may be desired, this effect is usually deleterious to most gene therapies.[112] For example, it is well-documented that the delivery of siRNA can elicit an excessive cytokine release and inflammation partly through TLR-dependent pathways. It has been found that common chemical modifications used to improve siRNA stability can help reduce this immune activation.[109] The choice of gene delivery vehicle can also affect the immunogenic properties of nucleic acids. In the case of siRNA complexes, some vehicles, such as many lipid-based systems, do not inhibit the siRNA from stimulating the immune system,[109] while some polymeric vehicles allow siRNA to effectively evade immune activation.[113] In addition, it is possible the vehicle itself may activate TLR-based defenses. One study found that PEI within PEI-based siRNA polyplexes acted as a TLR5 agonist, which was used to promote therapeutic anti-tumor immune activation.[114] Such findings suggest that the polymeric components of polyplexes must also undergo extensive testing to determine if they have unforeseen immunostimulatory properties.

The complement system is another component of the innate immune system that must be considered in assessing the immunostimulatory properties of gene delivery vehicles. Complement proteins in blood serum can recognize foreign material either directly or through antibody binding and, upon doing so, can initiate a proteolytic cascade within the complement system that ultimately triggers inflammation, phagocytosis of the foreign material, and rupturing of bacterial membranes. The three activation pathways of the complement system are known as the classical pathway, alternative pathway, and lectin pathway.[115] Along with avoiding hemolysis or altering blood coagulation, nonviral gene delivery vehicles must not activate the complement system to be considered hemocompatible.[116] Complement activation has been observed for liposomes, naked phosphorothioate oligonucleotides, and polyplexes, as well.[112,116,117] While naked polycations such as PLL, poly(amidoamine) (PAMAM) dendrimers, and PEI can all strongly activate the complement system, this activation is greatly diminished by charge neutralization with nucleic acid cargo.[117−119] In addition, it was found that complement activation was strongly dependent on polymer chain length, with cationic oligomers showing a weak activation.[117,118] No complement activation was seen for cyclodextrin-based cationic oligomers complexed with siRNA, which were administered to non-human primates.[120] Investigations such as this show how the careful formulation of polyplex systems can successfully avoid complement activation in vivo.

2.3.4. Challenges in Organ Targeting. Genetic cargoes are not uniformly distributed throughout the body. The liver, for instance, is a common location for nanoparticles to accumulate due to the clearance of circulating nanoassemblies by the liver sinusoidal endothelial cells, a highly vascularized structure that is a key part of the reticuloendothelial system.[121] The liver is also responsible for the metabolism and detoxification of xenobiotics as well as reabsorption of chylomicrons, which have similar dimensions to synthetic nanoparticles.[122] Therefore, targeting gene delivery vehicles to organs other than the liver represents a considerable challenge. Siegwart and coworkers engineered a strategy to reliably deliver mRNA payloads to extrahepatic organs by tuning the surface charge distributions of lipoplexes.[123] A similar strategy could be developed with polymeric vehicles to improve extrahepatic organ-specific delivery. While local or regional administration of polyplexes simplifies some of the complexities presented by organ-targeting, they are still beset with operational difficulties. For instance, skeletal muscle tissues are amenable to intramuscular injections, yet these highly vascularized tissues are often surrounded by other cell types (endothelial, epithelial, and adventitial cells), which makes DNA transfer inaccessible unless the tissue is damaged[124] or if minimally invasive polyplex injections are performed directly into the muscle.[125] Even though skeletal muscle is often injected locally or electroporated to promote transfection, smooth muscle layers and vasculature are often too thin for reliable injections.[126] Hence, electroporation or ex vivo transfection with subsequent grafting to the host is required, which can limit efficacy. Organ targeting with systemically-administered polyplexes impose stringent design constraints, requiring precise modulation of chemical and physical properties of the polymeric vehicle. Additionally, nanoparticles that extravasate from the blood must reach cells of interest through the interstitial space, which is a viscous, dynamic, and complex matrix of biomacromolecules. Larger nanoparticles (larger than 60 nm) cannot diffuse through the extracellular matrix of most tissues.[127,128] Mitragotri and coworkers have written a comprehensive review of the penetrative propensity of nanoparticles across cell and tissue barriers.[129]

Improved targeting of polyplexes can be achieved by the modification of parameters such as size, charge, or the incorporation of targeting ligands to deliver nucleic acids to remote destinations.[130] For targeting to be effective in systemically-injected polymeric vehicles, polyplexes often need to accommodate both stealth functionalities (to reduce non-specific interactions with serum proteins) and targeting ligands (for cell-specific binding).[131,132] How do we reduce non-specific interactions with proteins and yet ensure a multivalent display of specific cell-binding moieties that bind to target cells with high selectivity and affinity? We discuss methods to incorporate these functionalities in a complementary fashion in Section 3.4.

2.3.5. Cytotoxicity. Cellular toxicity is a key performance metric for gene delivery materials. For a gene delivery vehicle to be efficacious, transfection efficiency must ideally be maximized, and cytotoxicity minimized; otherwise, high cytotoxicity can result in tissue/organ damage in patients. However, highly efficient polymeric gene delivery vehicles often exhibit high cytotoxicity, a trade-off often seen with gene delivery vehicles. Thus, most gene delivery systems attempt to

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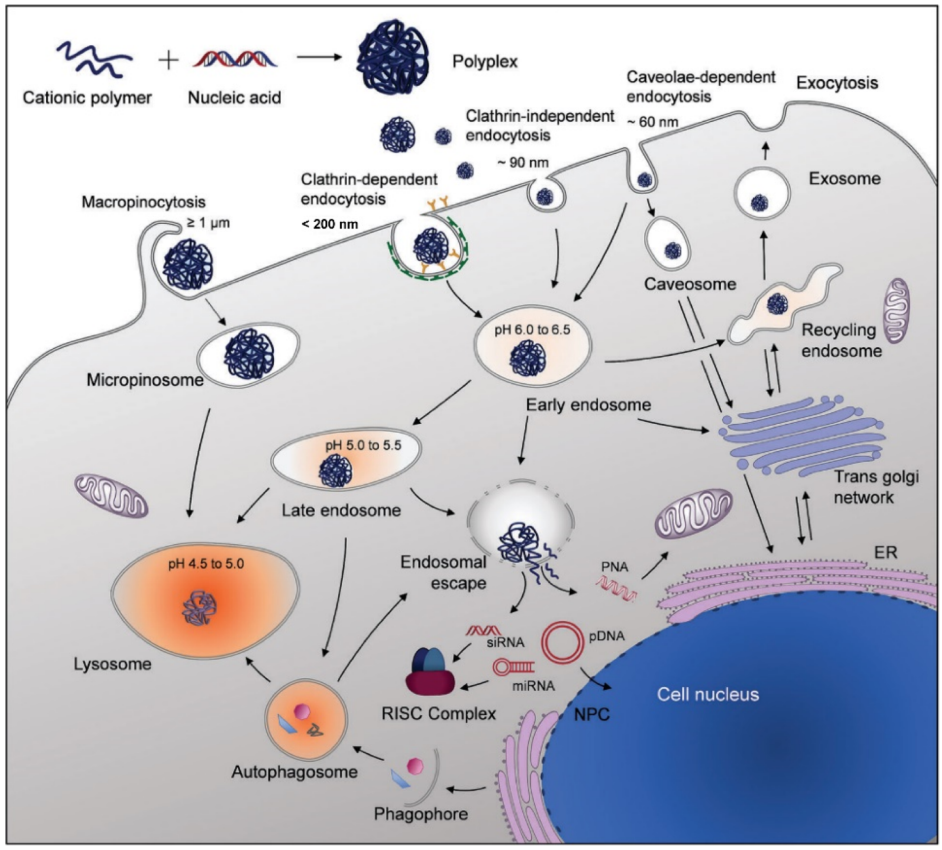

Figure 4. Possible endocytosis and intracellular trafficking pathways taken by polyplexes that represent intracellular barriers. While some cargo, such as siRNA, only needs to reach the cytoplasm, other cargo, such as pDNA, must be trafficked to the nucleus. In addition to nuclear uptake, the polymeric vehicle must shuttle the cargo during cellular binding, endocytosis, endosomal escape, intracellular transport, and unpackaging for a successful transfection of a given cell type. Reprinted with permission from ref 146. Copyright 2018 Royal Society of Chemistry.

strike a fine balance between achieving efficient transfection with limited toxicity. Note, that, since cytotoxicity is a broad term for cell death, this concept can be split into more specific categories, including apoptosis, necrosis, necroptosis, and autophagy, encompassing both programmed and unprogrammed mechanisms.[133] However, discussions about specific cytotoxicity pathways are beyond the scope of this Account.

Cationic polymers/moieties have been implicated as a major contributor to cellular toxicity, likely as a result of their interactions with negatively charged membranes and proteins.[134,135] As an example, cationic PEI-based polyplexes have been observed to exhibit varying levels of cytotoxicity, correlated with factors such as molecular weight,[136] polymer length,[137] permeation of the cellular and/or nuclear membranes,[138] mitochondrial interactions/depolarization,[139,140] and the presence of free polymer chains.[138] In particular, there is evidence that mitochondrial integrity is strongly associated with polyplex cytotoxicity mechanisms since depolarization will disrupt the redox homeostasis of the cell.[139,140] Notably, the addition of hydrophilic functionalities such as PEG[141] or carbohydrate moieties[142] to cationic polymers have been shown to ameliorate toxicity during transfection. The reduced cytotoxicity of these polymeric delivery vehicles is potentially due to their lower nuclear permeability.[143] Shorter glycopolymers have also been shown to induce cell death more slowly than longer polymers.[137] It has also been suggested that polymer degradation products may serve as a source of toxicity (e.g., through the generation of reactive oxygen species), though this hypothesized mechanism is dependent on the polymer structure.[137]

In this section, we provided snapshots of extracellular barriers, ranging from immune responses to reticuloendothelial system clearance, serum-induced aggregation, cell death, and targeting challenges. Balancing the conflicting design requirements imposed by these biological phenomena is a steep challenge that demands multifunctionality, precise design, and adaptability, all of which are hallmarks of polymeric materials.

2.4. Intracellular Barriers

Irrespective of whether they are deployed for in vivo or in vitro settings, all polymer-based gene delivery vehicles must overcome a series of intracellular barriers to successfully deliver their nucleic acid cargo. The series of barriers that must be overcome depends on the ultimate destination of the nucleic acid cargo; RNA-based cargoes only need to reach the cytoplasm to perform their therapeutic function, while plasmids and gene-editing constructs must be trafficked to and enter the nucleus.[144] The obstacles described in this section are outlined in the general order in which they may be encountered and include (1) cellular binding, (2) endocytosis, (3) endosomal escape, (4) intracellular transport, (5) unpackaging, and (6) nuclear uptake.[22,145−147] The difficulty in overcoming each of these barriers depends on many factors including the type of polymer, nucleic acid identity, therapeutic application, cell type, and pathway variations between/within cells, just to name a few (Figure 4). A large body of research has been amassed to determine how different polymeric

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vehicles overcome these barriers. PEI has been considered the gold standard in polymeric gene delivery for over two decades, presumably due to its ability to achieve endosomal escape through the proton sponge effect (which is discussed in detail in Section 2.4.3). PEI has served as the mechanistic model and the impetus for the development of next generation amine-containing polymers capable of escaping the endosome. As the prototypical polymeric transfection reagent, the field has gone to great lengths to understand the intracellular mechanisms of PEI as a model. Therefore, the following section explores intracellular barriers as studied through PEI-based models, which serve as the basis for understanding the delivery mechanisms of next-generation polymers as described in subsequent sections.[146,148] The focus on PEI shows the challenges the field faces in conclusively determining intracellular trafficking mechanisms. In addition, many of the lessons learned from these mechanistic studies of canonical polycations have been leveraged to create more sophisticated polymer-based systems with improved abilities to overcome these intracellular barriers.

2.4.1. Cellular Uptake. For polycationic gene delivery vehicles that lack a targeting moiety for a specific receptor on the cell surface, such as unfunctionalized PEI, the conventional wisdom is that these cationic polyplexes bind to the cell surface via non-specific electrostatic interactions.[46] The negatively-charged glycocalyx, which varies widely in composition and density, consists of a brushlike layer of oligosaccharides, called glycosaminoglycans, that are anchored to the cell surface as proteoglycans and glycolipids. Several viruses are known to rely on glycosaminoglycan binding for internalization and infection.[149] Polymeric vehicles that contain targeting moieties aim to increase the specificity of gene delivery systems primarily through their biodistribution. For example, specific targeting is frequently achieved through the conjugation of preexisting endogenous ligand−receptor interactions (for instance, folate/folate receptor); however, these can come with disadvantages such as non-specific binding to nontarget tissue expressing the receptor, competing circulation of endogenous ligands, or background from soluble receptors.[130]

Baldeschwieler et al. showed that proteoglycans were crucial components for the binding and uptake of the PLL-based polyplexes.[150] Studies utilizing enzymatic degradation and genetic knockout of glycosaminoglycans, among others, supported the hypothesis that PEI also relies on binding to proteoglycans, especially heparan sulfate, for internalization.[151−155] Behr et al. proposed a model of PEI polyplex uptake that is dependent on binding to the most common form of heparan sulfate proteoglycans called syndecans. Their work suggested that the syndecans cluster and condense around the bound polyplex, in a process aided by cholesterol, leading to syndecan phosphorylation and actin-dependent engulfment of the particle.[152] Several studies, however, show that the role of glycosaminoglycans in promoting PEI-based transfection is far more nuanced. For example, Durocher et al. found that different types of syndecans can have opposing roles in relation to PEI-based gene transfection, with some syndecans causing a reduction in gene expression.[156] Some studies have shown how glycosaminoglycans can be deleterious to successful transfection in part by destabilizing the polyplexes.[99,157] More recently, work by James et al. suggests that the role of heparan sulfate proteoglycans in mediating the successful transfection with PEI has less to do with promoting electrostatic binding but more through the ability of HSPGs to order lipid rafts and promote hydrophobic interactions between the lipid rafts and polyplexes.[158] As exemplified by these studies, many mechanistic underpinnings of PEI-based transfection are not fully settled due to the myriad challenges in characterizing the intracellular interactions of polyplexes. In this case, elucidating the role of glycosaminoglycans in PEI uptake is made difficult in part by the heterogeneity and variability of glycosaminoglycans on different cell types and tissues.[145]

2.4.2. Endocytosis. After it binds to the cell surface, a polyplex must be internalized by the cell in order to deliver its genetic cargo. Because of their large molecular weight and charged surfaces, polyplexes are most often internalized actively through endocytosis.[159] The success of the transfection for a particular cell type can depend on the endocytosis pathway.[160] The most well-characterized endocytosis mechanisms, which include clathrin-mediated endocytosis, caveolae-dependent endocytosis, macropinocytosis, and phagocytosis, have been the most closely examined routes in regard to gene delivery.[161] Clathrin-mediated endocytosis is the main method of internalizing extracellular and membrane components, and it is accomplished by the formation of clathrin-coated pits (60−120 nm in diameter)[162] in an actin- and dynamin-dependent manner. Caveolae are bulb-shaped invaginations (60−70 nm in diameter) within lipid rafts that contain the structural proteins cavins and caveolins.[163] The density of caveolae on the cell surface varies widely between cell types. Budding of caveolae is dynamin-dependent and a highly regulated process, which allows for the endocytosis of bound material and its trafficking along classical endocytic routes, transportation to other organelles, or even transcytosis.[164,165] Macropinocytosis is a non-specific, growth factor-induced method of endocytosis that allows for the uptake of extracellular fluid in irregular-shaped macropinosomes, ranging between 0.5 and 10 μm in size, by actin-dependent evagination and ruffling of the plasma membrane.[164] In contrast to macropinocytosis, phagocytosis (mostly employed by immune cells) requires a solid particle (>0.5 μm in size) to initiate endocytosis.[166]

The contribution of less-characterized pathways to gene delivery, including clathrin-independent pathways such as CLIC/GEEC, flotillin-dependent, Arf6-dependent, and RhoA-dependent endocytosis, is an active area of research.[159] These endocytosis routes coexist within mammalian cells, and while some cargo is internalized exclusively by one route, most cargoes utilize multiple pathways.[167]

A variety of uptake pathway-specific inhibitors are available that can assist in determining the primary endocytosis pathways utilized by polyplexes.[168,169] For example, chlorpromazine and amantadine inhibit clathrin-mediated endocytosis, filipin III and genistein inhibit caveolae-mediated endocytosis, dimethylamiloride inhibits micropinocytosis, dynasore inhibits dynamin, and cytochalasin D depolymerizes actin.[170,171] The contribution of each pathway toward the uptake for a specific polyplex formulation can be determined by treating cells with inhibitors (individually) and subsequently measuring the internalization of polyplexes (e.g., using fluorescent tags). These molecules are easy to incorporate into cell culture assays and have been utilized in numerous polyplex studies.[172−177] Unfortunately, these inhibitors are often non-specific and may not entirely block one pathway, resulting in off-target effects and potentially inducing cytotoxicity.[147,169] As an alternative to small-molecule inhibitors, methods such as RNA interference have been utilized to target and downregulate the expression of

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specific pathway proteins, such as clathrin heavy chain and caveolin-1, for the purpose of studying polyplex uptake.[178]

2.4.3. Endolysosomal Navigation and the Proton-Sponge Hypothesis. With few exceptions, the endocytosis of a given polyplex by any of the routes described above will lead to an entrapment of the polyplex in the degradative endolysosomal pathway and its exclusion from the cytoplasm. Following endocytosis and its arrival at the early endosome, polyplexes can be recycled back to the cell surface[179] or be carried forward into late endosomes (pH 6.0−4.8), which is gradually acidified by vacuolar-type H^+-adenosine triphosphatase(V-ATPase) proton pumps. Late endosomes eventually merge with lysosomes, whose acidic lumen (pH ≈ 4.5) and high hydrolase content facilitate the degradation of the cargo.[180] Endosomal entrapment is a severe bottleneck in gene delivery,[146,181] and considerable energy has been devoted to developing and modifying polymer-based systems to overcome this barrier.[46,147] Over two and half decades ago, when the ability of PEI to promote efficient transfection was discovered, it was proposed that PEI managed to avoid endosomal degradation by acting as a "proton sponge" (Figure 5A).[182,183] Ever since, the proton sponge hypothesis has served as a theoretical basis for the development of polymeric vehicles that can escape or endure endosomal entrapment. The theory states that the amino groups of PEI, which have a broad buffering capacity in the pH range of endosomes (pH 4−7),[184] act as potent "proton sponges" during the ATPase-driven acidification of endosomes. Buffering against this acidification causes a passive influx of chloride ions that causes osmotic swelling of the endosome leading to its disruption and subsequent release of the polyplex.[182] In addition, it was postulated that, during this process, the polymer itself swells, like a sponge, due to an increased charge−charge repulsion to aid in endosomal rupture.[183] This proton sponge theory has been thought to apply to other polymers that exhibit broad buffering capacities such as PAMAM[185] and poly(*N*,*N*-dimethylamino-2-ethyl methacrylate) (PDMAEMA).[186] As such, this theory is cited widely for explaining the efficacy of new transfection vectors.

The results from more than two decades of work, however, have not managed to verify the proton sponge hypothesis, since supporting evidence for the mechanism has been mixed and heavily debated. Mounting evidence is pointing toward alternative mechanisms of PEI's ability to escape endosomes, including direct membrane penetration, which has been examined in-depth in a review by Schubert et al. and subsequently summarized here (Figure 5B).[146] One key aspect of the debate regarding the proton sponge mechanism is the evidence tying buffering capacity to endosomal escape. While studies have shown that having a buffering capacity contributes to PEI's ability to promote transfection,[188−192] buffering capacity alone does not serve as the sole parameter contributing to endosomal escape and efficient transfection efficiency. Another key point of debate revolves around the accumulation of PEI in certain endolysosomal vesicles. It has been observed that PEI polyplexes are found in early endosomes and undergo acidification (pH ≈ 6), and some studies have found that PEI polyplexes are trafficked to the lysosomes,[193−196] but others do not find this colocalization.[190,197] Schubert et al. suggest that the inconsistencies in these studies of intracellular distribution are due to the complexity and differences across trafficking fates of various uptake mechanisms.[146,198] In addition, while some studies have

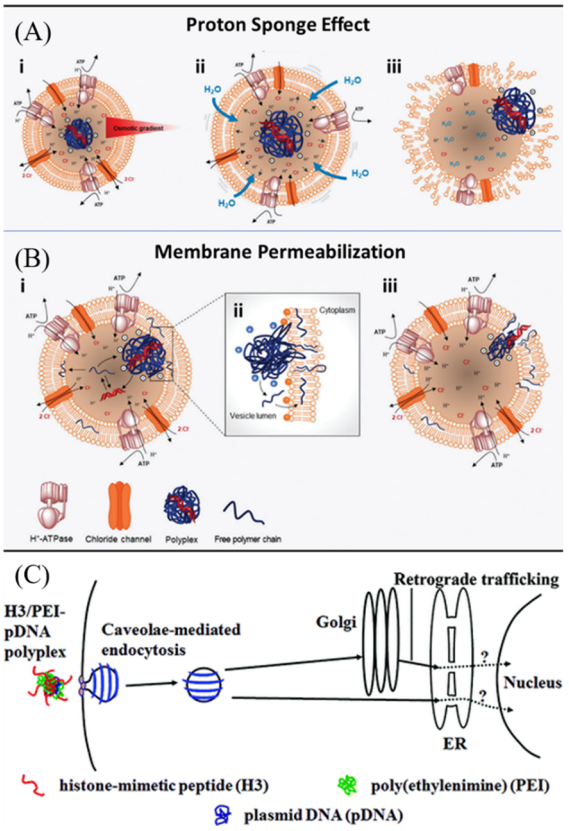

Figure 5. Possible mechanisms for the endosomal escape of PEI-based polyplexes. (A) The proton sponge hypothesis suggests the following steps: (i) Polyplexes buffer the endosome during its ATPase-driven acidification process. (ii) This causes an influx of protons and chloride ions, which increases the osmotic pressure. (iii) The pressure buildup leads to a rupture of the endosome, allowing the polyplex to escape. (B) An alternative theory of endosomal escape, the membrane permeabilization theory, suggests a slightly different mechanistic hypothesis: (i) Free PEI chains are present alongside the polyplex. (ii) These molecules intercalate into the endosomal membrane. (iii) Membrane defects and/or nano-holes are formed that allow for the escape of the polyplex without a full rupture of the endosome. Reprinted with permission from ref 146. Copyright 2018 Royal Society of Chemistry. (C) The retrograde transport hypothesis posits that a caveolar transport of PEI-based polyplexes can eliminate the need for endosomal escape. Reprinted with permission from ref 187. Copyright 2012 American Chemical Society.

observed endosomal buffering in line with the proton sponge hypothesis,[190,192,199] others have found a lack of buffering by PEI in the endolysosomal system and have questioned the mechanism.[184,189,197] Lastly, doubt has emerged that the osmotic pressures engendered by a proton sponge are enough to elicit a rupture of the endosome. While some calculations suggest that the expansion of endosomes by osmotic swelling does not meet the critical threshold[200] necessary to induce rupture,[184,201] another calculation[202] suggests rupture is possible only within a certain range of free polymer content within the endosome.

2.4.4. Alternative Hypothesis 1: Direct Membrane Permeabilization. Despite the controversy regarding its mechanism, the burst-release of PEI polyplexes from endosomes has been observed directly.[194,203,204] Interestingly, it was observed that the endosome remained intact after releasing its contents. This result suggests that the polyplex promotes

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release not by a large-scale rupture or lysis, as suggested by the proton sponge hypothesis, but through direct permeabilization of the endosomal membrane[22,204,205] (Figure 5B) in a manner not dissimilar to the membrane-lytic mechanisms underlying a viral infection.[206] This membrane permeabilization that allows for the leakage of proteins and dyes has been observed with PAMAM dendrimers[207−210] as well as PEI.[211,212] The membrane disruption caused by PEI may not only help with endosomal escape but could also be the cause of its well-known cytotoxicity.[137,139,213]

Although it has been suggested that the PEI polyplex itself causes membrane penetrations,[22,204] there is growing evidence that it is actually free polymer chains not bound to the nucleic acid cargo that is allowing for membrane penetration and endosomal release.[205,214] Many studies have demonstrated that the presence of free PEI chains are critical in promoting gene delivery.[215−218] At the N/P ratios (the ratio of ionizable amine groups to phosphate groups within nucleic acid payloads) necessary for transfection, the majority of PEI polymer chains (∼60−90%) exist as free polymers in solution[219,220] and may exist in an equilibrium between free and bound states, similar to what has been observed with PAMAM dendrimers.[221−224] Depending on its length, free PEI chains have been shown to promote release from endosomes and even assist in the translocation of genetic cargo through the nuclear membrane.[225] The solution behavior of free PEI is pH-dependent,[226] and its ability to destabilize the membrane barrier is greatly enhanced at a low pH.[227] In addition, since PEI has an exceptional ability to interact with and translocate anionic lipids,[228,229] Won et al. suggests that the preferential ability of PEI to perforate mature endosomes is due to their higher anionic lipid content.[230] They achieved direct visualization of PEI adsorption and permeabilization of model lipid vesicles consisting of a mixture of neutral and anionic lipids.[230] Lastly, Banaszak-Holl et al. quantified this permeabilization by free PEI chains in patch clamp measurements of whole HEK293 cells. They concluded that PEI caused persistent nanoscale hole formation via a detergent-like membrane disruption mechanism (known as the carpet model), and its potency was correlated to its charge density.[231] Many supporters of the membrane permeabilization mechanism of PEI hypothesize, however, that the proton sponge effect may play a synergistic role in assisting membrane permeabilization if it is indeed occurring.[204,214,232] It is also key to recognize that the endosomal escape mechanism of any given polycationic reagent may depend greatly on polymer composition and the particular cell type being transfected, which makes universal mechanistic claims exceedingly difficult.[146] In conclusion, we agree with Schubert et al. that, despite there being a popular and long-standing explanation for the ability of PEI to escape the endosome, two decades worth of research has failed to verify that the proton sponge effect is the most critical parameter for endosomal escape and improving therapeutic nucleic acid delivery performance. Evidence is mounting that endosome permeabilization by the direct interaction of PEI chains with the endosomal membrane plays a critical role in the endosomal escape of polyplexes and should play a featured role in the prevailing mechanistic theory of its functionality.

2.4.5. Alternative Hypothesis 2: Retrograde Transport Via the Golgi and the Endoplasmic Reticulum. It is commonly observed that PEI polyplexes undergo both clathrin- and caveolae-dependent endocytosis.[175,177,178,187,198,233−235] Most groups who observed this, however, concluded that caveolae-dependent endocytosis is either entirely[178,198,234] or mostly accountable[175,177,187,235] for transgene expression. Although caveolae can interact with early endosomes and partake in classical endocytic routes,[165,236] it has been shown that endocytosed caveolae are capable of bypassing lysosomal compartments and directly merge with organelles such as the Golgi and endoplasmic reticulum (ER).[163,237−240] The ER is contiguous with the inner and outer membranes of the nucleus,[241] and it has been shown that several cargoes, such as proteins, can enter the nucleus upon arrival at the ER.[242,243] In the case of some toxins and viruses, ER localization can contribute to cytoplasmic release.[244] In these cases, the efficiency of the caveolae-dependent delivery has been attributed to the ability of caveolae-dependent endocytotic vesicles to bypass the rapid degradation of the endolysosomal system. In the context of polymeric delivery, Sullivan et al. (Figure 5C) and Reineke et al. showed that PEI-pDNA complexes that underwent caveolin-dependent endocytosis could bypass endosomal degradation by retrograde transport.[187,235] It appears that retrograde transport can also offer a compelling alternative to both the proton sponge and the direct membrane permeabilization hypotheses.

Others, however, have found that fluid-phase endocytosis (such as macropinocytosis) can be important for the uptake and expression of PEI polyplexes as well.[245,246] In addition, Zhuang et al. found that PEI polyplexes can be endocytosed via a route that is clathrin-independent, caveolin-independent, dynamin-dependent, and flotillin-dependent.[154] One reason such discrepancies can arise is the preference of endocytotic routes for certain size ranges and the heterogeneous size distributions of PEI polyplexes.[233,234,247−249] The route of endocytosis is also strongly cell-type dependent,[233,234,245] and inhibition of one endocytotic route can lead to compensatory increases in others, since cells are often employing multiple endocytotic routes in tandem.[177,198] As exemplified by PEI, defining the endocytosis mechanism for any given polymeric system is a challenge due to the intricacy, codependence, and highly variable nature of endocytotic pathways within and between cells.

2.4.6. Intracellular Transport. Although escaping the endosome is an important barrier to overcome for all polymer-based gene delivery, the timing of the escape is also an important parameter to consider for some genetic cargo. In the case of large DNA cargoes, escaping from endosomes far away from the nucleus has been considered detrimental due to its poor ability to reach the nucleus via diffusion.[189] While small oligo DNAs can diffuse through the cytoplasm efficiently, diffusion of DNA greater than 250 bp is highly restricted in cytoplasm, and plasmids greater than 3000 bp appear immobile.[204,250] The actin cytoskeleton plays a significant role in inhibiting DNA motility.[251] Therefore, it is thought to be advantageous for DNA to stay within its endocytic vesicles for long enough to use it as a shuttle to the nucleus but not for so long that degradation of the genetic cargo in the endolysosomal system occurs.[146] Imaging and microtubule inhibition studies have shown that, upon endocytosis, vesicles containing polyplexes are actively transported via microtubules towards the nucleus.[199,252] PEI polyplexes were shown traveling with a linear speed of 10^{-1} $\mu m \cdot s^{-1}$ in COS-7[253] and HUH-7[254] cells and accumulated in the perinuclear space within minutes,[253] reducing the distance needed for the plasmid to reach the nucleus. Outside PEI, Reineke and coworkers tracked the filopodia-driven transport of polyplexes

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formulated from the cationic glycopolymer Glycofect and concluded that these complexes were trafficked over long distances (13 μm) along filopodial projections at a velocity from 0.003 to 0.07 $\mu m \cdot s^{-1}$.[255]

There is debate about the timing of DNA release by polycations, and it is unclear if polyplexes outside of endosomes are efficiently trafficked. There is evidence, however, that some naked plasmids (i.e., plasmids uncomplexed from the polycation) can utilize intracellular machinery in the cytoplasm to complete the race to the nucleus and allow for nuclear uptake. Plasmids have been shown to be actively transported on microtubules, along with actin to a lesser degree,[256] by recruiting molecular motors, transcription factors, and importins to facilitate movement.[257] This recruitment, and subsequent transport, is sequence-specific.[258] Dean et al. showed that plasmids containing binding sites for cyclic adenosine 3′,5′-monophosphate response-element binding protein, present in the cytomegalovirus promoter, significantly increased microtubule transport rates and nuclear accumulation of the plasmid.[259] Interestingly, stabilizing microtubules via acetylation, either with inhibitors or mechanical manipulation, can greatly increase rates of nuclear localization and improve gene delivery.[259−261]

2.4.7. Unpackaging. Although it is not entirely clear what stage in the transfection process is optimal for the unpackaging of the nucleic acid cargo, the preferred location/time of this occurrence likely varies heavily on the type of polymer, cells, pathway utilized, and nucleic acid type. It is generally agreed, however, that unpackaging must occur at some point to allow for the nucleic acid to perform its ultimate function. A fine balance must be achieved so that the polymer properly protects the nucleic acid from degradation in the extracellular and intracellular space while releasing it at the optimal time and place.[262] Premature release in the degradative endolysosomal system[263] or intracellular space can lead to degradation of the cargo due to nuclease activity. Naked plasmid DNA has a half-life of ∼50−90 min in the cytoplasm of HeLa and COS cells.[264] For this reason, it is suggested that polyplexes should be programmed to release DNA near the nucleus or inside the nucleus.[201] Simple parameters of the polycation can be tuned to achieve the right balance of protection and release including the polymer length,[265,266] charge density,[267,268] and structural rigidity.[269,270] The release performance can also be improved with the incorporation of chemical moieties that allow for intracellular degradation of the polymer.[262,271,272]

While great progress has been made in "smart" stimuli-responsive polymers (described in Section 3.6), it is still valuable to understand how and to what degree materials like PLL or PEI manage to release their cargo. Lauffenburger et al. found that PLL polyplexes could reach the nucleus intact but were unable to unpack (or unpackage) their cargo to allow for gene expression.[265] Others have also attributed PLL's poor transfection efficiencies to its inability to unpack nucleic acid cargo.[273−276] Chloroquine, a lysosomotropic antimalarial used widely in gene delivery,[181,277,278] has been commonly used in conjunction with PLL to improve its transfection properties.[189,279,280] Chloroquine's mode of action is usually attributed to its ability to promote endosomal escape,[181] but several studies have shown that chloroquine can improve transfection efficiencies of strong-binding polycations, such as PLL, by competitively binding and releasing the nucleic acid cargo.[279,281] In contrast, PEI unpackages much more efficiently than PLL[273] and does not require chloroquine for efficient transfection.[190] Studies have shown that PEI polyplexes can relinquish DNA cargo in the presence of competitive polyanions present in the cellular environment including glycosaminoglycans,[275,276] RNA,[282] and cytosolic proteins.[283] It is unclear, however, whether competitive binding causes unpackaging in the cells, and if so, what macromolecule is ultimately responsible.[146,232] The intracellular location of polyplex unpackaging is also unclear. Chen at al. used fluorescence resonance energy transfer to quantify PEI unpacking kinetics in the endolysosome, cytoplasmic, and nuclear compartments and found that the unpackaging of PEI begins in the endo/lysosome and continues at a similar rate in the cytosol.[284] While others have also observed PEI unpackaging in the cytosol,[285] several others have observed intact polyplexes in the nuclei of cells (Section 2.4.8) and witnessed unpackaging occurring after nuclear uptake.[273] It is not clear whether unpackaging before or after nuclear uptake is optimal for transcription and to what degree the polyplex must be unpackaged. Surprisingly, Fajac et al. showed that transcription of plasmid can still occur within loosely bound PEI polyplexes (N/P = 5−15), and was only inhibited when the DNA was fully compacted (N/P > 20).[286]

Importantly, this work suggests that, on the one hand, that a complete dissociation of the polycation from the nucleic acid cargo may not be required for an efficient transgene expression. On the other hand, Pack and coworkers have reported a 58-fold increase in delivery efficiency, merely by weakening PEI-DNA interactions through acetylation of primary amines within PEI. Despite significant losses in buffering capacity caused by acetylating up to 57% of primary amines, they still observed polyplex unpackaging within HEK293 cells via fluorescence resonance energy transfer.[268] Therefore, the increased buffering capacity of other amino-containing polymeric reagents does not necessarily correlate to improvement in transgene delivery, and a balance between DNA-polymer binding and buffer capacity must be engineered.[287−290]

2.4.8. Nuclear Membrane Penetration and Active Nuclear Transport. The nucleus of the cell is contained by a phospholipid bilayer envelope that consists of an outer membrane, which is continuous with the endoplasmic reticulum, and an inner membrane, which encloses the nucleoplasm. The inner and outer membranes are separated by the perinuclear space and are fused at many sites by proteinaceous pores, called nuclear pore complexes (NPCs).[291] Nuclear pore complexes are large macromolecular assemblies (120 MDa) that are constructed from multiple copies of ∼30 proteins called nucleoporins.[292] NPCs control the bidirectional transportation of cargo, such as proteins and mRNA, in and out of the nucleus in a highly selective manner. While small molecules and ions can passively diffuse through the 9 nm pores of NPCs, larger cargo (up to 39 nm in diameter) requires active transportation through the nuclear pore complex.[293] Large proteins bound for the nucleus, for example, have small peptide tags called nuclear localization signals (NLS) that recruit nuclear shuttle proteins, called karyopherins, which shuttle the cargo through the nuclear pore complex.[294] Although there are many types of NLS tags, the prototypical NLS is the monopartite NLS derived from the SV40 large T antigen NLS containing the lysine-rich sequence PKKKRKV.[295] Different types of NLS signals can recruit a variety of karyopherins, including importin α, importin β, and exportins, that are used to shuttle different macromolecular

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cargoes. Most commonly, an NLS will bind to the importin α-subunit of an importin α/β dimer or directly to the importin β, which will then translocate the complex through the nuclear pore complex.[294] The release of the cargo is mediated by the Ras-related small GTPase Ran, which regulates the directionality of cargo transport into and out of the nucleus.[296]

While viruses have evolved the ability to harness the nuclear import machinery to transfer genetic cargo into the nucleus,[297,298] polyplex-based systems have been shown to be severely hampered by the nuclear membrane barrier.[299] Therefore, polyplexes greatly benefit from the breakdown of the nuclear membrane that occurs during the cell division. PEI-based polyplexes show a 30- to 500-fold increase in transfection efficiency when introduced to cells shortly before cell division (G2/S vs G1 phase),[300] which can be achieved chemically via a double thymidine block synchronization strategy, among others.[301−303] The nuclear barrier of non-dividing cells is more persistent, however, since their nuclear membrane does not break down, and the transport of DNA through the nuclear pore complex is necessary.[304] The uptake of DNA, both plasmid and oligos, through the nuclear pore complex is energy-dependent and highly dependent on the cargo size.[305,306] Wolff et al. showed that the size limit for passive diffusion of dsDNA into the nucleus was between 200 and 310 bp, while DNA between 310 and 1500 bp required an active transport.[307] The nuclear uptake of a 900 bp DNA cassette was improved via a covalent attachment of the SV40 T antigen NLS, a strategy also employed by Behr et al. to improve the transgene expression of an end-capped DNA reporter construct.[308] The covalent[309,310] and noncovalent attachment[311,312] of NLS peptides to plasmids has been employed with mixed results.[313] Interestingly, researchers have found success through the use of nuclear proteins, such as high-mobility-group proteins and histones, as gene delivery vectors themselves, since these cationic proteins are trafficked to the nucleus and naturally compact DNA.[314,315] In addition, the glycosylations of vectors and plasmids have also been used to improve nuclear uptake via a glyco-dependent mechanism involving nuclear lectins.[316] Figure 6 summarizes several strategies for increasing nuclear uptake.

It has been found, however, that naked plasmid DNA itself can promote active nuclear uptake by the virtue of having the correct sequence.[318] DNA nuclear targeting sequences are promoter regions of DNA that bind transcription factors in a sequence-dependent manner. Of a handful of DNA nuclear targeting sequences identified to be effective with all mammalian cell types, the SV40 promoter is the most well-known.[317] This 72 bp sequence binds at least 10 different transcription factors ubiquitously expressed in mammalian cells.[319] Binding of the transcription factors to the DNA recruits importins that allow for active uptake of the DNA through the nuclear pore complex.[317] On the basis of this mechanism, it would seem that any eukaryotic promoter region that can bind transcription factors should be able to promote nuclear uptake, but this is not the case.[320] Although DNA nuclear targeting sequences seem to bind a large array of proteins, a specific subset of transcription factors, importins, and chaperone proteins are necessary to promote nuclear uptake,[321−323] which not all promoters may recruit or utilize.[317] Another well-established DNA nuclear targeting sequence includes the binding site for the nuclear factor $\kappa\beta$ (NF$\kappa\beta$) transcription factor, which is induced through stimuli such as the addition of tumor necrosis factor-α (TNF-α).[324,325]

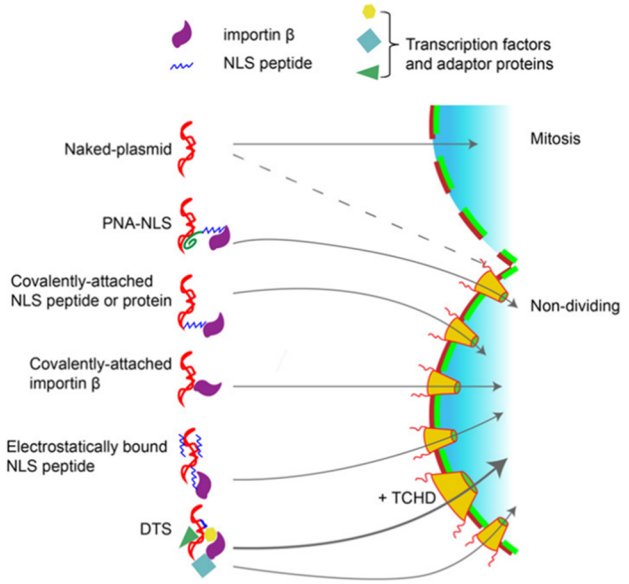

Figure 6. Examples of strategies employed for increasing nuclear uptake of plasmids. The expression of plasmids in dividing cells is far higher than in non-mitotic cells due to the breakdown of nuclear membrane, which allows for enhanced nuclear uptake. To increase the nuclear transport of non-dividing cells, an attachment of nuclear localization signals (NLS) or inclusion of DNA nuclear targeting sequences (DTS) are employed to harness importins to allow for a shuttling of the plasmid through the nuclear pore complex. Reprinted from ref 317 with attribution under the Creative Commons Attribution License 4.0 (CC BY).

In addition, glucocorticoid receptor binding sites have also been used to promote the nuclear uptake of DNA.[326,327] The introduction of glucocorticoids, such as dexamethasone, induces a conformational change in the glucocorticoid receptor, which promotes its transport into the nucleus.[299] Dexamethasone dilates the nuclear pores, which can also promote the nuclear uptake of large plasmid DNA and increase the transfection efficiency.[302,328,329]

For researchers endeavoring to improve the nuclear uptake of DNA cargo with polymer-based vehicles, it would be helpful to understand what levels of nuclear uptake are typically achieved with standard PEI-based transfections. Using quantitative polymerase chain reaction measurements, Szoka et al. detected as few as 75 and as many as 50 000 plasmid copies (<5% of the applied dose) in the nuclei of transfected cells but found that levels above 3000 plasmids/nuclei yielded marginal returns in transgene expression.[330] According to a study of Escande et al., PEI enhances nuclear uptake compared to naked DNA. They showed that complexation of circular DNA with PEI increased nuclear uptake by 10-fold (from 0.1 to 1%) after microinjection into the cytoplasm.[331] In fact, plasmid still bound to PEI has been observed in the nucleus[273,284,325,332,333] and was typically seen 3.5−4.5 h after transfection.[273,325,333] Midoux et al. claim that entire polyplexes (70−300 nm in diameter) may pass through NPCs, which are typically exclusive of particles that size.[325] Although the mechanism of this polyplex translocation through the nucleus in non-mitotic cells is not clear,[334] work by Reineke et al. suggests that the permeabilization of the nucleus by PEI may play a role.[137,143] More work, however, is needed to fully understand the role of PEI in nuclear uptake of DNA cargo. Any endeavor to maximize the nuclear transport of a polymer-

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based vehicle must consider many variables including: differences in cell types, stages of division, pathways utilized in uptake, the timing and location of unpackaging, and transport requirements for each type of nucleic acid cargo.

We conclude our discussion of biological concepts pertinent to gene delivery with a few directions for future research. We emphasize the need to exploit advances in intracellular imaging. For instance, light sheet fluorescence microscopy[335] can visualize polyplex trajectories within live cells as well as model organisms such as the zebrafish. This way, the intracellular polyplex distribution among different organelles can be acquired with a high spatiotemporal resolution through live cell imaging instead of fixed specimens, shedding light on polyplex itineraries within cells and animals. We also note that insufficient attention has been devoted to measuring the immunogenicity of polyplex delivered through in vivo modalities. While a histological examination of tissue samples is growing more prevalent, we also believe that characterizing the expression of pro-inflammatory and anti-inflammatory markers induced by a polyplex administration will be illuminating. Overall, the application of more sophisticated biological characterization techniques can resolve several enigmas that still confound the elucidation of polymeric gene delivery mechanisms.

3. CHEMICAL DESIGN OF POLYMERIC CATIONIC VECTORS

The promise of precise molecular engineering of polymeric materials is often cited as the main advantage to using them in many fields but particularly in nonviral gene delivery. It is no surprise that, as the field of polymer synthesis continues to advance, more diverse polymeric vectors are reported for their potential in gene therapy applications. The constant invention and refinement of new polymerization techniques coupled with the synthesis of novel functional monomers continues to expand the ever-growing catalogue of synthetic and semisynthetic macromolecules available. The introduction of reversible deactivation radical polymerizations in the early 2000s has permitted the synthesis of previously inaccessible well-controlled polymers that incorporate a larger variety of chemically interesting monomers. Techniques such as reversible addition−fragmentation chain-transfer (RAFT) polymerization,[336−338] nitroxide-mediated polymerization (NMP),[339,340] and atom transfer radical polymerization (ATRP)[341,342] allow the synthesis of polymers with tailored molecular weights and low molecular weight dispersity, while using previously inaccessible monomers,[343] initiation pathways,[343] and biologically friendly solvents.[344−346] These techniques reduce the termination events present in conventional free radical polymerization, granting polymeric molecules in which tailored end groups are incorporated in most of their chains. Apart from the opportunities to incorporating beneficial end groups (e.g., targeting moieties for cell-specific gene delivery) this level of end group control also allows for the synthesis of controlled block copolymers through these techniques. In gene delivery, these versatile and robust polymerization methods allow for the incorporation of cationic, hydrophilic, hydrophobic, and targeting functional groups as monomers or end groups.[347] In addition to the surge in controlled radical polymerization techniques, other polymerization methods continue to be developed for the synthesis of nucleic acid delivery vectors. For instance, polymerization methods using click chemistry,[348,349] azide−alkyne cycloaddition,[350] anionic polymerization,[351] cationic polymerization,[352] and ring-opening polymerization[353] have also been reported for the synthesis of polymeric vectors.

The tenet of polymeric gene delivery design is the incorporation of positive charges distributed along the macromolecular structure. These charges are responsible for the polyelectrolyte complexation of polycations and negatively charged nucleic acids into polyplexes; it has been proposed that the favorable entropic changes due to the release of counterions from the polymer and nucleic acid chains are the driving force for this complexation.[354,355] Paradoxically, the positive charges that allow complexation are also responsible for some of the cytotoxicity concerns that prevent a widespread use of polymeric vectors.[134,135] Several chemical strategies have been employed to mitigate some of the inherent drawbacks of polymeric cations and enhance their delivery efficiency: (1) engineer the type of charge groups used for polycation synthesis, (2) modulate the polymer architecture and molecular weight, and (3) tailor the polycation chemical composition through the introduction of hydrophobic, hydrophilic, or stimuli-responsive moieties (Figure 7).

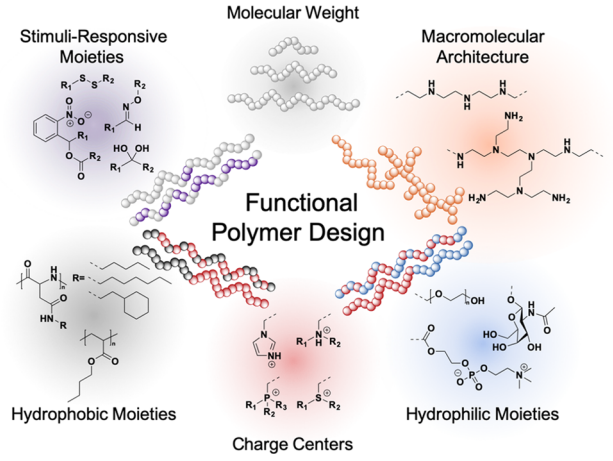

Figure 7. Schematic summary of the factors considered during the design of functional polycations with tailored properties for the delivery of therapeutic nucleic acids.

A large breadth of literature has been dedicated to studying how modifying each of these aspects affects the biological processes involved in gene delivery and ultimately how they affect the transfection performance. It remains challenging to ascertain how effectively gene delivery vehicles can be translated across diverse cell types. This section is focused on describing classic and novel polycations with a variety of architectures and compositions that are used for gene delivery while highlighting how their specific molecular design affects their performance as gene delivery vectors.

3.1. Polymer Architecture

Polymers that are used for nucleic acid delivery are chemically and structurally diverse, and herein we describe the fundamental terms that define these structures. Polymers are macromolecules that are defined chemically and topologically by their composition (i.e., the type and number of (co)-monomers they contain) and their architecture (i.e., the spatial arrangement in which those monomeric units are linked together to form the polymer chains) (Figure 8). Homopolymers incorporate only one type of monomer, while

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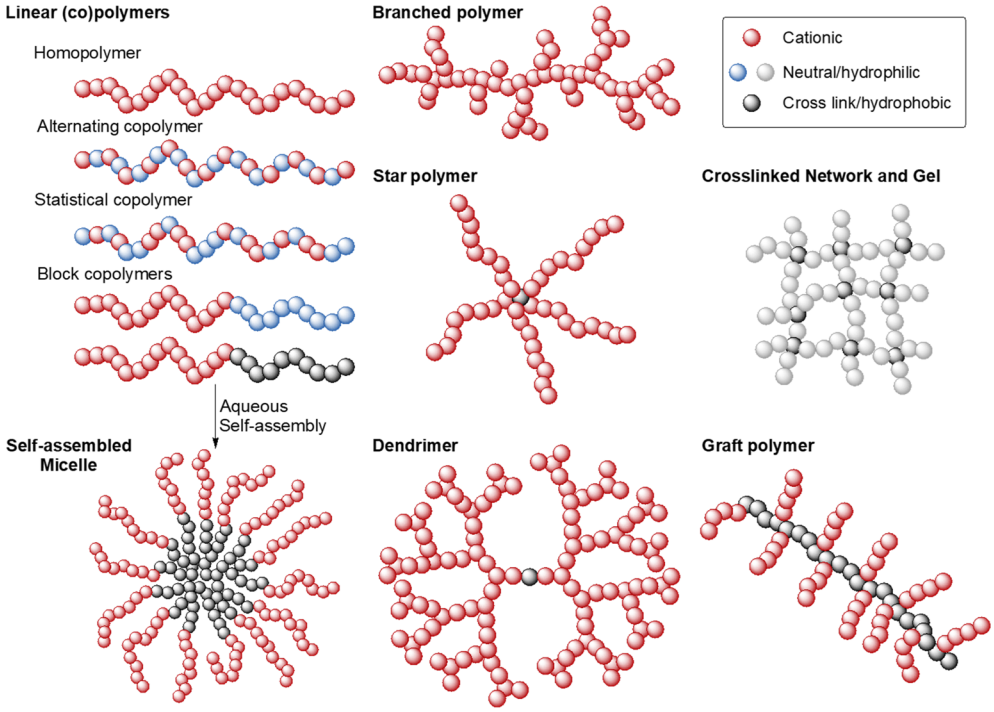

Figure 8. Typical polymer architectures and self-assembled structures are defined based on the monomer identity and spatial arrangement (cartoon to display orientation in space; size not drawn to scale).

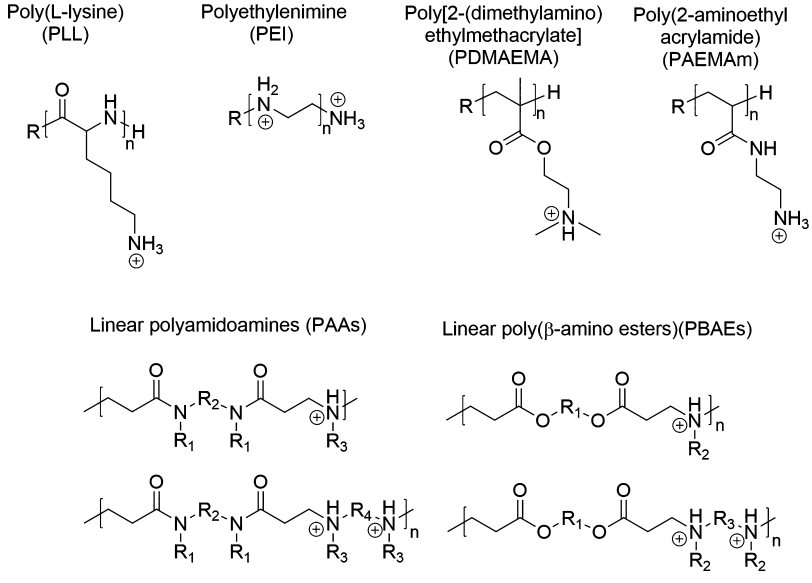

Figure 9. Chemical structures of common linear cationic polymers used as vectors for gene therapy.

macromolecules with two or more monomer types result in statistical, alternating, gradient, or block copolymers. Statistical copolymers incorporate the different repeating units along the polymeric structure with an organization that reflects their reactivity. Alternating copolymers are a specific case of statistical copolymers that incorporate two types of monomeric units in an alternating pattern. Finally, block copolymers display defined segments, or "blocks", that comprise only one type of monomeric unit. In terms of architecture, linear polymers are composed of monomers bound only to two other monomers to form the polymer chains. As highlighted in Figure 8, monomers and crosslinkers with the ability to be chemically bound to more than two monomers enables the synthesis of macromolecules with radiating chains, resulting in (co)polymers with dendrimer, branched, star, and graft architectures, as well as polymeric networks and gels. Besides the topologies accessible through covalently linking monomers in different spatial arrangements, other topologies can be created via supramolecular assembly of macromolecules. For example, amphiphilic copolymers (i.e., polymers that contain hydrophilic and hydrophobic monomers) can self-assemble into structures such as micelles, worms, and vesicles (Figure 8).

3.1.1. Linear. Linear polycations are the most commonly studied polymeric nucleic acid delivery vehicles.[356] PEI, PLL, PDMAEMA, poly 2-aminoethylmethacrylamide (PAEMA), poly(amidoamines) (PAAs),[357] and poly(β-amino esters)

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(PBAEs)[358] have all been widely explored as linear polycations for the delivery of various payloads (Figure 9).[359,360] PEI and PLL are common commercially available "off-the-shelf" materials that contain amino groups, which can be protonated at physiological pH.[49] Because of their high availability, these materials were among the earliest structures explored by researchers in the field.[182,361] These structures are often used as positive controls and have been widely chemically modified to optimize the performance of a number of specific applications (vide infra). Linear PEI derivatives are marketed as jetPEI® by Polyplus-transfection® SA for both in vitro transfection reagents and in vivo applications. Inspired by the chemistry and performance of these structures, other common systems have been created via radical polymerization routes to house pendant amine structures. For example, PDMAEMA is readily synthesized to create several homopolymer and copolymer architectures at different lengths that have been extensively explored as polycationic vectors. The tertiary amino groups in PDMAEMA have pK_a values of ∼7.5, indicating that they are only partially protonated at physiological pH (7.4) and ionic strength (150 mM).[360,362]

Linear polycations exhibit structural differences such as (i) cation identity (e.g., primary amines in PLL, secondary amines in PEI, and tertiary amines in PDMAEMA), (ii) cation position (e.g., along the backbone in PAAs and PEI versus pendant in PLL and PDMAEMA), and (iii) cationic density (i.e., nitrogen-to-carbon atomic ratios). These structures, however, all share a distinctive feature when used as delivery vehicles: polyplex formulations based on these polymers exhibit moderate-to-high transfection efficiencies in vitro depending on the cell type and particularly at high molecular weights and formulation ratios (see Sections 3.2 and 6.3 for a detailed discussion). This is attributable to their strong binding and protection of the nucleic acid payloads and their ability to interact with the cellular membrane. While this non-specific interaction with cell membranes can be beneficial to performance, unfortunately, it can elicit a high cell toxicity (half-maximal inhibitory concentration (IC_{50}) values on the order of 10 of $\mu g \cdot mL^{-1}$).[49,362] To overcome the delivery hurdles of linear polycations, several strategies such as the evaluation of other architectures (Sections 3.1.2−3.1.4), tailoring of the polycation molecular weight (Section 3.2), introduction of different functional groups such as alternative charged centers (Section 3.3), hydrophilic (Section 3.4) and hydrophobic moieties (Section 3.5), and stimuli-responsive moieties (Section 3.6), have been explored and are further discussed below.

PAAs and PBAEs represent a somewhat different class of linear polycations.[357,358,363] These polymers are synthesized via the Aza-Michael addition of primary or (bis)secondary amines to multifunctional acrylamides (for PAAs) and acrylates for (PBAEs). Their uniqueness arises from their modular syntheses. A plethora of different functional groups, contained in the amine or acrylate/acrylamide monomers, can be incorporated seamlessly into the polycationic structure (see the R_1−R_4 substituents in Figure 9). The large number of monomers available for the synthesis of PBAEs have afforded more than 2000 PBAEs that have been explored as gene delivery vectors.[358,364] In contrast to PAAs, PBAEs contain degradable ester groups along their polymer backbone, which can contribute to the cargo release. Also, because of the modularity of PAAs and PBAEs, modifications to lower the cytotoxicity of their formulations, such as the introduction of hydrophilic moieties and the modification of the polymer end groups, can be easily achieved. The synthesis, properties, and use of these highly modular polycations in different biomedical applications, including gene delivery, have been recently reviewed.[232,357,358,363]

Linear block copolymers that link polycationic homopolymers with non-ionic hydrophilic blocks that condense nucleic acids into nanometric polyplexes are sometimes called polyion complex (PIC) micelles. These nanometric polyplexes formed by electrostatic complexation (rather than by amphiphilic self-assembly, see Section 3.5.2) place the nucleic acid cargo in the assembly core and provide a hydrophilic protective corona. PEG, as well as hydrophilic acrylamide, acrylate, and methacrylate polymers have been used as the hydrophilic coating. PICs were first reported in 1996, where mixtures of PEG-*b*-PLL diblock copolymers and ASOs formed relatively monodisperse aggregates.[365] Since then, PICs have been used as delivery vehicles for DNA,[366−369] siRNA,[91,370−382] ASOs,[376,383,384] ssRNA,[382] antisense ODNs,[384] and mRNA.[385] PICs, their formation, and applications in gene delivery have been summarized in recent reviews.[386−388]

As alternatives to conventional PIC micelles (where the hydrophilic block is covalently bound to the polycations), Kataoka and coworkers have shown that PEGylated antisense ODNs[384] or siRNA[389] form similar PICs when mixed with PLL homopolymers. The addition of targeting moieties to PICs to enhance their performance and provide cell-specific delivery has also been explored. PICs have been functionalized with cRGD peptides,[370,371,379,381] antibodies to target pancreatic cells,[377] lactose groups for enhanced delivery to HuH-7 cells,[384,389] and glucose groups for a systemic delivery of ODNs to the brain.[390]

In addition, the transfection efficiency of PIC micelles can be improved by the introduction of stimuli-responsive properties (Section 3.6).[91,378,380,391−393] For instance, Belamie et al. reported siRNA delivery to mesenchymal stem cells with endosomal pH-triggered release. Simultaneous complexation of siRNA with either PLL or PEI homopolymer polycations and PEG-*b*-poly(methacrylic acid) (PMAA) diblock copolymers formed tripartite PIC micelles that disassemble upon protonation of PMAA at lysosomal pH conditions (Figure 10A).[91] In another example, the complexation of pDNA with a PEG-*b*-poly{*N*-[*N*′-(2-aminoethyl)-2-aminoethyl]-aspartamide} (PEG-*b*-P[Asp(DET)]) diblock copolymer, synthesized by coupling the blocks through a disulfide group, afforded PIC micelles with intracellularly cleavable PEG coronas. (Figure 10B).[391] Triblock copolymers with thermoresponsive properties have also been employed in the formulation of PIC micelles. Miyata et al. showed the complexation of ASOs with a triblock terpolymer containing poly(2-ethyl-2-oxazoline) (PEtOx), poly(2-*n*-propyl-2-oxazoline) (PnPrOx), and PLL, containing a PnPrOx midblock that exhibits a lower critical solution temperature. Triblock micelles were able to outperform diblock micelles that did not contain the PnPrOx midblock, when used as a serum-stable delivery agent for a cancer therapeutic.[383] The presence of the thermoresponsive PnPrOx midblock prevented nucleic acid degradation by nucleases and polyanion exchange with glycosaminoglycans (GAGs) at physiological temperature.[392]

Polyion complex micelles offer a simple method to introduce hydrophilic coatings into polyplexes (a concept that is further explored in Section 3.4). Their chemical versatility has been demonstrated through the incorporation of targeting, cross-

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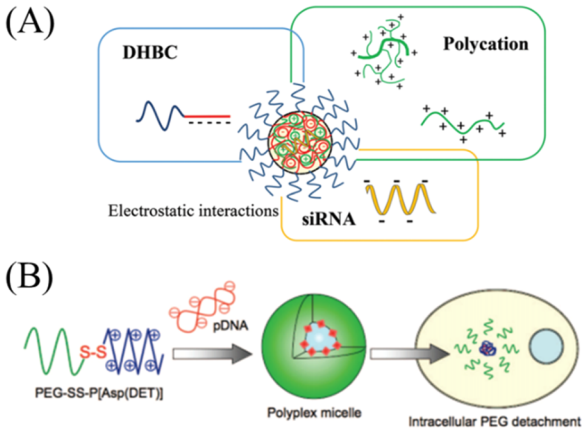

Figure 10. (A) PIC micelles with acid-induced disassembly of their cores. Reprinted with permission from ref 91. Copyright 2017 Royal Society of Chemistry. (B) PIC micelles based on PEG-ss-P[Asp-(DET)] degradable diblock copolymers undergo PEG cleavage in the reducing intracellular environment. Reprinted with permission from ref 391. Copyright 2008 American Chemical Society.

linking, and stimuli-responsive moieties that have allowed them to be used for the delivery of many therapeutic nucleic acids.

3.1.2. Branched (co)Polymers and Dendrimers. Branched polycations having secondary polymer chains budding from a primary polymer backbone in a tree branch-like structure are also a class of widely studied nucleic acid delivery vehicles. These polycations can be divided into branched (co)polymers and dendrimers. Branched (co)-polymers possess randomly distributed branches along their structure with broad molecular weight distributions. Dendrimers, on the other hand, are well-defined molecules with fractal branching radiating from a core. Branched PEI, branched PBAEs, as well as PLL, PAMAM, and poly-(propyleneimine) (PPI) dendrimers (Figure 11) have all been widely explored as gene delivery vectors. The use of branched (co)polymers for gene delivery presents two main advantages over dendrimers or linear polymers: (1) these polymers often incorporate different types of amine groups (with different pK_a values) within the branching points, the backbone, and the end groups, which can be protonated at varying pH values and (2) branched polymers can be easily modified and synthesized at low cost.[394,395] In general,

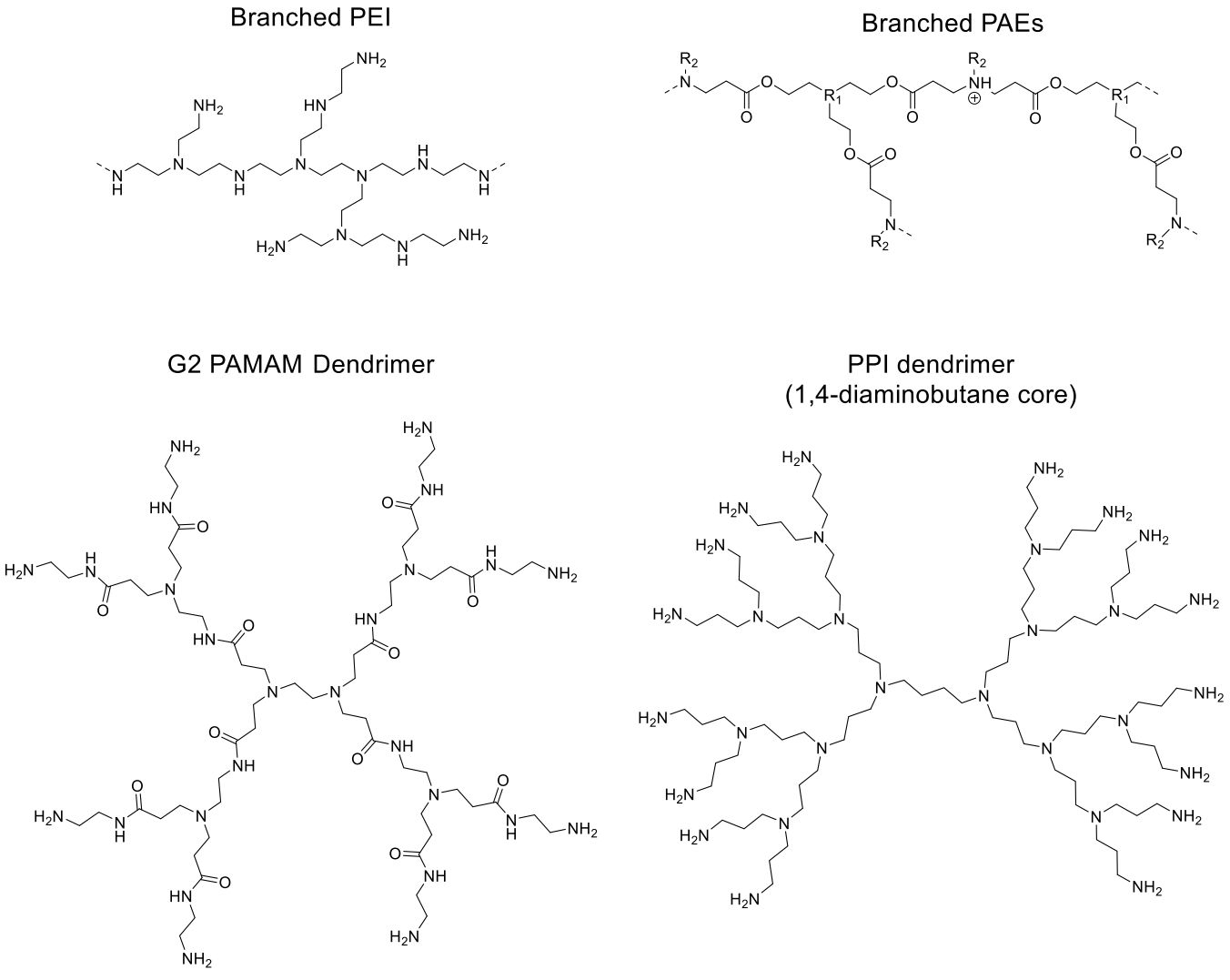

Figure 11. Chemical structure of polycationic branched polymers and dendrimers commonly used for the delivery of nucleic acids.

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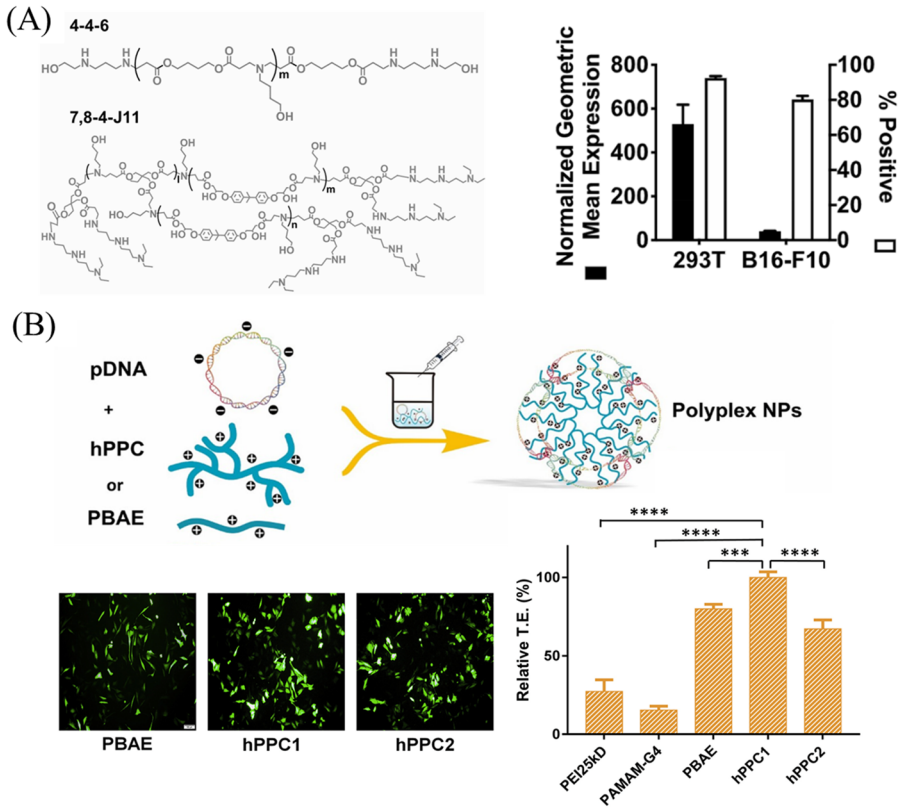

Figure 12. (A) Linear (4-4-6) and branched (7,8-4-J11) PBAEs used to transfect HEK293T and B16−F-10 cells, respectively. Adapted with permission from ref 405 with attribution under the Creative Commons Attribution License CC BY-NC-ND 4.0. (B) Polyplex formulations (N/P ratio of 75) based on linear (PBAE) or hyperbranched (hPPC1-2) poly(β-aminoesters) outperform PEI and PAMAM dendrimer controls in the transfection of HeLa cells. Reprinted with permission from ref 406. Copyright 2020 Elsevier.

branched polymers with increasing degrees of branching and molecular weights have shown enhanced cellular internalization but at the cost of higher cytotoxicities and higher variability due to larger dispersity indices.[396,397]

Branched PEI, one of the most widely studied polycation classes for gene delivery, is composed of primary, secondary, and tertiary amines. The presence of these different amine groups (with different pK_a values) endows branched PEI systems with an efficient nucleic acid binding ability and broader buffering capacity when compared to polycations based on just one class of amine cation. This architectural feature likely contributes to the high performance of branched PEI vectors.[395] Branched PEIs with high molecular weight have shown greater transfection efficiency and nucleic acid binding than those with low molecular weights. A high molecular weight, however, is correlated to a greater toxicity towards cells due to the increase in charge density on the polymer, which causes cell membrane disruption.[396,398] Branched PEIs with low molecular weights exhibit lower toxicity but are less efficient at binding DNA, and thus chemical modifications such as end-group functionalization[285,399] and incorporation of degradable linkages/crosslinks have been explored to improve the transfection efficiency of these vectors.[400−402]

Similar to their linear counterparts, branched PBAEs are synthesized via a one-pot Michael addition of primary or secondary amines to multifunctional acrylates.[358,363] Branched PBAEs effectively condense DNA, display lower cytotoxicity in comparison to PEI, and display biodegradability due to their ester linkages.[358] Recent studies showed that, compared to their linear counterparts, branched PBAEs display higher transfection efficiencies, with high molecular weight hyperbranched PBAEs displaying simultaneously a higher transfection efficiency and lower cytotoxicity.[403,404] PBAEs have been recently reported as vectors for the delivery of plasmids for gene editing therapies.[405−407] Green et al.[405] reported linear and branched PBAEs that are optimized for the transfection of HEK293T or B16F10 cells, respectively (Figure 12A). It was shown that polyplexes formulated with these PBAEs have the capacity to co-deliver two plasmids encoding Cas9 endonucleases and sgRNA, respectively, to perform either 1-cut knockout or 2-cut gene deletions. Hu and coworkers reported that polyplex formulations based on linear and hyperbranched PBAEs outperformed a 25 kDa PEI and a PAMAM G4 dendrimer control in the transfection of SiHa and HeLa cells with green fluorescence protein (GFP) encoding plasmids (Figure 12B).[406] Similar formulations were then used for the delivery of CRISPR/Cas9 encoding plasmids targeting HPV16 E7 oncogenes.

The multiple end groups of dendrimers have shown utility for their application in drug and gene delivery.[408] Dendrimers are synthesized using repetitive sequences in which each layer, called a generation, is grown in a stepwise manner from the core. This sequence guarantees a regular branched structure that is well defined. The end groups of dendrimer macromolecules for gene delivery often contain primary amines, thus presenting a highly charged corona at physiological conditions, leading to efficient nucleic acid binding and enhanced cellular internalization. The nucleophilic amine end groups allow for a further chemical modification allowing the incorporation of

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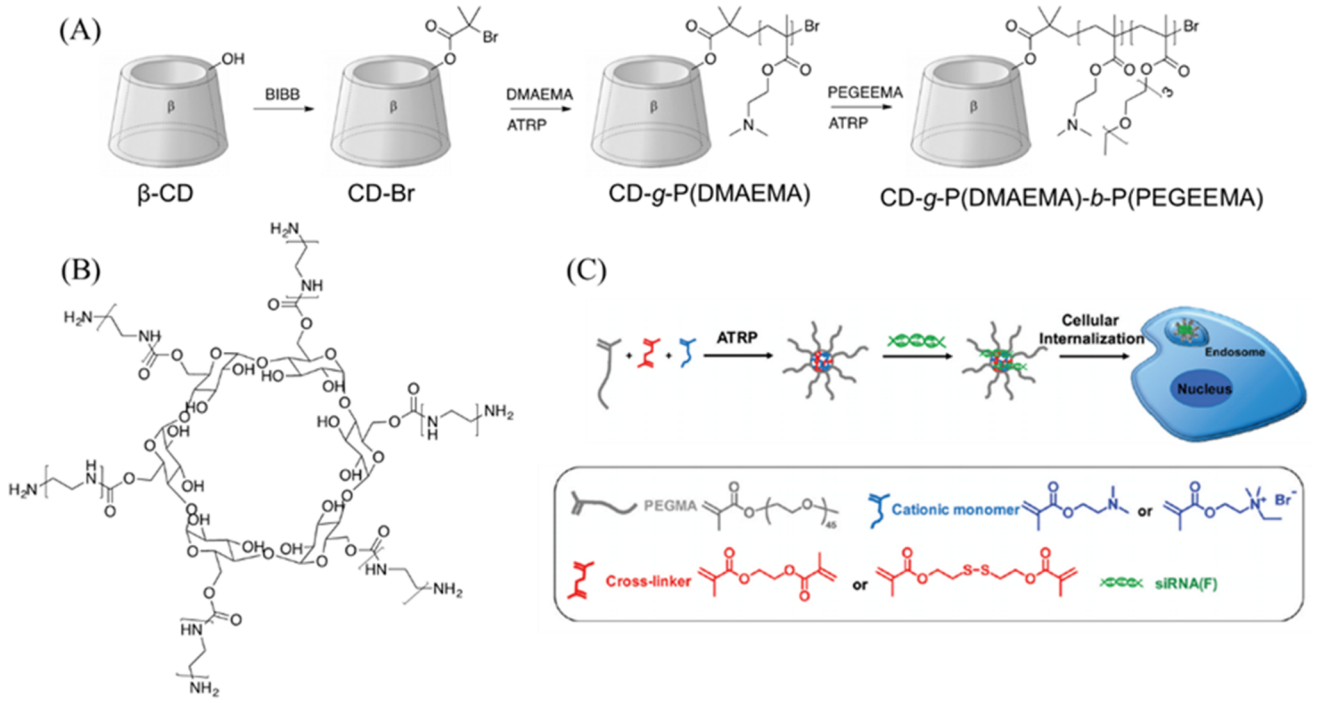

Figure 13. (A) Synthesis of CD-*g*-P(DMAEMA)-*b*-P(PEGEEMA) star polymers. Adapted with permission from ref 429. Copyright 2009 American Chemical Society. (B) Chemical structure of a α-CD-OEI star polymer. Reprinted with permission from ref 428. Copyright 2007 Elsevier. (C) Arm-first cationic cross-linked star polymers with degradable cores. Reprinted with permission from ref 430. Copyright 2011 American Chemical Society.

targeting moieties to increase specific internalization and/or addition of hydrophilic units to reduce toxicity.

PAMAM dendrimers, the most studied dendrimer for gene delivery applications, contain hydrogen-bonding amide and tertiary amine groups in their cores and display primary amine end groups as their corona. The highly charged primary amine end groups are responsible for the toxicity, and their modification has been explored as a tool to reduce toxicity,[409] increase circulation time,[410,411] or improve targeting ability.[412,413] The molecular weight, size, and number of end groups in PAMAM dendrimers grows rapidly with each generation. For instance, a Generation 3 (G3) PAMAM dendrimer weighs 5147 g·moL^{-1} and contains 24 terminal amine groups, while a G6 dendrimer weighs 43 451 g·moL^{-1} and contains 192 terminal amine groups.[185] The in vitro transfection efficiencies and toxicities of PAMAM dendrimers are highly generation-dependent and results vary depending on the type of cell line used.[414] Because of their high transfection efficiencies, intact and "activated" G6 PAMAM dendrimers marketed as SuperFect® and Polyfect®, respectively, are sold by Qiagen as transfection reagents for a broad range of cell lines including COS-7, NIH/3T3, HeLa, 293, and CHO cells. A thorough analysis on the use of PAMAM dendrimers for biomedical applications including gene transfections was recently reported by Giarolla et al.[414] PLL[415] and PPI[416−418] dendrimers have also shown promise as gene delivery vectors, especially because of their reported ability to escape the endosomes after cellular internalization. Similar to PAMAM, PLL and PPI dendrimers consist of spherelike structures decorated with primary amines that maintain a good ability to be internalized into cells after complexation with nucleic acids.

Because of their highly charged corona, dendrimer vectors show high cellular internalization, but toxicity remains a limiting factor moving forward. Overall, dendrimers are unique vectors due to their well-defined structures. Further review of the application of dendrimers for gene therapy can be found elsewhere.[419,420]

3.1.3. Star. Star polymers are a class of branched polymers in which linear polymer "arms" radiate out from a common branching point or "core". Polymer arms are synthesized through the same techniques used to synthesize linear polymers. Controlled polymerization techniques permit the synthesis of star polymers with targeted molecular weights, grafting densities, and end-group chemistries. Star polymers present increased charged density, compared to linear polymers of the same chemical composition, by covalently linking several linear arms to the core, making them an interesting synthetic platform for gene therapy. Star polymers also possess an increased number of end groups that can be chemically modified. The synthesis of PDMAEMA-based star polymers by group transfer polymerization and their applications in pDNA delivery were reported in the early 2000s.[421] Other types of cationic and hydrophilic polymers, such as oligoethylene imine (OEI), PAEMA, and poly(ethylene glycol)ethyl ether methacrylate (PEGEEMA), have also been used as arms in the synthesis of star polycations with low molecular weight dispersity.[347] Star polymers, with cationic peptide arms, showed good biocompatibility during gene delivery.[422,423] The use of α-, β-, and γ-cyclodextrin (CD) as cores in star polymers[424−427] has gained popularity due to their biocompatibility and the development of several synthetic routes that allow for the conjugation of polymers to the hydroxy groups present in CDs.

The length, composition, and number of arms in star polymers determine their properties and gene delivery efficiency, and thus synthetic strategies that allow for the control of each of these parameters have been explored. When considering cationic arm length, Reineke and coworkers

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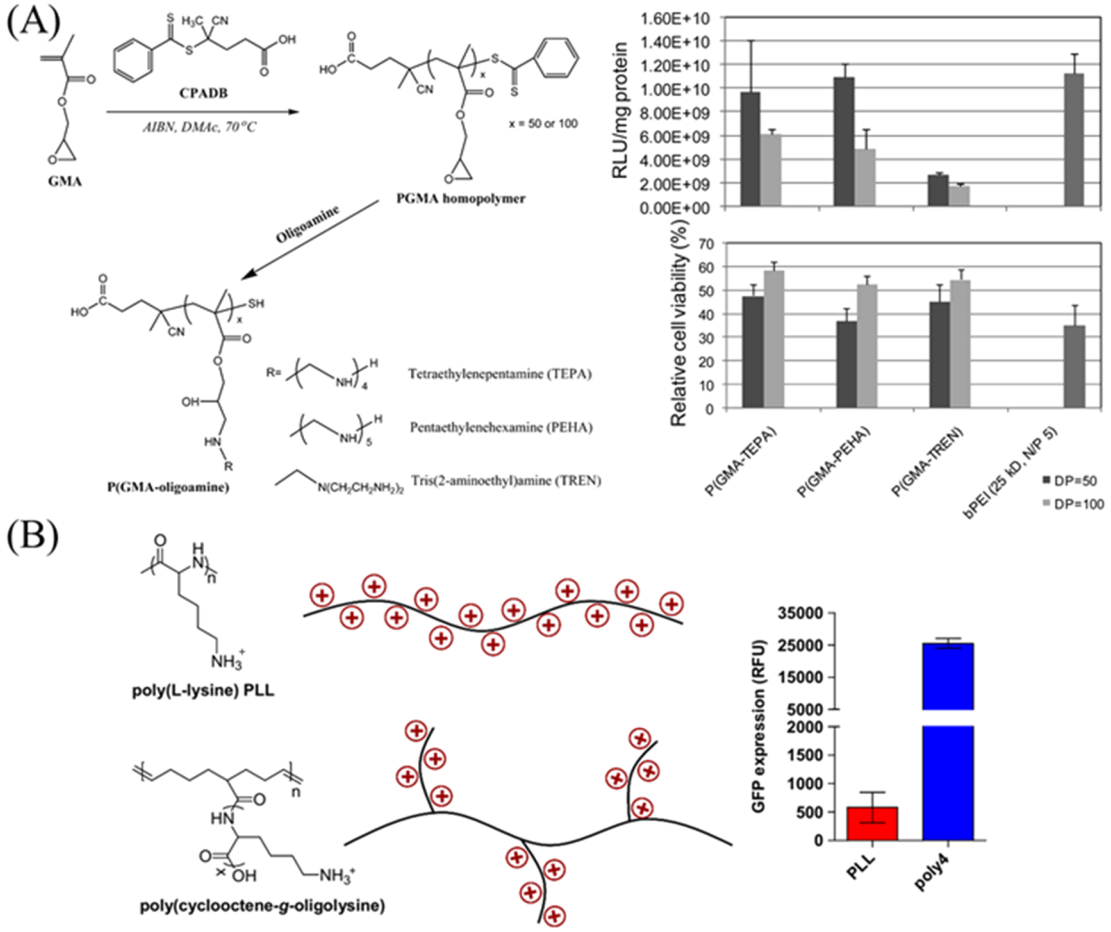

Figure 14. (A) Oligoamine-grafted PGMA (P(GMA-oligoamine)) exhibits similar cell transfection efficiencies (RLU) of HeLa cells to branched PEI, while maintaining higher cell viabilities. Reprinted with permission from ref 443. Copyright 2013 American Chemical Society. (B) Poly(cyclooctene-*g*-oligolysine) polymers showed enhanced transfection efficiencies in COS-1 cells (GFP expression) when compared to linear PLL. Reprinted with permission from ref 454. Copyright 2011 Elsevier.

synthesized a series of discrete star polycations based on a β-CD core termed "click clusters".[424] These macromolecules were synthesized through the selective functionalization of the primary alcohol groups in β-CD with azido groups and subsequent coupling with alkyne-functionalized OEI dendrons through a copper-catalyzed 1,3-dipolar cycloaddition. The OEI arms varied in length between one and five ethylene amine units, and the star polycations with arms containing four or five units showed the highest pDNA transfection efficiency in HeLa and H9c2 cells (at least 1 order of magnitude of luciferase relative luminescence units (RLU) higher than the other polycations at N/P of 20), which was comparable to controls jetPEI® and SuperFect®. This high level of transfection was achieved while maintaining low cytotoxicity (>0.8 fraction cell survival in both cell lines) compared to the poor viability seen for the controls (<30% viability for both controls in both cell lines). Similarly, Li et al. synthesized α-CD-OEI star polymers with linear and branched OEI arms containing 1−14 ethylene imine units (Figure 13B).[428] Star polymers with longer (14 ethylenimine units) branched arms revealed at least 1 order of magnitude higher transfection efficiency (luciferase expression measured as RLU) with HEK293 and Cos7 cell lines than the other analogues and a 25 kDa branched PEI control. Similar trends were observed in both the presence and absence of serum.

In terms of the composition of the arms, Neoh and coworkers reported the synthesis and transfection efficiency comparison of star copolymers that contained either PDMAEMA homopolymer arms or PDMAEMA-*b*-PEG diblock copolymer arms. To synthesize these star (co)polymers, β-CD was modified with ATRP-initiator groups. The resultant multifunctional initiators were used in the polymerization of PDMAEMA arms that were subsequently chain-extended with a PEGEEMA block (Figure 13A). When compared to linear high molecular weight PDMAEMA and PEI controls, the star polymers with PDMAEMA and block PDMAEMA-*b*-PEG arms (at N/P 20-30) displayed an approximately fivefold higher transfection efficiency with HEK293 cells. Similar luciferase transfection efficiencies with a decrease in cytotoxicity were observed in comparison to a PDMAEMA homopolymer star.[429]

Chemical modifications of star polymers allow for the improvement of their delivery. An end-group modification of star polycations to incorporate targeting ligands such as hyaluronic acid,[431] folic acid,[432,433] and adamantyl groups[434] allowed formulations that actively target specific cell receptors or tumor delivery. Polyplex formulations based on PEGylated polycationic star polymers show improved colloidal stability, decreased toxicity, and increased blood circulation times.[423,429,435,436] Additionally, the incorporation of degradable moieties such as disulfide linkages[430] (Figure 13C) and

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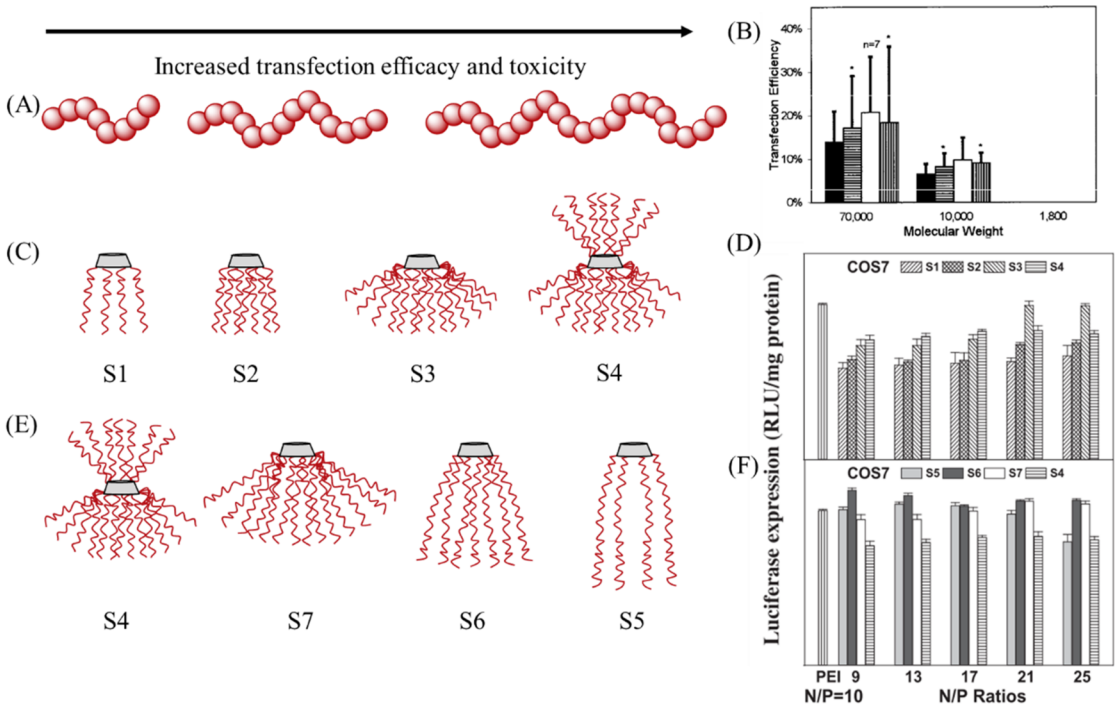

Figure 15. (A) Representation of general trend: increasing molecular weight increases transfection efficacy and toxicity. (B) Increasing the molecular weight of PEI results in higher transfection efficiency. Reprinted with permission from ref 457. Copyright 1999 John Wiley and Sons. (C) In star polymers, it is possible to increase the number of arms while keeping the molecular weight of arms consistent. (D) In the case of PDMAEMA, a higher number of arms within star polymers was found to increase transfection efficacy. (E) In another variation of star polymers, we can increase the molecular weight of arms while reducing the number of arms such that the molecular weight of the star polymer remains constant. (F) Adopting the design from (E) was also found to increase transfection efficacy. (D, F) Reprinted with permission from ref 468. Copyright 2013 Elsevier.

acid-labile functional groups[437] in the star cores provided routes for polymer degradation and nucleic acid release.

3.1.4. Graft Copolymers. Polycationic graft copolymers—also called brush- or comb-like polymers—link several cationic polymer chains (or combs) into a single macromolecule. The combs are typically short oligocations that, on their own, display poor transfection efficiencies but, when grafted to a common polymer backbone, afford macromolecules with large charge densities and enhanced delivery performance. Graft polymers with PDMAEMA,[438−440] PEI,[441] PEG-*b*-PEI,[442] oligoamines,[441,443] oligopeptide combs,[444−454] and other structures have all been explored as gene delivery vehicles. Several key features that dictate the properties and efficacy of these polymers include the type, amount, and length of the polycationic grafts.

Synthetic approaches using the grafting-to approach have been exploited, where the cationic combs are attached to preformed polymeric backbones. For instance, Pun and coworkers synthesized a library of graft polymers via a post-polymerization of poly(glycidyl methacrylate) (PGMA) homopolymers with tetraethylenepentamine (TEPA), pentaethylenehexamine (PEHA), and tris(2-aminoethyl) amine (TREN).[443] Graft homopolymers containing TEPA and PEHA combs with a degree of polymerization of 50 have been explored for the transfection of HeLa cells with DNA polyplexes at N/P ratios of 10 (Figure 14A). These structures have shown a similar performance to a control 25 kDa branched PEI; the use of degradable linkers between the backbone and the cationic grafts have been explored as a strategy to reduce the toxicity and enhanced the release of the nucleic acid cargo.[440]

A graft (co)polymer with cationic oligopeptide combs has also been synthesized via a grafting-through polymerization of oligopeptide macromonomers. Pun and coworkers have synthesized a series of vinyl-terminated cationic oligopeptide monomers that can be copolymerized with *N*-(2-hydroxypropyl)methacrylamide (HPMA) via a conventional free radical and RAFT polymerization to afford brush copolymers with pendant oligopeptide combs.[444,445,447−450] The first brush copolymer contained oligolysine (K_{11}) combs, which delivered pDNA to HeLa cells with transfection efficiencies similar to a linear PLL control yet with a lower cytotoxicity.[444] Harnessing the modularity of this synthetic approach, several iterations of these brush copolymers were synthesized aiming to improve their efficiency. Polymers with optimum oligolysine length (K_{10}),[445] incorporating neutral (glycine) and different cationic (arginine and histidine) peptides in the oligopeptide sequences, were explored.[449,452] Additionally, brush polymers with oligopeptides linked to the polymer backbone created with a degradable linker[448,451] or ligands for cell-specific delivery have also been studied.[447] Emrick and coworkers have prepared comb-peptide polymers through ring-opening metathesis polymerization (ROMP) of cyclooctene-oligopeptide macromonomers that afford comb-like cationic delivery systems (Figure 14B).[446,453,454] A pentalysine-comb cyclooctene polymer with a molecular weight of ∼30 kDa showed a more than twofold greater pDNA transfection efficiency of COS-1 cells when compared to jetPEI®, SuperFect®, and linear PLL controls.[453] The polyplex formulations based on these comb polymers showed a lower efficiency when compared to Lipofectamine 2000 (33K vs 49K relative fluorescence units) but showed lower

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cytotoxicity (99% vs 67% COS-1 cell viability). Analogous polycyclooctene polymers containing di-, tri-, tetra-, and pentalysine grafts have also been evaluated in the transfection of C_2C_{12} cells, where the tetralysine-containing comb polymer variant displayed greater GFP expression levels than the other variants.[454] Copolymerization of the tetralysine comb polymers with a cyclooctene macromonomer containing a nuclear localization signal peptide greatly increased the performance of these systems. The DNA binding ability of the tetralysine-containing comb polymers can be modulated through copolymerization with a hydrophilic zwitterionic sulfobetaine-cyclooctene monomer. To modulate association, copolymers with ∼17 mol % of the sulfobetaine monomers have shown a weaker binding affinity when complexed with DNA than the tetralysine-comb homopolymers; subsequently, this translated to a twofold increase in delivery efficiency with SCOV3 cells.[446]

Overall, in this section, we have discussed how the study of polycations with multiple architectures have been a central pillar of the field of polymer-mediated nucleic acid delivery. Initially limited to the used of "off-the-shelf" polycations, the field has exponentially grown in parallel to the development of new techniques that allow for the synthesis of macromolecules with diverse architectures. It is important to note that architecture is only one variable to improve the delivery efficiency of cationic polymers; often changes in molecular weight and polymer composition are simultaneously examined, and these strategies will be discussed further in the following sections.

3.2. Polymer Molecular Weight

Polymer molecular weight plays a key role in optimizing transgene expression and delivery, with both higher and lower molecular weight polymer vectors possessing pros and cons. The molecular weight of polymers employed for gene delivery influences two key factors related to the success of a gene delivery vehicle: transfection efficacy and cytotoxicity.[45,47,455,456] These effects have been evaluated in PEI,[457,458] PLL,[459] PDMAEMA,[460] PAEMA,[461] and other polycations. High molecular weight polymers possess improved transfection efficiencies, in part due to increased interactions with the cell membrane (Figure 15A).[186,457,462−464] However, these enhanced membrane interactions are problematic and lead to cytotoxicity, which often increases when increasing the molecular weight of polymeric delivery vehicles.[462,465,466] Alternatively, lower molecular weight polymers show a reduced cytotoxicity and dissociate more readily from DNA leading to improved cargo unpacking.[265] These trends in transfection efficacy and cytotoxicity tend to hold in the range of 1−100 kDa;[455−457,462−464] however, certain polymers display cutoff ranges where these trends no longer apply. For example, Mikos and colleagues found that PEIs of 1800, 1200, and 600 Da showed no increase in transfection efficacy compared to naked pDNA indicating that, for PEI, there is a minimum threshold molecular weight to see such trends.[457]

High molecular weight polymers exhibit higher transfection efficiencies yet increased toxicity consistently across different architectures including dendrimers,[467] stars,[468] and linear polymers. For example, Xu et al. examined the effects of molecular weight as well as arm number and length on the transfection efficiency for a series of PDMAEMA star polymers.[468] When the arm length was held constant, increasing the number of arms (which consequently increases the molecular weight of the star polymer) simultaneously improved transfection efficiency and increased toxicity towards HepG2 and COS7 cells (Figure 15B). In addition, when molecular weight was held constant, star polymers with longer but fewer arms had a higher transfection efficacy and more toxicity (Figure 15C). For example, at N/P = 9, increasing the number of arms from 4 to 21, while keeping the molecular weight constant at 50 kDa, increased cell viability over 15% but decreased luciferase expression more than 10-fold in both cell lines. It is hypothesized that stronger interactions between these longer arms and the cell membrane lead to higher cellular delivery but also to higher toxicity. These observations are like those discussed above for linear polymers.

Although the trends of molecular weight on transfection properties discussed above hold true for many structures, there are examples where no trends or contrary effects are observed.[469−473] Volonterio et al. observed that when using PAMAM dendrimers of different generations (2, 4, and 7), with higher generations having increasing molecular weights, there was no trend in pDNA transfection efficacy in HeLa cells.[469] This seemingly contradictory observation could be due to the wide range of N/P ratios studied, which were in the range of 5−75. In another example that conflicts with the prevalent trend that increasing molecular weight increases transfection efficacy and cytotoxicity, Reineke et al. synthesized a series of diblock glycopolymers containing a non-ionic hydrophilic glycopolymer block composed of 2-deoxy-2-methacrylamido glucopyranose (MAG) units, and a *N*-[3-(*N*,*N*-dimethylamino)propyl]methacrylamide (DMAPMA) cationic block.[470] They evaluated the effect of the molecular weight of each block on pDNA transfection efficiency and cytotoxicity and found that increasing the DMAPMA block molecular weight decreased the pDNA internalization and transfection efficacy yet also increased toxicity in HEPG2 cells. Interestingly, the MAG block length had no effect on the transfection efficacy or toxicity in the systems studied. The block length effects can also be dependent on the type of nucleic acid being delivered; Reineke and coworkers synthesized a series of three P(MAG)-*b*-poly(*N*-(2-aminoethyl) methacrylamide) P(MAG)-*b*-P(AEMA) diblock glycopolymers, where the degree of polymerization of the AEMA block was 21, 39, and 48, respectively.[471] They showed that, when these diblock copolymers were used to transfect HeLa cells with pDNA, polymers with a shorter AEMA block led to lower cell internalization but a higher luciferase expression. In contrast, when using these diblock copolymers as siRNA delivery vectors to induce luciferase knockdown in U-87 cells, only the polymer with the longer AEMA showed a gene knockdown statistically different from an siRNA-only control.

Overall, we have summarized the key concepts and trends that relate the molar mass of polycationic vectors to their performance. Although particular trends are observed for specific polycationic systems, the lack of a general structure-property relationship that can be applied to all polymeric vectors (or even the contradictory observations between studies on the effects of molecular weight in gene delivery) implies that molar mass will still be one of the key parameters that needs careful optimization when designing new polycationic systems. This is especially true for star, branched, graft, and self-assembled vehicles, where molecular weight is intrinsically tied to other properties, such as degree of

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branching, number of arms, number of end groups, and aggregation number.

3.3. Selection of Charged Groups

3.3.1. Nitrogenous Cations. The benchmark design for synthetic gene delivery vectors centers in the incorporation of cationic charges into macromolecules that can electrostatically bind to nucleic acids (Figure 16). Typically, these cations

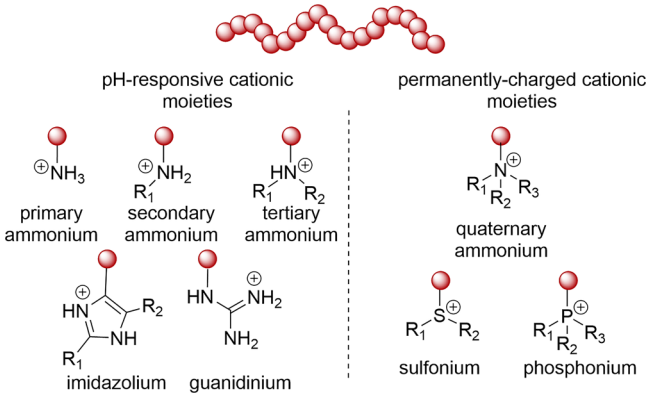

Figure 16. Structures of common cationic moieties used in gene delivery. Note that imidazolium cations can be linked to the polymer through R_1 and R_2 as well.

consist of nitrogen-based moieties incorporated into the polymer chains by direct polymerization or by post-polymerization modifications. Additionally, they can be incorporated into a variety of different repeat units based on PEI, acrylates, acrylamides, sugars, peptides, and more. Nitrogenous cations, such as ammonium (from primary to quaternary), imidazolium, and guanidinium, as well as combinations of these within the same polymer structure, are predominantly used throughout the nonviral gene delivery literature. The type of amine-based cationic center determines the pK_a of the resulting polymer, and therefore it dictates the percentage of protonated amines. Additionally, finding the "right" pK_a is often cited as a way to improve an endosomal escape through the proton sponge effect (Section 2.4).[183] For polymers containing alkyl-substituted amines, the type of amine (primary, secondary, and tertiary) does not directly dictate the gene delivery performance. For instance, Leong et al. found that the amine type surrounding a hyperbranched poly(amino)ester with a tertiary amine backbone had little effect on the transfection efficacy, cytotoxicity, or degradation rate of the gene delivery vehicle.[474] This is likely because the pK_a is dependent on both the number of substituents and the type of substituent. Furthermore, for many alkyl-substituted amines, the pK_a (~8−11) is generally too high to see significant differences between the number of amine substituents (primary, secondary, tertiary). The pK_a values of the amine groups can be lowered by further adjusting the surrounding chemical environment so that it is in the range of physiological conditions. For example, P[Asp(DET)] and poly{*N*-(*N*′-{*N*″-[*N*‴-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}-2-aminoethyl)aspartamide} (P[Asp(TEP)]) both have pK_a values of ~6, likely as a result of their closely packed amine groups.[374] PICs of the polyaspartamide analogues and siRNA displayed low toxicity and high endosomal escape, likely due to the low pK_a, which could be tuned by optimizing the length of the alkyl spacers between amines.

Beside alkylamines, there are other nitrogenous cations that have pK_a values close to physiologically relevant pH conditions. Imidazolium cations have a pK_a of ~7, depending on the functional groups surrounding the heterocycle. Pun and colleagues compared histidine and lysine as two amino acids in HPMA-*co*-oligoamino acid brush polymers.[475] It was observed that when oligohistidine, an imidazole-containing amino acid, was incorporated at high enough amounts in the statistical copolymer (>0.53 mmol histidine/gram polymer), the vector had greater transfection efficacy compared to the lysine-only derivative. Interestingly, inhibition studies showed internalization through the caveolar endocytic pathway, which does not rely on endosomal buffering capabilities as much as other pathways. This is cited as the reason why histidine incorporation only improved transfection efficacy by a maximum of three- to fivefold. Long and coworkers incorporated imidazolium into polyesters for DNA transfection to HeLa cells observing successful transfection and insignificant toxicity compared to untreated cells.[476] Additionally, quaternization of imidazole-containing polymers can be performed via post-polymerization modifications. Long et al. observed that 25% quaternization of poly(1-vinylimidazole) with 2-bromoethanol was optimal for increased pDNA binding, higher transfection, and minimal cytotoxicity.[477]

The percentage of protonated amines determines, in part, transfection efficacy. This can be modified by monomer pK_a design (as described above) or by copolymerization of cationic and non-ionic monomers. Fischer et al. compared linear PEI homopolymers and statistical copolymers containing PEI and poly(2-ethyl-2-oxazoline) (PEtOx), synthesized through hydrolysis of PEtOx for DNA delivery.[478] They observed that the density of PEI units was the most important factor in determining the ability of the polymer to bind to DNA and—consequently— transfection efficacy, rather than the total number of PEI units. They observed that higher PEI unit density led to higher efficacy. However, cytotoxicity improved with lower PEI density polymers with an equivalent number of PEI units.

Besides providing the necessary positive charge to complex DNA, other nitrogenous cations present additional benefits when incorporated in polymeric gene delivery vectors. For instance, guanidinium is an especially attractive cation due to its ability to hydrogen bond with phosphate anions and guanine, both particularly useful for nucleic acid delivery.[479−487] Pun et al. synthesized brush copolymers based on oligolysine macromonomers copolymerized with HPMA.[449] Comparing the original brush to an analogue containing guanidinylated lysine groups, it was observed that the guanidinylated analogues had improved HeLa cell transfection efficacy. Stenzel et al. observed that micelles containing zwitterionic side groups, with guanidium and carboxylate groups, had high cellular uptake and low cytotoxicity.[486] Benzimidazole is another promising nitrogenous cation. Algul and colleagues observed that small-molecule analogues of benzimidazole improved the transfection efficacy with a GFP-expressing plasmid likely due to its ability to enhance cell penetration.[488] They found that the analogue with the highest LogP value and three chloro groups had a 3.5-fold increase in the transfection efficacy of mammalian cells compared to the positive control and commercially available transfection reagent X-tremeGENE HP®, although with a slightly higher toxicity.

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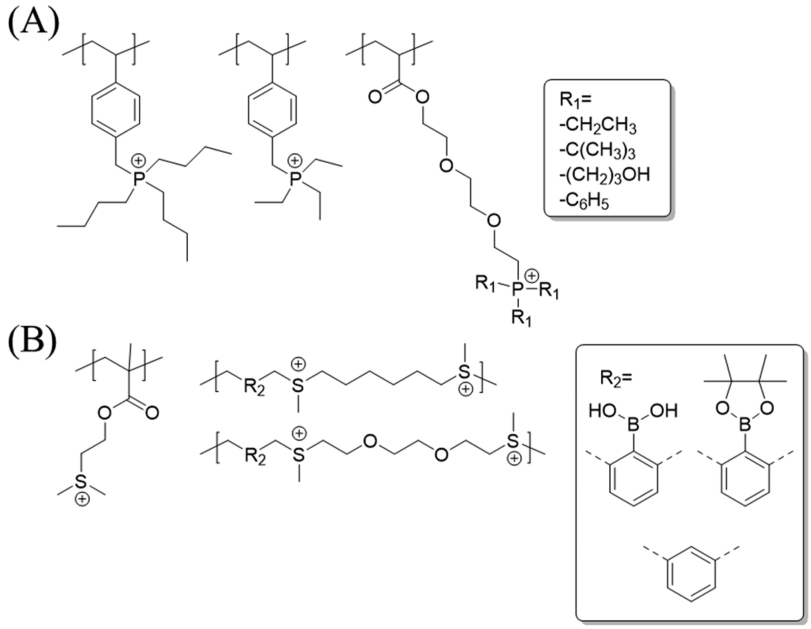

Figure 17. Chemical structures of polycations for gene delivery based on (A) phosphonium and (B) sulfonium non-nitrogenous cations.

3.3.2. Non-Nitrogenous Cations. Additionally, a limited number of polycations containing charge centers based on phosphorus and sulfur heteroatoms have been reported for their use in gene delivery (Figure 17). The relatively scarce use of these non-nitrogenous polycations in the field is thought to be due to the few available synthetic pathways for their preparation as well as concerns for the chemical instability of the cationic moieties and their precursors in biological relevant media.[489,490] Despite these barriers, the need for more efficient and non-toxic delivery vectors encourage the use of these type of cations, which show the promise of better cytotoxicity profiles and higher transfection efficiencies when compared to nitrogenous analogues.[491] Non-nitrogenous cations present differences in partial charge distribution between the heteroatom and adjacent carbon atoms due to the varying electronegativity in nitrogen, sulfur, and phosphorus atoms, which is thought to influence the binding of nucleic acids.[489,492] Recent reviews on the synthesis of phosphonium-containing polyelectrolytes[493] and, in particular, their application in gene delivery[489] are available; therefore, the focus herein is to summarize recent examples and key studies that highlight the advantages and nuances of using these cations in gene delivery.

Long et al. reported the use of phosphonium-containing polycations as nonviral gene delivery vectors.[103,174] Poly(triethyl-(4-vinylbenzyl)phosphonium chloride) (PTEP) and poly(tributyl-(4-vinylbenzyl)phosphonium chloride) (PTBP)[174] homopolymers as well as block copolymers of PTBP with either poly(oligoethylene glycol methacrylate) (POEGMA) or poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC)[103] were synthesized by direct polymerization of the phosphonium-containing styrenic monomers. PTBP homopolymers showed enhanced DNA binding and transfection efficiency when compared to their ammonium analogues, at N/P ratios from 2 to 10, for in vitro transfection of HeLa cells with pDNA.[174] Polyplexes formed between pDNA and POEGMA-*b*-PTBP or PMPC-*b*-PTBP diblock copolymers showed enhanced colloidal stability compared to polyplexes formed with PTBP homopolymers and displayed similar transfection efficiencies and cell viability to jetPEI® formulations when delivered to HepaRG cells.[103] In another example of direct polymerization of phosphonium-containing monomers, Mantovani et al.[494] reported the synthesis of a library of polyphosphonium polymethacrylates using RAFT polymerization and their use for RNA delivery. A comparison of polymers with different cations (triethyl alkyl ammonium vs triethyl alkyl phosphonium) and spacers (i.e., the alkyl group between cation and polymer backbone) revealed stronger binding with siRNA with a phosphonium polycation made with a trioxyethylene spacer. Polyplexes formed between siRNA and this polycation showed high siRNA uptake and low cytotoxicity but an undetectable GFP knockdown in 3T3 cells.

Post-polymerization modification strategies have also been employed to introduce cationic phosphonium groups into polymeric structures. Fréchet et al.[495] reported water-soluble phosphonium-based polycations based on a two-step post-polymerization modification of polyacrylic acid. Esterification of poly(acrylic acid) (PAA) with triethylene glycol monochlorohydrin and posterior quaternization of the side chains with different tris(alkyl) phosphines granted a library of phosphonium-based polycations. The best-performing polymer contained triethyl phosphonium pendant groups, and it exhibited stronger siRNA binding, lower cytotoxicity, higher gene knockdown, and better serum tolerance than an analogous polymer with triethylammonium pendant groups. Similar examples of post-polymerization modification with tris(alkyl) and tris(aryl) phosphine have also been reported for the synthesis of phosphonium-based carbosilane dendrimers[496,497] and branched copolymers with poly(ethylene glycol acrylate) (PEGA).[498] An alternative post-polymerization modification strategy is the conjugation of pre-synthesized phosphonium moieties into polymeric backbones. This strategy has been realized through alkylation,[499] amidation,[500] or photoinitiated thiol−yne addition[501] to conjugate pre-synthesized phosphonium groups into PEI, poly(aminopropyl-methacrylamides), and degradable polyphosphoester block copolymers, respectively.

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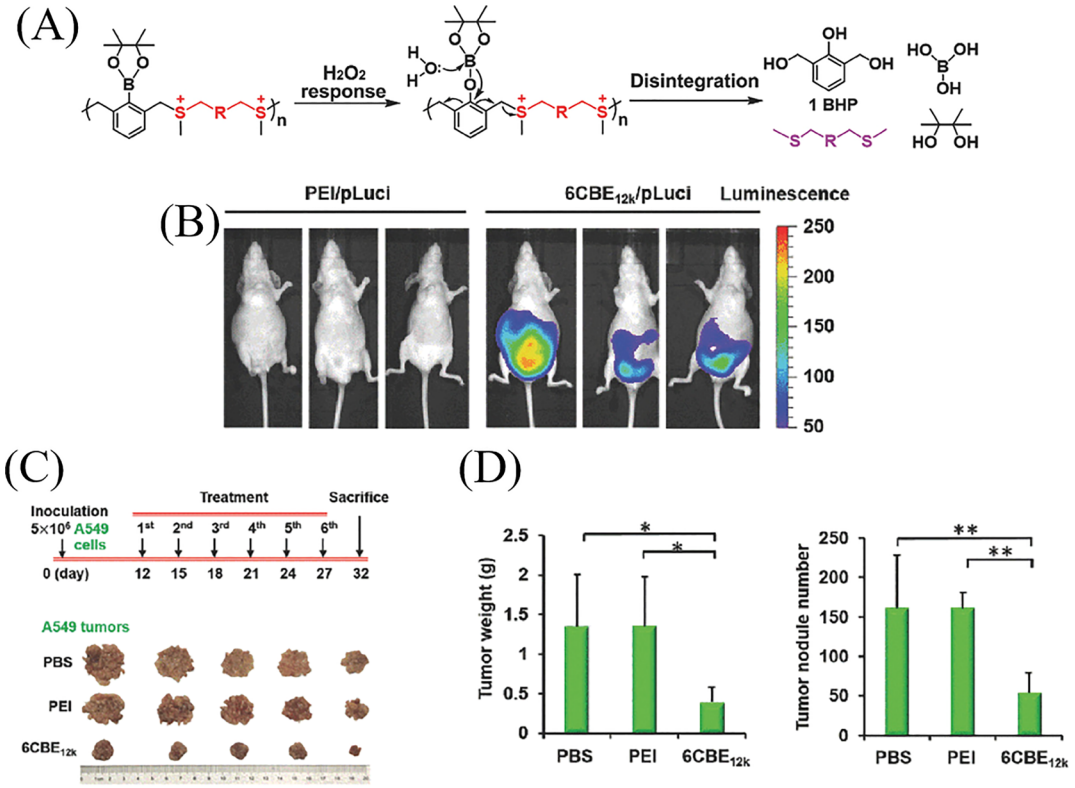

Figure 18. (A) Upon degradation in the presence of ROS, sulfonium-based polycation 6CBE12k degrades into neutral, non-nucleophilic, small molecule thioethers. The degradation provides a mechanism for intracellular pDNA release, (B) enhanced transfection, and (C, D) inhibiting tumor growth and dissemination. Reprinted with permission from ref 503. Copyright 2017 John Wiley and Sons.

In addition to phosphonium-based polycations, polymers with tertiary sulfonium moieties are also an alternative to nitrogenous polycations. Matyjaszewski et al. reported the synthesis of sulfonium-containing poly(meth)acrylates for their use in siRNA delivery.[502] Their approach is based on thioether-containing (meth)acrylate monomers that can be alkylated either before or after polymerization to produce macromolecules with tertiary sulfonium moieties as pendant groups. ATRP polymerization using a PEG macroinitiator granted neutral-*block*-cationic water-soluble block copolymers. The ability of these polymers to complex siRNA was a function of the length of the cationic polysulfonium block. Polyplexes based on these polymers showed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) knockdown in vitro in MC3T3s cells. Similarly, Long et al. reported the conventional free radical as well as RAFT polymerization of thioether-containing methacrylate monomers as intermediates in the synthesis of sulfonium-containing homo and diblock copolymers.[490] The sulfonium-containing polyelectrolytes, obtained via a post-polymerization alkylation of the thioether side chains with methyl iodide, contain ∼90% sulfonium repeating units and were explored as pDNA delivery vectors. These sulfonium-based polyelectrolytes complexed pDNA at charge ratios greater than 1, formed colloidally stable polyplexes (in water, serum-free, and serum-containing media), but showed lower transfection efficiencies than Jet-PEI in HeLa cells. The absence of a proton-sponge effect, due to the lack of a protonatable species, is cited as a potential reason for the lower efficiencies, hinting to the need of incorporating extra functionalities to sulfonium-based polycations for pDNA delivery.

The use of sulfonium-based polycations can bring additional advantages to the gene delivery field since some of these macromolecules are inherently degradable. For instance, Shen et al. reported sulfonium-based polycations with the ability to degrade into neutral fragments in the presence of reactive oxygen species (ROS), as a mechanism to release DNA intracellularly (Figure 18A).[503] This was achieved by combining sulfonium cations, incorporated in the polymer backbone, with ROS-responsive phenylboronic acid and esters. Poly(thioethers) varying in the length and composition of spacers connecting sulfur atoms to the rest of the polymer were synthesized. Post-polymerization, these poly(thioethers) were alkylated with methyl triflate, affording polycations with sulfonium ions in the backbone. Non-degradable versions without the boronic ester group were also synthesized. These polycations were shown to efficiently bind DNA at charge ratios higher than 2 and showed degradation in the presence of H_2O_2. Polyplexes based in the best-performing polymer, $6CBE_{12k}$, a 12 kDa polymer synthesized with a hexyl spacer between the sulfonium cations, showed a 2−3 orders of magnitude higher transfection efficiency than a control 25 kDa PEI, when tested in vitro in HeLa cells, A549 cells, and NIH3T3 fibroblasts in the presence of 10% fetal bovine serum. The transfection efficiency of $6CBE_{12k}$ polyplexes in ROS species-depleted HeLa cells, treated with either diphenyleneiodonium or ascorbic acid, decreased ∼50% with respect to untreated cells, showing the importance of ROS-mediated degradation in these systems. The antitumor efficiency of a formulation with the suicide gene pTRAIL was tested in vivo in two different mice models (i.p. inoculated mice with A549 and HeLa). The sulfonium-based polyplexes showed a

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statistically significant reduction of tumor size and weight, in contrast to controls with PEI, where no reduction was observed (Figure 18C,D).

Modulating the charge content and type in polyplex formulations has been regularly utilized as a strategy to improve their efficacy. We further discussed the implications of this strategy and present a body of literature that expands on this and questions the very need of these charges for efficient gene delivery in Section 5.3. Nonetheless, it is in the nature of the polymer chemistry field to continue to diversify the types of macromolecules that can be synthesized, and we thus expect the preparation of novel polycations based on N, P, S, and other heteroatoms and their utilization as gene delivery vectors will continue to be an active area of research.

3.4. Introducing Hydrophilic Moieties

3.4.1. PEGylation. Colloidal stability is a crucial design parameter especially for in vivo gene delivery. Delivery vehicles could self-aggregate due to poor stability characteristics allowing for an expeditious clearance by macrophages in a size-dependent manner. The high ionic strength of physiological environments is a major factor that can cause delivery vehicles to become colloidally unstable and aggregate.[132] Beyond systemic clearance, cellular internalization, which is often a delivery bottleneck, is also sensitive to polyplex aggregation and instability. Additionally, PEI and other amine-containing cationic gene delivery vehicles are inherently problematic for in vivo applications due to their inherent cytotoxicity and the presence of protein-mediated fouling and aggregation. Negatively charged proteins found within the blood (e.g., albumin) can adhere to the nanoparticle vehicle, while creating a surface for protein fibrillation leading to the adherence of more proteins, such as opsonins.[504,505] Opsonins are readily recognized by receptors bound on macrophages that facilitate phagocytosis and clearance of foreign materials or pathogens.[506] Protein-serum fouling and poor colloidal stability leads to a rapid systemic clearance of gene delivery vehicles. As a consequence of protein fouling (opsonization) or polyplex aggregation, the RES is able to clear the foreign particles rapidly. Furthermore, protein fouling can also lead to particle aggregation causing particle entrapment in capillaries of the RES.[119,507,508] Aggregation or particle size increase can significantly affect the clearance of the particle by compounding the specific clearance with non-specific clearance routes. Additionally, van der Waals, electrostatic, and hydrophobic forces can also promote further protein aggregation with the vehicles in vivo.[507,509] Protein fouling, regardless of whether it proceeds rapidly or gradually, will inevitably lead to clearance from the blood by macrophages and necessitates mitigation.[510] Systemic clearance should be minimized in order to maximize delivery of vectors.

PEG has a long track record of being a viable option for addressing the challenges associated with in vivo applications of gene delivery vectors. PEGylation affords systems with a hydrophilic non-ionic inert corona that inhibit protein interaction, giving rise to its "stealth sheath" properties, described in 1977 by Davis et al.[511] Since then, the application of PEG has expanded, and its FDA "generally recognized as safe" designation has allowed for an expedited processing of medical applications, including for gene delivery.

There are several factors that need consideration when PEG is incorporated into a gene delivery system. In general, PEG chains offer steric repulsion that counteracts other intermolecular forces that drive proteins to adhere to positively charged complexes and encourages fibrillation. This repulsion also stems from a large excluded volume and a dense hydration cloud. The hydration cloud is produced by the hydrophilic nature of PEG, which grants a layer of two to three water molecules per PEG unit.[512] The efficiency of this "shielding effect" from proteins and other detection avenues can be tailored through a selection of the PEG molecular weight and architecture, as well as optimizing the grafting density.[100,513] Molecular weight and density can improve the steric repulsion of PEG up until a threshold—~5 wt % or at least 2000 Da—which would result in a significant shielding at the lowest PEG content.[513,514] For polymer brushes, density also plays a role in the PEG conformation. A more densely-packed PEG segment will resemble more of a comb structure, whereas a lower density is depicted as a "mushroom" shape. A higher density of PEG chains across a smaller backbone length will force the chain to extend, leading to fewer available conformational changes (Figure 19).[515] The reduction in conformations will inhibit proteins that are larger than the interbrush spacing from penetrating the hydrophilic shield and would enable binding to the cationic segment or surface.[515,516] However, if the protein is smaller than the overlap spacing between PEG brushes, there is little resistance against protein aggregation. The reduction in entropy following the loss of excluding water molecules from

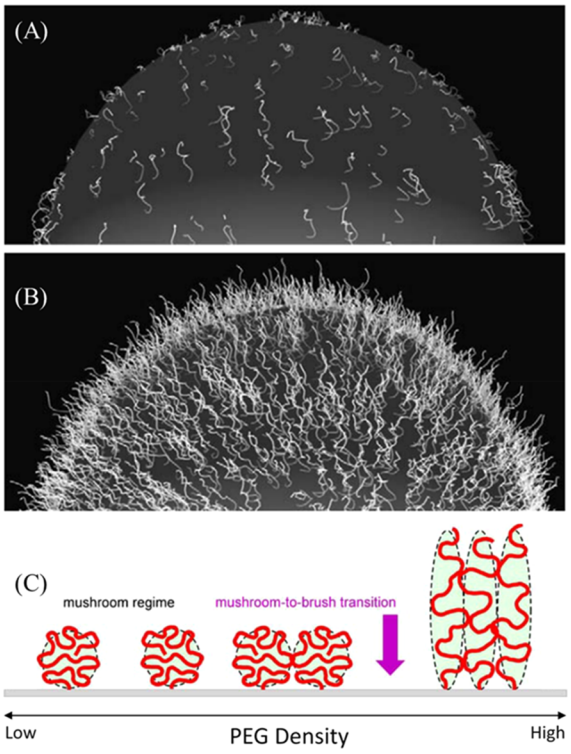

Figure 19. Schematic diagrams of PEG configurations on the upper hemisphere of a polymeric nanoparticle. (A) The low surface coverage of PEG chains leads to the "mushroom" configuration where most of the chains are located closer to the particles surface. (B) The high surface coverage and lack of mobility of the PEG chains leads to the "brush" configuration where most of the chains are extended away from the surface. Reprinted with permission from ref 513. Copyright 2006 Elsevier. (C) The mushroom-to-brush transition is highlighted, from a surface view, where PEG density forces chains to extend. Reprinted with permission from ref 518. Copyright 2006 Springer Nature.

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the PEG brushes is much lower compared to mushroomlike PEG chains.[516,517]

Kataoka and coworkers demonstrated the importance of a higher PEG density (i.e., the crowdedness of the stealth shield) in prolonging the systemic circulation of PLL rods.[519] More recently, they have shown how PEGylating cationic micelles affects their stability under shear.[520] The shear stresses in blood flow impair the efficient fouling protection provided by PEGylation, which they suggested could be mitigated by crosslinking the polycation chains through disulfide bonds. Liang et al. further expanded on the effects of blood shear flow on PEGylated carriers by demonstrating that a denser incorporation of a PEG protective layer will withstand a higher shear flow before becoming perturbed and exposing the cationic core to serum proteins.[521] A critical shear flow can be quantified via properties such as surface tension, PEG grafting density, and the elasticity, which agrees well with the work from Liang's group.[522] At low shear flows, the PEG is disturbed exposing the DNA/cationic core to protein, resulting in complex aggregation. At higher flow rates, the force deforms the complexes into smaller sizes thus preventing further aggregation. Finally, at the extreme end of high shear rates, the core of the micelle is forced to restructure and organize to incorporate protein aggregates within the core highlighted (Figure 20). Overall, the transitions between these regions can

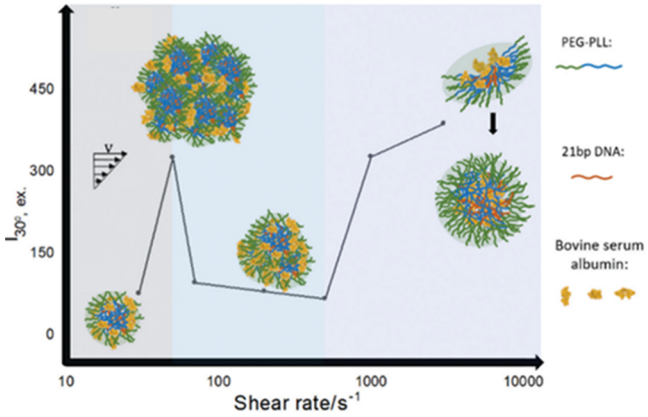

Figure 20. Schematic and graphical display of shear induced deformation and aggregation of PEGylated complexes in the presence of serum. Reprinted with permission from ref 521. Copyright 2020 Royal Society of Chemistry.

be tailored by increasing the PEGylation density. The grafting density and molecular weight of the PEG hydrophilic sheath are two key parameters to consider while improving polyplex resistance towards protein fouling.

In addition to preventing protein aggregation, PEG provides complexes with a hydrating and charge-screening layer from other complexes in the same system.[523,524] PEGylated polyplexes have shown a reduction in their zeta potentials.[525−527] This charge screening leads to reduced polyplex aggregation. Hanes et al. were able to determine the grafting density or surface coverage of the PEG block via zeta-potential readings.[528] Further screening from PEG is beneficial to reduce contact with other charged entities such as extracellular DNA nucleases, heparin, heparin sulfate, or mucus.[529−531] Limiting the interactions with these macromolecules minimizes the likelihood of payload degradation, loss, or immobilization. In an early instance of incorporating PEG into gene delivery vehicles, PEG was shown to offer improved colloidal stability and reduced aggregation and immunogenicity to a PEI gene delivery vehicle.[532] Wagner et al. coupled 5 kDa PEG derivatives to the primary amino groups of PEI, which demonstrated a reduction of interactions with blood components and an improved colloidal salt stability.[533] PEGylation strategies have also been applied to other common cationic blocks, such as PLL,[365,534,535] PDMAEMA,[536] and polyspermine.[537] Park and Healy grafted a lactide-PEG block onto a lysine polymer to enhance DNA binding and protection.[538] This study demonstrated the importance of incorporating PEG to protect against DNase I degradation, an enzyme detrimental to the cargo. DNase I degradation is reliant on fitting into the minor groove of DNA; the lactide-PEG block hinders proper alignment and displays improved resistance towards DNase I compared to lysine homopolymer.[538]

Advances in polymer architecture have helped overcome the challenge of proper PEG spacing. PEG spacing is a parameter that will dictate if small proteins are to be able to penetrate and adhere to the delivery vehicle. Polymerizing PEG brush monomers or using more complex PEG architectures like multifunctional end groups of PEG resembling a star, dendritic, or bottlebrush shape helped reduce the spacing between PEG chains.[539,540] Arima et al. showed a sevenfold longer blood half-life when they compared PEGylated fourth- and third-generation polyamidoamine dendrimers. In addition, PEGylation of a fourth-generation dendrimer yielded a product with negligible cytotoxicity.[541]

PEGylation has also been used to directly modify nucleic acids. Zhang and coworkers synthesized a densely packed PEG bottlebrush vector containing covalently bound siRNA. The PEG bottlebrush was synthesized through ROMP copolymerization of a norbornenyl PEG monomer and a functional norbornene monomer that allowed for the introduction of azide groups post-polymerization. The brushes were then functionalized with siRNA containing clickable dibenzocyclooctyne groups.[542] The resulting non-cationic vehicle displayed excellent protection of nucleic acid cargo from degrading enzymes and protein fouling while allowing for cellular uptake and delivery of cargo to desired tumor cells.[542] Nuclease degradation was monitored by fluorescence masking with an antisense RNA strand. Yet, when ribonuclease III was added to quench the binding, the bottlebrush displayed a prolonged half-life compared to control groups. Using dense PEG coatings is of particular interest in mucous-membrane gene delivery.[100,543] PEGylation, once used as a mucoadhesive, can be tailored to allow fast penetration and reduced immobilization in viscous media and mucus, allowing for the use of polymeric gene delivery to target the lungs,[544] brain,[545] vaginal tissue,[546] or ocular tissue.[547,548] For example, Hanes et al. synthesized a PLL-*b*-10 kDa PEG polymer, which was found to effectively deliver genetic cargo in vivo to the brain, eye, and lungs.[544] However, they found this system is immobilized in the sputum of cystic fibrosis patients. Immobilization was also observed in the vitreous humor of the eyes or spinal fluid of the brain where the viscous properties match the mucus found in lungs of cystic fibrosis patients. To improve the mucus penetration of their gene delivery system, they compared different lengths of PEG blocks (2, 5, 10 kDa). As a control, they formulated a non-ionic polystyrene-*b*-2 kDa PEG (PS-*b*-PEG). They found that the shorter PEG block diffuses more quickly, most likely due to the smaller size fitting through the

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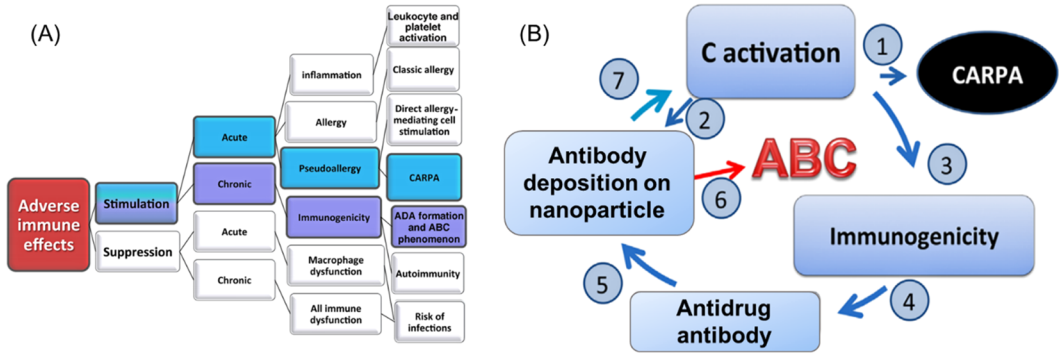

Figure 21. (A) Pathways for adverse immune effects to nanoparticles. The highlighted pathways are specific for PEG and the immunogenic consequences for using PEGylated materials. (B) Positive feedback cascade of PEGylated material activating the complement system (C activation) leading to CARPA and ABC. Reproduced with permission from ref 564. Copyright 2017 John Wiley and Sons.

pores of the mucus network. Interestingly, the PS-*b*-PEG penetrated and diffused the most in the mucus suggesting that an even higher density of PEG is required to penetrate mucus and reduce interactions between the mucus and the PLL.[544]

Although careful PEGylation of cationic polymer gene delivery vehicles can overcome many of the challenges associated with in vivo delivery of these vehicles, one serious issue remains—the marked decrease in cellular internalization that comes with PEGylation. Wagner and coworkers showed that moderate PEGylation could enhance transfection of PEI polyplexes; however, they also showed that further increasing the extent of PEGylation could decrease the uptake of the polyplexes.[247] Other groups have shown a similar trend of PEG incorporation inhibiting the uptake of gene delivery vehicles.[285,549,550] The same phenomena that accounts for a reduction of protein fouling and particle aggregation also reduces the ability of PEGylated gene delivery vehicles to be internalized efficiently. Many researchers refer to this problem as the PEGylation dilemma. This apparent reduction of gene delivery efficacy has been addressed in the field through the incorporation of active targeting groups or cell binding motifs. Targeting groups accessible by the cell help to facilitate internalization without losing the stabilizing and anti-fouling properties of the PEG groups. For example, the incorporation of aptamers,[551,552] antibodies,[553,554] cell-penetrating peptides,[530,555] peptides,[556,557] and other targeting ligand moieties[558] has been shown to increase the internalization of nanoparticles. Others have specialized in adapting responsive PEGylated systems to be responsive to environmental stimuli, like pH, where PEG chains are cleaved from delivery vehicles in response to external triggers, thereby overcoming the shortcomings of PEGylation.[559−561]

Recent evidence also shows that PEGylation can elicit two potential responses upon administration, shown in Figure 21.[549,562−564] The first is a chronic immunogenic response leading to an accelerated blood clearance (ABC) of PEGylated systems. The second is an acute pseudoallergenic response leading to complement activation-related pseudoallergy (CARPA) and hypersensitivity reactions to PEGylated systems. Either of these responses should lead to concerns of the safety and efficacy of PEGylated materials. Szebeni et al. provide an excellent discussion and review of such phenomena surrounding PEGylated material.[563]

Much of the early attention given to the ABC phenomena was towards PEGylated liposomes.[565,566] Not to be overlooked, this knowledge should be translated to the design of PEGylated polymeric nanoparticles.[567,568] Kiwada and coworkers showed that the spleen has an integral role in the phenomena when they performed a splenectomy in rats and measured levels of immunoglobulin M (IgM) and G (IgG).[569] Rats that were splenectomized before being injected with PEG-containing liposomes showed the same levels of IgM as the control vehicle, whereas the injected group had an eightfold greater elevated IgM level. Recently, they also investigated the role IgM takes in the clearance of PEGylated complexes. They discovered that both IgM and marginal zone containing B-cell (MZ-B cells) activation are required for splenic cells to be able to associate with PEG complexes.[570] IgM in serum-free environments, however, does not facilitate the adhesion and removal of PEG liposomes. Kiwada and coworkers reported that IgM binds to the PEG complexes. In the presence of serum, the complement system is activated, where the formation of an immune complex containing the PEG liposomes, IgM, and complement proteins can be recognized via the MZ-B cell's complement receptors. A serious issue arises for the incorporation of PEG to a delivery vehicle if a patient already possesses anti-PEG IgM; studies have shown patients have displayed anti-PEG antibodies without PEGylated nanoparticle exposure.[571,572] This is an alarming revelation, since patients who have never been subjected to PEGylated nanoparticles could elicit an unwanted severe immune response or have the PEGylated therapeutic rapidly eliminated. Lai and coworkers demonstrated that anti-PEG antibodies can be temporarily sequestered with freely circulating high molecular weight (40 kDa) PEG in addition to PEGylated therapeutics for at least 48 h.[573] Gabizon and Szebeni recently shared their expertise on avoiding complement activation, the dangerous phenomenon associated with PEGylated nanomedicines, and reviewed clinical and experimental data relating to ABC.[563] Furthermore, Truong and coworkers published a review that details other factors that affect the immunogenicity of PEG in humans and animals.[574]

An additional problem that PEG encounters with long-term therapeutics is oxidation. Although PEG is touted as a safe compound due to its low toxicity profile, reactive oxygen species—such as hydroperoxides and peroxide free radicals—are generated from the metabolism of polyethers, which can be problematic.[575] These free radical byproducts can lead to oxidative stress causing tissue damage, reminiscent of age-related and neurological diseases.[576] Likewise, payloads containing peptides, proteins, or DNA are known to be susceptible to peroxide radicals.[577,578] Oxidative damage to

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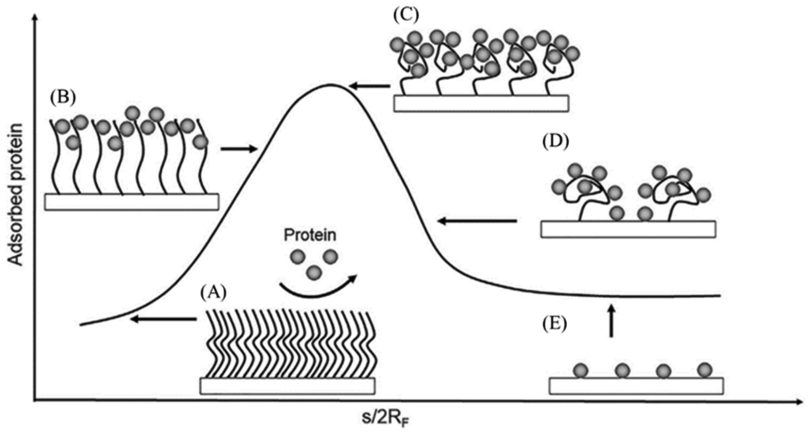

Figure 22. Depiction of protein adsorption depending on grafting density of poly(sulfobetaine methacrylate) (P(SBMA)) zwitterionic brushes. (A) At the highest grafting density, proteins are unable to penetrate and adhere to the polymer. (B) With reduced grafting density, the polymer chains are extended, but the protein can penetrate and adhere to the polymer. (C) At intermediate grafting densities, the polymer is flexible and exposes more of its charges, allowing for more sites for protein adherence. (D) At the lowest grafting densities, the polymer is perceived as a mushroom and can be self-coiling, which can hide and hinder protein adherence. (E) A minimum limit of protein adherence occurs even without the charge found with P(SBMA), where protein adheres to the surface of the nanoparticles. Reprinted with permission from ref 589. Copyright 2020 John Wiley and Sons.

DNA represents the prevalent form of DNA damage within human cells.[579] Thus, a complete pharmacokinetic analysis of the metabolic byproducts is needed before PEG is incorporated into a genetic vehicle due to the potential damage of the cargo or a neutralization of the therapeutic effect. Kumar and Kalonia presented an effective vacuum method to remove the majority of peroxide free radicals formed before implementing commercially available PEG polymers.[580] When the majority of peroxides formed from PEG polymers is removed, the cargo is less likely to be degraded by the vehicle. As stated before, PEG is generally regarded as safe, and only at exceedingly high doses has PEG been seen to present adverse effects. Taupin et al. have reviewed extensively the toxicity, metabolism, and clearance of PEG while addressing the previous concerns.[581]

Overall, PEG excels at protecting and stabilizing polyplex formulations and increases their blood circulation half-lives. Its incorporation can be tailored to meet specific characteristics through its molecular weight, density of chains, and architecture, and it continues to be a popular choice among researchers—especially in combination with targeting moieties—for its biocompatibility profile, stability, and fast-track FDA approval record. However, the oxidative stress induced by PEG and its immunogenic effects must be taken into consideration, especially for therapeutic applications involving long-term use.

3.4.2. Zwitterionic Moieties. To circumvent the potential immunogenicity and reduction in internalization triggered by PEGylation, researchers have turned to alternative hydrophilic moieties. Zwitterionic polymers are one such class of polymers with the potential to replace PEG as the hydrophilic moiety of choice when designing polycations for nonviral gene delivery. Unlike PEG, zwitterionic polymers are made of neutral monomers composed of stoichiometrically equivalent amounts of positively and negatively charged ions. Schlenoff has extensively and concisely presented arguments and data that postulate the mechanism for zwitterion's anti-fouling properties.[582] Briefly, zwitterionic molecules provide favorable environmental interactions via four distinct mechanisms: watery surface, structuring of water, steric effects, and ion-coupled forces. Like PEG, zwitterionic molecules are effective at attracting water molecules and creating a dense hydration cloud.[583,584] The hydrophilicity of zwitterionic molecules is driven via strong dipole interactions rather than perturbed hydrogen bonding as seen with PEG.[585−587] This facilitates the ordering of water molecules to resemble bulk water in a thermodynamically favorable way. Thus, the perturbation of water molecules during protein adhesion imposes a greater thermodynamic penalty. Others have used similar strategies for polymer design by increasing the grafting density of zwitterionic monomers for enhanced anti-fouling and colloidal properties for brushlike polymers.[588,589] Ahmed and Leckband found a non-monotonic correlation between the amount of protein adsorbed and grafting density for poly(zwitterionic) brushes (Figure 22),[589] which contrasts with the linear correlation present for PEGylated surfaces.

Recent examples of zwitterionic incorporation leading to minimal protein fouling has rapidly increased the focus on zwitterionic molecules to enhance anti-fouling behavior, salt stability, and biocompatibility.[590−593] Figure 23 shows three zwitterionic monomers that gave rise to polymers that demonstrate minimal protein fouling: sulfobetaine methacrylate (SBMA), carboxybetaine methacrylate (CBMA), and 2-

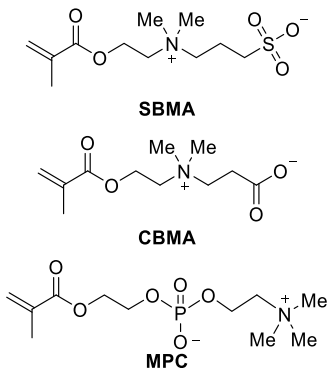

Figure 23. Commonly used zwitterionic monomers sulfobetaine methacrylate (SBMA), carboxybetaine methacrylates (CBMA), and 2-methacryloyloxyethyl phosphorylcholine (MPC).

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methacryloyloxyethyl phosphorylcholine (MPC).[594,595] Notably, the betaine derivatives have a more established history and are easy to synthesize. Conversely, MPC monomers originally difficult to synthesize have been optimized to produce an inexpensive and pure product able to undergo a controlled radical polymerization.[596,597] The phosphorylcholine functional group of MPC resembles the lipid head group of the cell membrane, which can be advantageous for both antifouling and cell membrane associations.[434,594,598] MPC was recently shown to alleviate concerns arising with PEG. Repeated administration of MPC complexes in a murine model showed minimal histologic or immunogenic side effects while simultaneously showing a twofold increase in internalization compared to jetPEI®, a commercial transfection reagent.[599,600] The work carried out by Giorgio and Duvall was inspired by previous work that demonstrated high molecular weight zwitterionic polyplexes that showed enhanced biocompatibility and uptake compared to PEG analogues.[593] As an added benefit, zwitterionic polyplexes showed enhanced resistance to destabilization from increasing salt concentration. Zwitterionic polymers are known to be unstable or form collapsed coils in water but gain stability with increasing ionic strength from salt ions.[601] Zwitterionic polymers are not a catch-all replacement for PEGylation. Most recently, Giorgio and Duvall demonstrated a smaller therapeutic window for MPC than PEG containing polyplexes, requiring a deeper mechanistic understanding of differences between zwitterionic polymers and PEG.[602]

Erfani and coworkers highlight the effects of zwitterions and their interactions with biomolecules, noting key behavioral differences arising between zwitterions and PEG derivatives in aqueous media.[603] A key behavioral difference between PEG and zwitterionic polymers is their protective action: with well-hydrated and extended polymer chains, the zwitterionic polymer is able to inhibit both aggregation of complexes and degradation of its payload. Like PEG, both the molecular weight and grafting density of the zwitterionic polymers need to be considered, but the salt concentration and the zwitterionic self-association need to also be considered to provide superior hydration screening.[604−606] Jiang's research group, which has applied zwitterionic polymers to solve numerous biomaterial challenges, has employed both molecular simulation and experiments to shed light on the roles of zwitterionic charge density, composition, and architecture.[607−609] Reduction in the payload degradation of the delivery vehicle arises from salts found near the chains. Salts are primarily associated with the anti-polyelectrolyte effect between zwitterionic chains but also provide a stabilizing effect for proteins.[603] Polymer architecture can tune and amplify the stabilizing effect of zwitterions and is reviewed by Erfani and coworkers.[603]

Responsive polymers have been designed to respond to their environment and, upon the application of the right environmental trigger, degrade into zwitterionic materials. The goal for producing a zwitterionic end-product is to reduce the toxicity of the system. Like many cationic polymers, the cationic nature needed for condensing the genetic material is often a downside due to its inherent toxicity. By engineering a polymer and producing a labile end group, Jiang and Carr addressed this concern by synthesizing a carboxybetaine ester diblock polymer containing a quaternary amine able to condense DNA, a tertiary amine able to buffer its environment, and an end group able to undergo hydrolysis to form a zwitterionic polymer resulting in minimal toxicity.[610] This proof-of-concept study showed that this polymer system was able to produce a 20-fold increase in transfection efficiency compared to branched PEI without the associated cytotoxicity. Similar methodologies were carried out to produce a DNA vaccine platform, whose major complication for success was its associated toxicity.[611] Jiang and Carr further optimized their responsive polymer system by comparing the spacer length between the cationic moiety and the anionic moiety, as well as their monomer end group needed for hydrolysis.[612] They found that a single carbon spacing was sufficient to shift the pK_a of the tertiary amine within the endosomal pH and that an ethyl ester end group provided an order of magnitude higher transfection while remaining nontoxic. Notably, they synthesized an ultraviolet (UV)-labile end group to determine the kinetics of DNA release from their polyplexes. After their complexes were irradiated with UV light for 1 h, ∼73% of the DNA was released, demonstrating the benefit of switchable polymers for effective release of DNA upon cellular entry. This work highlights the benefit of switching potentially cytotoxic cationic polymers into nontoxic zwitterionic polymers for effective gene delivery.

In addition to poly(zwitterions), polyampholytes are starting to be examined for their protein antifouling and colloidal stability properties.[613] Like zwitterionic polymers, polyampholytes are charge-neutral ionic polymers that contain both cationic and anionic groups. However, unlike zwitterionic polymers, polyampholytes may not contain both cationic and anionic entities within the same monomeric unit and may not be charge-neutral at the repeat-unit level. There has been very little work with incorporating alternating singly charged monomers into a polymer delivery vehicle for gene therapy, but the benefits of incorporation may already be apparent, as seen in zwitterionic monomer incorporation. With carefully designed sequences of singly charged monomers, a plethora of options exists for realizing the desired spatial organization of positively and negatively charged groups along the polymer.[518,614] Emrick and Jayaraman et al. showed the resulting relationship of distributing zwitterionic polymers throughout a cationic comb polymer.[446] At 50 mol % incorporation of zwitterionic polymers, the total polymer still maintained its cationic nature while providing high levels of genetic cargo delivery (double the amount compared to the control) and viability (>97%).[446] When this charge is maintained without a screening effect from the zwitterionic polymer being incorporated, this polymer is able to complex the DNA. Furthermore, the incorporation of zwitterionic polymers into the delivery system weakens the strength of the DNA binding. The DNA binding can be optimized through stringent incorporation and control over monomer addition to facilitate both reliable protection of the genetic cargo as well as cargo unpackaging upon cellular import.

In summary, the incorporation of zwitterionic into polycationic vectors affords colloidally stable polyplexes with suitable cytotoxicity profiles. Their use, limited by the small number of commercially available zwitterionic monomers, will continue to grow as a response to the growing biosafety concerns of the use of PEGylated polymers.

3.4.3. Carbohydrate Monomers. One final class of hydrophilic moieties that confer colloidal stability and enhanced targeting to polycationic vectors is carbohydrate monomers. These monomers carry glycan moieties that can be incorporated either in a polycation backbone or as pendant

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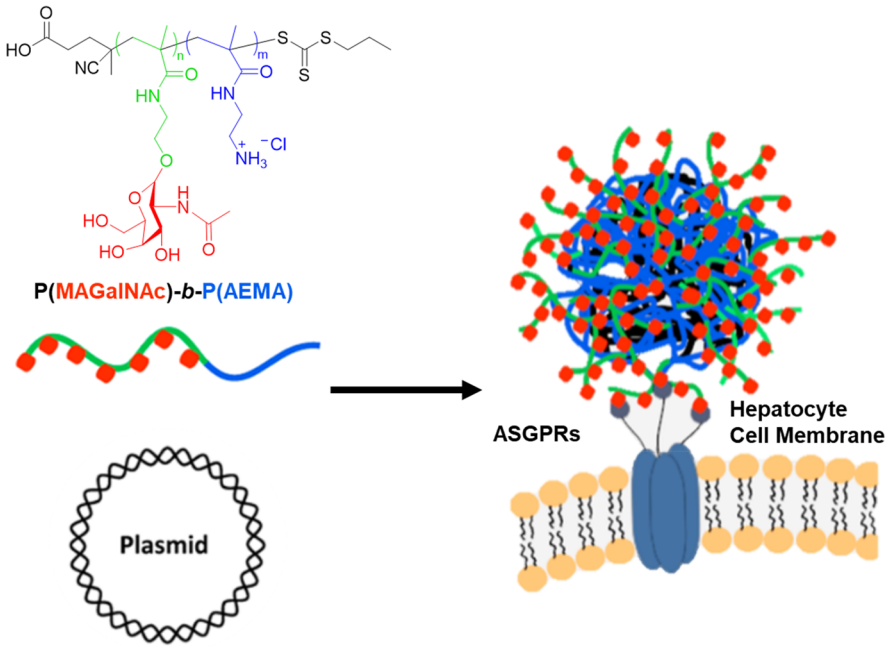

Figure 24. P(MAGalNAc)-*b*-P(AEMA) diblock glycopolymers display high affinities to ASGPRs on liver hepatocytes, allowing for a liver-targeted gene delivery. Adapted with permission from ref 622. Copyright 2016 American Chemical Society.

groups. Like PEG, the hydrophilic nature of carbohydrate-derived glycopolymers arises from their hydrogen-bonding capability. Similar to ether linkages in PEG, carbohydrates can form a dense hydration cloud from their abundant hydroxyl groups, providing enough steric and hydration repulsion towards proteins and aggregation between complexes. Acetylation of the hydroxyl groups is thought to extinguish the hydrophilic nature of the glycopolymers, reducing its overall colloidal stability. Previous incorporation of sugar moieties into polymers were avoided due to its tedious requirement of protection and deprotection of hydroxyl groups during polymerization. The development of a variety of methods allowing for the synthesis of glycopolymers including controlled radical polymerizations and without the need of using protected monomers[415,615−617] has helped the development of this class of polymers for several applications. Yu and Kizhakkedathu developed glycopolymer brushes for protection against protein interactions.[618] These carbohydrate-containing monomers mimic the glycocalyx of cell membranes. The glycocalyx has many cellular processes, but an important trait worth mimicking is the prevention of non-specific cell or protein interaction.[619] The modified surface substrate with glycopolymers showed super-hydrophilicity via low contact angles (10°).[618] When placed in protein solutions containing bovine serum albumin and fibrinogen, the glycopolymers provided excellent protection from protein adsorption. Molecular dynamics simulations showed that hydroxyl-rich glycopolymers bind water molecules tightly, further justifying the resistance displayed towards protein adsorption and the need for maintaining the hydroxyl group's integrity after polymerization.[620]

Beyond similar traits to PEG, glycopolymers offer the additional benefit of biocompatibility due to the composition being of naturally occurring sugar moieties. The degradation of glycopolymers can be easily metabolized into important biomolecules found within cells. Narain et al. used this concept and formed hyperbranched statistical copolymers of aminoethyl methacrylate (AEMA) with sugar-based monomer 2-lactobioamidoethyl methacrylamide.[621] Polyplexes formed from these glycopolymers and siRNA elicited minimal toxicity, most likely due to the acid-catalyzed degradation of the 2,2-dimethacryloyloxy-1-ethoxypropane branches in the delivery vehicles. Small polymer fragments degrading in acidic conditions are then more readily processed by the cell due to its recognizable sugar structure. Ahmed and Narain demonstrated the enhancement of the delivery system's stability, toxicity, and delivery by incorporating carbohydrates within polymeric vehicles. After 3-gluconamidopropyl methacrylamide was statistically incorporated, regardless of high or low amounts, these copolymers outperformed their cationic homopolymers roughly twofold in transfection efficiency and provided minimal toxicity towards cells when compared to untreated cells. Additionally, the cationic homopolymers PAEMA and poly(*N*-(3-aminopropyl) methacrylamide) (PAPMA) with no carbohydrate substitution only had a viability of ∼20%.[615]

Unlike the challenges highlighted with PEG, glycopolymers may circumvent the PEGylation dilemma by allowing the delivery vehicle to interact and target native carbohydrate-binding domains (CBDs) present on the cellular surface.[487,622] By using a hydrophilic carbohydrate block made of 2-deoxy-2-methacrylamido glucopyranose (MAG), these polymers can offer a similar hydrophilic sheath shield providing the necessary steric effects that inhibit complex aggregation.[623,624] Furthermore, incorporating a methacrylamido *N*-acetyl-*D*-galactosamine (GalNAc) unit can promote a selective binding with asialoglycoprotein receptors (ASGPRs) found on hepatocytes shown in Figure 24.[622] Cationic diblocks synthesized with GalNAc as the hydrophilic block displayed similar colloidal stability as that of PEG-based analogues, as well as enhanced targeted gene delivery both in vitro and in vivo. During in vivo studies in mouse models, these diblocks accumulated in the liver at concentrations 70 times higher than those observed in the lungs. Additionally, polymer composition

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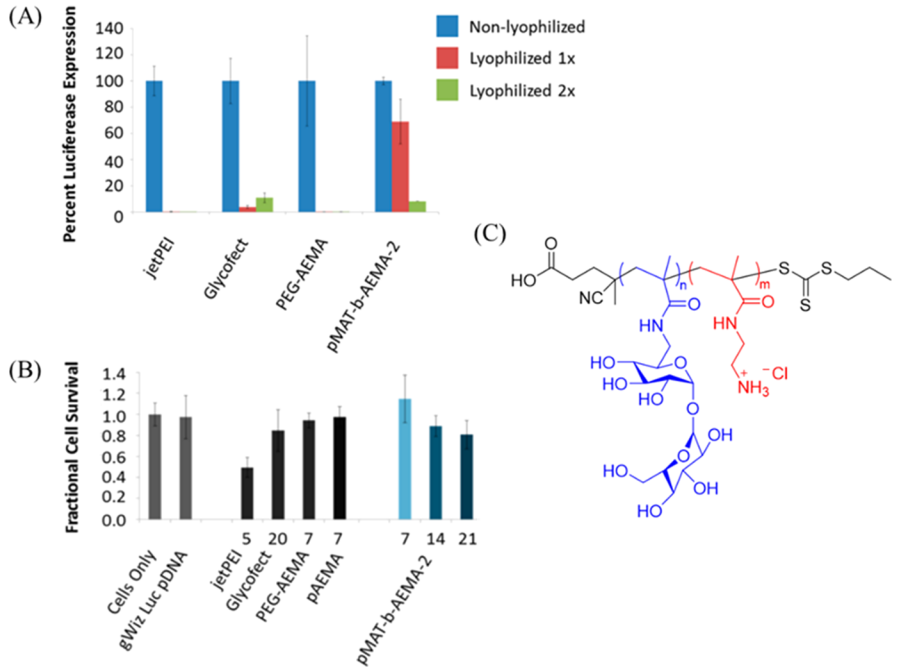

Figure 25. (A) pDNA polyplexes formulated from poly(methacrylamido trehalose)-*b*-PAEMA P(MAT-*b*-AEMA) diblock glycopolymers preserve a high transfection efficacy (∼60% luciferase expression in U87 cells) after lyophilization in contrast to controls formulated with jetPEI®, Glycofect, and a non-carbohydrate PEG-AEMA diblock copolymer. Reprinted with permission ref 105. Copyright 2016 American Chemical Society.(B) Cell survival was also superior to those of commercial controls. (C) Chemical structure of P(MAT-*b*-AEMA). Reprinted with permission from ref 104. Copyright 2012 American Chemical Society.

and morphology effects were determined by comparing block and statistical copolymers and terpolymers incorporating MAG and either one or both cationic monomers AEMA and DMAEMA.[289] It was found that block copolymers formed more stable complexes in protein-containing media compared to statistical copolymers. Yet luciferase gene expression was not inhibited, concluding both architectures could efficiently deliver their genetic payload indicating that these polymers are still able to promote cell entry at high rates unlike similar structures with PEG (which show a decrease). However, Narain et al. reported a slightly different trend within their study, which showed statistical glycopolymers made of 3-gluconamidopropyl methacrylamide outperforming diblock glycopolymers.[615] Even though both studies used HeLa cells, with differences in polyplex concentration and the sugar used it is hard to draw a direct comparison of performance between block and statistical polymer architecture. In a similar study, a diblock copolymer formed with MAG and AEMA showed effective colloidal stability in protein-containing media over time compared to leading industry standard transfection reagents, jetPEI®and Glycofect.[471] Furthermore, when compared to a PEG analogue with a similar molecular weight and architecture, the diblock glycopolymers from this study demonstrated better colloidal stability with increasing salt concentration, again highlighting glycopolymers' potential as a PEG alternative.[625]

Another carbohydrate that has been used in lieu of MAG is trehalose, a disaccharide of glucose. Trehalose has an established track record as a super-hydrophilic functionality incorporated into gene delivery vehicles with the added benefit of serving as a lyoprotectant.[104,105,626,627] Reineke and coworkers were first to produce trehalose-containing glycopolymers via click polymerization.[627] Their work demonstrated the stability and efficiency to deliver nucleic acids to cells with a trehalose-containing polymer in serum or serum-free media. This work established that a short disaccharide like trehalose can provide a smaller, less bulky, alternative to PEG. Further reports focused on incorporating trehalose into gene delivery vehicles by formulating diblock copolymers of trehalose with varying degrees of AEMA.[104] This further gathered evidence of trehalose being able to act as a lyoprotectant, expanding from the paper's finding that poly(trehalose) is able to lower the energy of phase transitions between liquid and solid in an aqueous solution. The lowering of energy also allows for a minimal loss of biological function after resuspension, demonstrated by the uptake of polyplexes by U-87 glioblastoma cell and resulting in lower cytotoxicity compared to untreated cells.[104]

Further work from the Reineke group showed excellent colloidal stability of cationic-trehalose copolymer in both salt- and serum-containing media, while simultaneously promoting high gene delivery with low toxicity in vitro and in vivo.[105] Additionally, the trehalose-containing polyplexes were reconstituted after lyophilization displayed minimal differences in polyplex size, measured via dynamic light scattering (DLS) and transmission electron microscopy (TEM), without a loss in biological function (Figure 25).[104] The ability to reconstitute polyplexes from a dry preserved powder could promote the storage stability and making formulation preparation easier which would be advantageous for clinical translation and manufacture.

Overall, glycopolymers stand as a suitable bio-inspired alternative to PEG, which can be tailored with a variety of beneficial characteristics, such as serving as a lyoprotectant, a receptor target, or stabilizing agent. Recently, the Reineke group published a review article of work with cationic glycopolymers used for gene delivery highlighting their therapeutic benefits like degradability, targeting, and stabil-

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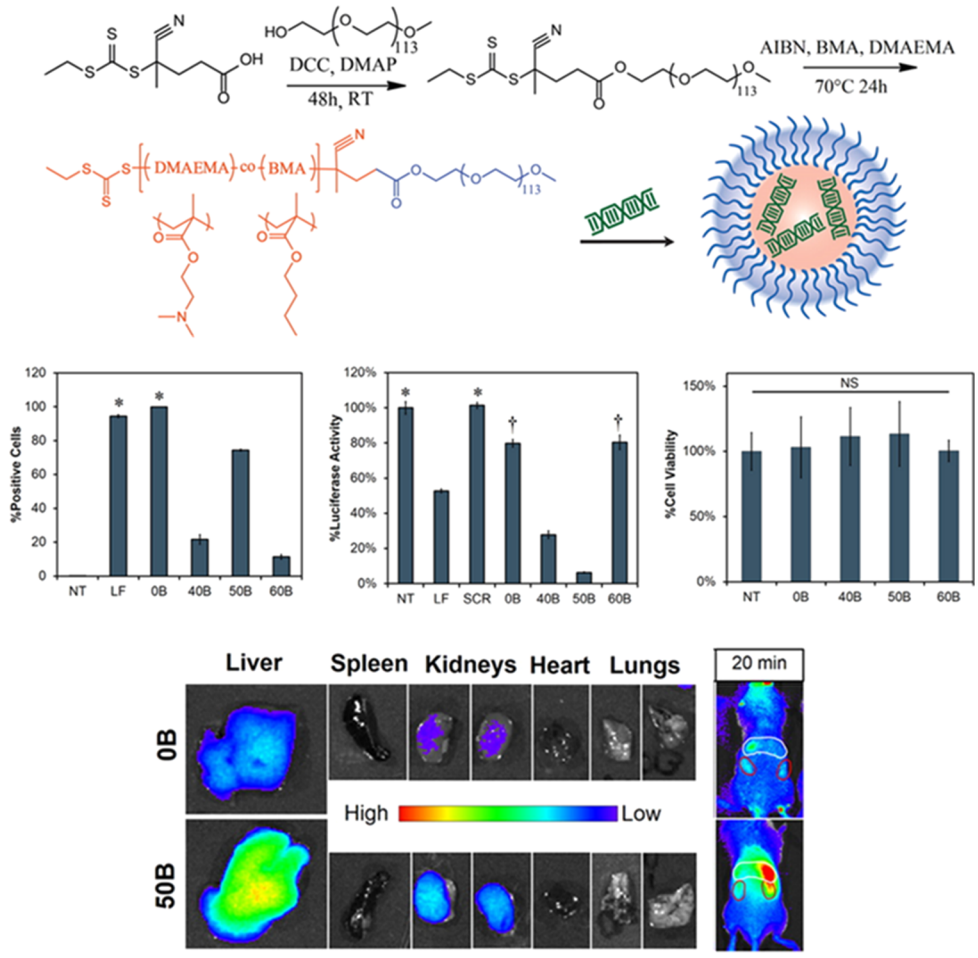

Figure 26. (top) Duvall et al. synthesized a library of PEG-*b*-P(DMAEMA-*co*-BMA) diblock with varying incorporation of the hydrophobic BMA (0-50B) comonomer in the cationic siRNA binding block. (middle) The diblock copolymer with 50 mol % of hydrophobic BMA (50B) showed optimum cell internalization, gene knockdown, and cell viability in vitro. (bottom) The 50B copolymer displayed enhanced tissue biodistribution in vitro due to longer circulation times and slower renal clearance. Reprinted with permission from ref 644. Copyright 2013 American Chemical Society.

ity.[628] The use of glycopolymers thus continues to be an active area of research in our laboratories as well as many others.

3.5. Introducing Hydrophobic Moieties

3.5.1. (Co)polymers with Hydrophobic Moieties. Introducing hydrophobicity has been utilized as a tool to fine tune polymeric vectors in an effort to increase their gene delivery efficiency.[492,629−633] As previously discussed, the requirements for nucleic acid complexation outside and inside the cell are seemingly contradictory. Outside the cell, the vectors must compact and protect DNA from degradation and remain stable against competitive binding from negatively charged proteins present in the plasma. Polymers must also facilitate cellular internalization, as well as endosomal escape. On the other hand, once in the cytosol, the vector must release the nucleic acids, doing this at the right time, for the transfection to occur. Nucleic acid binding must therefore be carefully optimized, and introducing hydrophobicity is one of the parameters in the polymer chemist's toolbox. The type (e.g., linear alkyl, cyclic alkyl, lipidic, aryl, cholesteryl) and content of hydrophobic moieties that are introduced into a vector are critical parameters that must be optimized in a case-by-case scenario; introducing a hydrophobic moiety simultaneously affects several of the various processes that are conducive to a successful transfection. The content of hydrophobic moieties in polymeric vectors has an upper limit since the aqueous solubility of the polymers and colloidal stability of the polyplexes must be ensured. Multiple reviews of the hydrophobic modification of polymeric cations and how it affects nonviral gene delivery are found in the literature.[492,629−633] Our focus in this section is to highlight the key concepts mentioned above with relevant and recent examples.

Incorporating hydrophobic groups into a polymeric vector induces hydrophobic−hydrophobic interactions with nucleic acids that modulate their binding.[492] Additionally, introducing hydrophobic moieties into polycations decreases their charge density, which helps prevent polyplex destabilization by negatively charged proteins present in serum.[634,635] For instance, Bajaj and coworkers show that a tailored hydrophobization of primary and secondary amines of low molecular weight PEIs (M_w 800−2000 Da) with cholesteryl groups afforded vectors that showed a high pDNA transfection efficiency (>60% GFP-positive HeLa cells) even in the presence of up to 50% serum during transfection.[636] This is critical since polycation-based polyplexes exhibit low transfection in the presence of serum, hindering in vivo applications. Polyplexes with an enhanced serum stability display longer circulation times and slower renal clearance.

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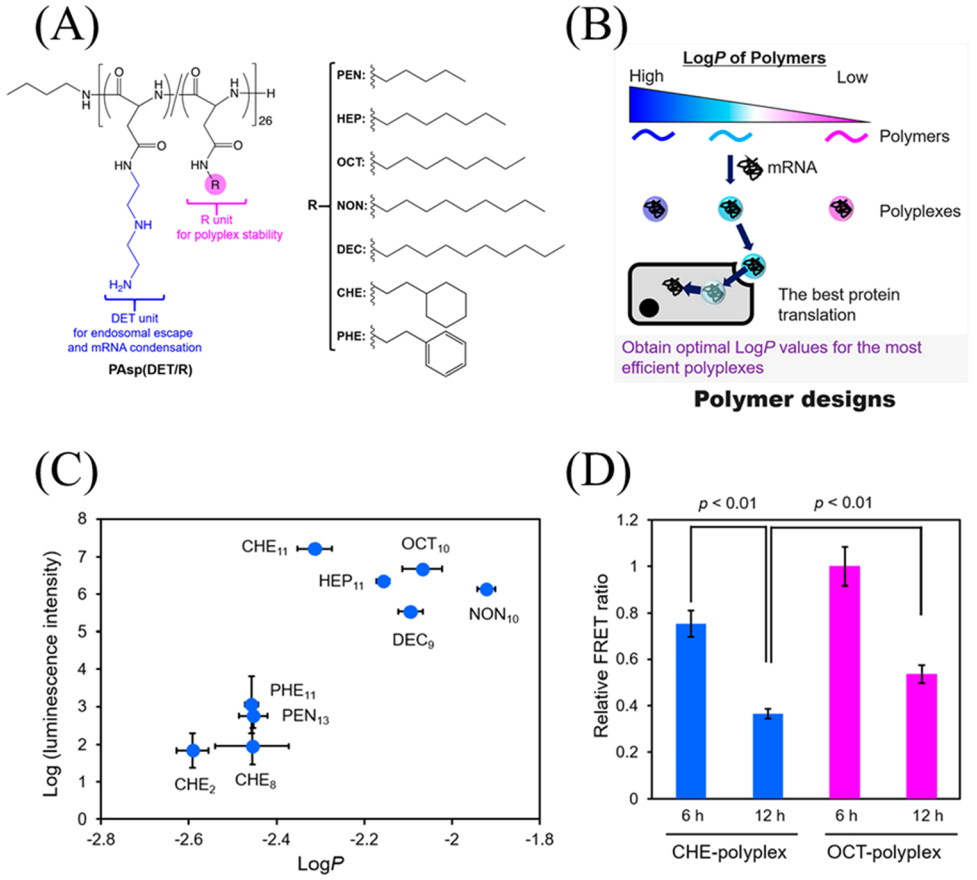

Figure 27. (A) Kataoka et al. synthesized a series of amphiphilic polyaspartamides containing various amounts and types of hydrophobic moieties. (B) They employed LogP as a parameter to measure polymer hydrophobicity and relate it to gene delivery efficiency. (C–D) Derivatives with 11 units of cyclohexyl ethyl (CHE11) hydrophobic pendant groups bind efficiently to mRNA and exhibit high luciferase expression efficiencies in cultured C2C12 cells and fast in vitro transcribed mRNA release within cells. Reprinted with permission from ref 647. Copyright 2019 American Chemical Society.

The different strategies that have been employed to introduce hydrophobic moieties into polycationic vectors fall into one of three categories: post-polymerization modification, copolymerization with hydrophobic monomers, and end-group modification. Low molecular weight PEI is reported as a prime candidate for introducing hydrophobicity to improve its efficiency.[637−643] As discussed above, PEIs of low molecular weight are less cytotoxic than PEIs of higher molecular weight but grant lower transfection efficiencies, which can be improved by different hydrophobic modifications. In studies focusing on the alkylation and acylation of PEI, both the type of the hydrophobic moiety and the length of the alkyl chains have been optimized extensively.

Introducing hydrophobic moieties has also been explored in other cationic polymer systems. For instance, Duvall et al. synthesized diblock copolymers composed of poly[(ethylene glycol)-*b*-[(2-(dimethylamino)ethyl methacrylate)-*co*-(butyl methacrylate)] (PEG-*b*-P(DMAEMA-*co*-BMA)) via a RAFT copolymerization of DMAEMA and BMA using a PEG macro chain transfer agent (macro-CTA) (Figure 26).[644] The obtained vectors showed promising results for the delivery of siRNA in vivo. The molar content of BMA in the nucleic acid forming block was varied from 0 to 75 mol %, and its effect on the formation of micelles, binding of siRNA, cell uptake, transfection efficiency, and cytotoxicity was explored. Cell uptake and transfection efficiencies were evaluated in NIH3T3 fibroblasts, and polymers with 50% BMA showed the greatest transfection efficiency. The high endolysosomal escape ability of this polymer and stability against heparan sulfate contributed to the high performance. In in vivo experiments in Balb/c mice models, the polymer vector with 50% BMA incorporation showed better peptidylprolyl isomerase B gene silencing in the liver, kidneys, and spleen compared with a diblock copolymer with no BMA. The same system was explored for the in vitro delivery of pDNA to MDA-MB1-231 human breast cancer cells and IMDBF dermal fibroblasts.[645]

Engbersen et al.[267] studied the effect of acetylation and benzoylation of bio-reducible poly(amido amines) on the in vitro transfection of COS cells. The polymers were synthesized via Michael addition polymerization of *N*-boc 1,4-diaminobutane with cystamine bis(acrylamide). After cleavage of the boc groups, the primary amines were modified with acetic anhydride or benzoyl chloride, targeting different substitution degrees. Polymers with larger substitution degrees exhibited a reduced charge density and enhanced buffering capacity as observed from lower pK_a values, when compared with unsubstituted polymers. Unlike the acetylated derivatives, the benzoylated polymers self-assembled into nanometric aggregates. The DNA transfection efficiencies with benzoylated PAMAs (poly(amidoamine)s) were higher than the acetylated polymers with comparable degrees of substitution, and moreover they were not affected by the presence of serum during transfection. The more hydrophobic benzoylated

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polymers exhibited both serum protection and enhanced endosomolytic properties, as evaluated via a hemolysis assay.

The quantification of polymer hydrophobicity through partition coefficients or retention times in a high-pressure liquid chromatography (HPLC) analysis is useful when comparing vector libraries that incorporate different types of hydrophobic units.[646,647] For instance, Miyata and Kim et al.[647] showed how the partition coefficient (LogP) can be used as a metric for the hydrophobicity of polymeric vectors during their optimization for gene delivery (Figure 27). A poly(β-benzyl-L-aspartate) parent polymer was synthesized via a ring-opening polymerization of a *N*-carboxy anhydride monomer. Polymers with different hydrophobic groups were synthesized via post-polymerization amidation of the parent polymer with diethylenetriamine and different aliphatic amines. Alkyl amines (from pentyl to dodecyl amine), cyclohexyl ethyl amine (CHE), and phenyl ethyl amine were used. The polymers were labeled with Alexa Fluor 647 to allow for the measuring of the partition coefficient into 1-octanol and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer mixtures using fluorescence spectroscopy. LogP values between −1.9 and −2.6 were observed. All polymers completely condensed luciferase coding mRNA at N/P values greater than 2 in 10 mM HEPES buffer. The polymers containing a CHE substituent with an intermediate value of LogP exhibited greater luciferase expression in mouse myoblast C2C12 and neuroblastoma Neuro-2a cells, when compared to all other polymers. These vectors exhibited an equilibrium of polyplex stability in the extracellular environment and efficient mRNA release after cellular uptake.

Although most studies focus on the optimization of the amount and type of hydrophobic moieties introduced, a recent study suggests that the topology (i.e., how the hydrophobicity is distributed along the polymeric chain) can affect how these polycations are internalized by cells. Perrier et al.[648] synthesized copolymers of di-boc-guanidinoethyl acrylamide with either hydrophilic hydroxyethyl acrylamide or hydrophobic *N*,*N*-dimethyl acrylamide via RAFT polymerization. The copolymers were synthesized in statistical, diblock, or tetrablock topologies. Comparing the statistical copolymer with the homopolymer of guanidinium ethyl acrylamide, it was found that introducing hydrophobicity increases the cell uptake into MDA-MB-231 and Caco2 cells. Regarding the microstructure, the statistical copolymer was internalized more than the diblock and tetrablock copolymers. Cellular trafficking studies revealed that polyplexes based on the statistical copolymer were internalized mainly via an endocytosis pathway, while the diblock copolymer was internalized via a combination of endocytosis and passive membrane crossing. That study suggests that the diblock topology results in polyplexes where the guanidinium groups are more compacted with DNA, thus reducing the overall cellular uptake, due to reduced interactions with the negative cell membrane but potentially allowing for a second mechanism of uptake due to well-defined hydrophobic blocks that can interact with the membrane.

One limitation of introducing hydrophobic moieties via a post-polymerization modification or copolymerization with hydrophobic moieties is the inherent decrease of the charge density, that is, the number of protonatable, or charged, repeating units per polymer chain. Although low charge density is not by itself a disadvantage for the transfection process,[634] synthetic strategies where hydrophobicity can be untethered from charge density are necessary to establish structure−property relationships. As a strategy to overcome this barrier, Khan et al.[649] reported the synthesis of amphiphilic homopolymers in which each repeat unit contains both a hydrophobic moiety and a cationic group via a polymerization modification of poly(glycidyl methacrylate). This strategy afforded a library of homopolymers with a variety of hydrophobic (e.g., aliphatic and aromatic) and cationic (primary amine and guanidine) groups. siRNA gene silencing experiments on HT-29-luc luciferase reporter cells showed that the polyplexes form with a polymer containing pentyl chains and amine cations, at a N/P of 4.5, was more efficient (∼80% luciferase reduction) than all other polymers in the library and linear (∼25% luciferase reduction) and branched (∼40% luciferase reductions) PEI controls. This optimum polymer showed a balance of siRNA binding, release, and low cytotoxicity, which contributed to its high performance.

3.5.2. Polycationic Micelles from Amphiphilic Block Copolymers. Water-soluble polymeric micelles have been widely used in in the field of drug delivery[650−652] and have seen a recent surge in their application for pDNA and siRNA.[653−660] Polycationic micelles, composed of amphiphilic block copolymers that contain cationic and hydrophobic blocks,[661] are core−shell-type nanoparticles that condense nucleic acids into complexes termed "micelleplexes".[662] Some polycationic micelles for gene delivery contain an additional hydrophilic non-ionic block that is incorporated for enhanced colloidal stability. Examples of polycationic micelles with PEI,[663] polypeptides,[664,665] PDMAEMA,[662,666−669] and quaternized PDMAEMA[670] shells, and various hydrophobic, core-forming blocks such as polybutadiene,[671] PS, poly(*n*-butyl methacrylate) (PnBMA),[662,672] and various polyesters such as poly(ε-caprolactone) (PCL)[669] and poly lactic acid (PLA)[664] have been explored. Each of these hydrophobic blocks offers different core properties due to their varying glass transition temperatures: PS forms stiff and glassy micelle cores, while poly(*n*-butyl acrylate) (PnBA) and PnBMA form a rubbery core at room temperature, which has been linked to differences in transfection efficiency.[673] In gene therapy, micelleplexes have been studied for the delivery of DNA,[663,666,671,674,675] siRNA,[654,665,676,677] and miRNA[678] and, recently, as vectors carrying preformed CRISPR/Cas9 ribonucleoproteins.[667]

The non-ergodic, process-dependent, self-assembly of block copolymer amphiphiles presents an opportunity to create a variety of topologies, since micelles with various morphologies, sizes, and aggregation numbers can be obtained through processing changes, even when using the same diblock copolymers.[679,680] Self-assembled micelles exist above a threshold amphiphile concentration termed the critical micelle concentration. For polymeric amphiphiles these critical concentrations can be as low as 10^{-6} to 10^{-7} M, indicating that the micelles remain stable during dilution making them promising candidates for an intravenous administration.[660] Each polycationic micelle is formed by hundreds of block copolymer chains, which, depending on the degree of polymerization of the cationic block, result in cationic shells with ∼10^3 to 10^4 charged groups per micelle. Polycationic micelles and their complexes with nucleic acids used for gene therapy are nanometric (10−100 nm),[652] which has been suggested to increase their internalization efficiency and binding capacity.[681] Polycationic vectors are highly tunable vectors whose size, critical micelle concentration, and aggregation number can be tailored by adjusting the block

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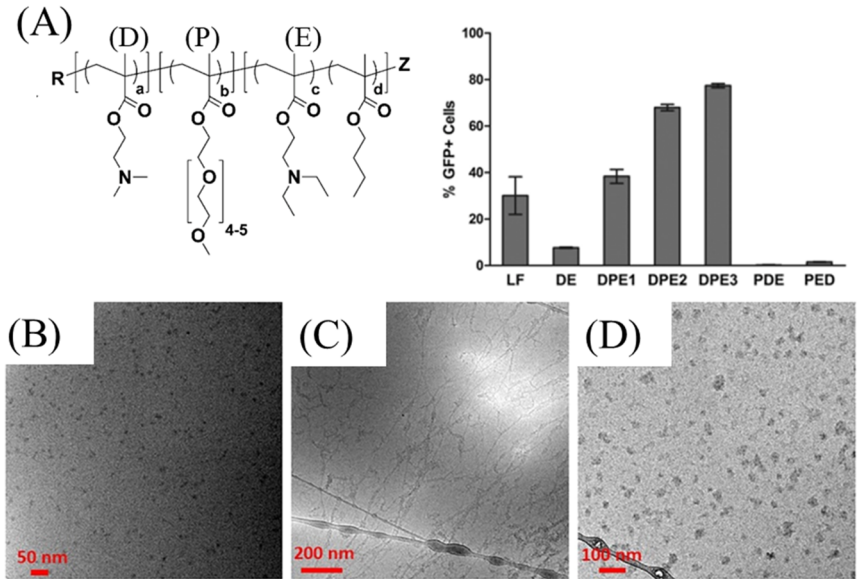

Figure 28. (A) Triblock copolymer blocking order (i.e., DPE, PED, and PDE) and length (DPE1-3) were optimized for the delivery of mRNA to RAW 264.7 macrophages (LF = Lipofectamine control). Reprinted with permission from ref 686. Copyright 2012 Elsevier. (B) Cryo-TEM images of micelles from QPDMAEMA-*b*-PLMA-*b*-POEGMA triblock copolymers and their complexes with (C) long DNA (2000 bp) and (D) short DNAs (113 bp). Reprinted with permission from ref 670. Copyright 2020 American Chemical Society.

copolymer molecular weight, incorporating additional blocks, introducing hydrophilic moieties either in a statistical or block-like fashion, or modifying the micelle end groups.[668,682−685]

Polycationic micelles formed from triblock copolymers containing a non-ionic hydrophilic block—in addition to a cationic and a hydrophobic block—have also been used as components for micelleplex formulations. As discussed in Section 3.4 the introduction of a hydrophilic block reduces toxicity, increases colloidal stability, and increases the circulation time of polyplex formulations.[674,682] These triblock copolymers can be synthesized with different blocking orders (i.e., the spatial organization of the three blocks) that influence the corona properties and, therefore, performance as gene delivery vehicles. Triblock copolymers synthesized with hydrophilic-cationic-hydrophobic,[665−667,682] hydrophilic-hydrophobic-cationic,[670,675] and cationic-hydrophilic-hydrophobic[686] blocking orders have been evaluated as nucleic acid delivery vehicles. For instance, Bryers and coworkers synthesized a series of triblock copolymers composed of cationic PDMAEMA (D), hydrophilic poly(ethylene glycol methacrylate) (PEGMA, P), and hydrophobic P(DEAEMA-*co*-nBMA) (E) blocks with different block lengths and blocking orders: D-P-E, P-E-D, and P-D-E. The performance of micelles formed from these triblock copolymers as mRNA vectors for the transfection of RAW 264.7 macrophages and DC2.4 dendritic cells was compared.[686] All polymers formed polycationic micelles with hydrodynamic diameters between 20 and 30 nm. mRNA micelleplexes formed with the DPE-triblock copolymers exhibit a better transfection efficiency (68% GFP+ cells) than the copolymers with the other two blocking orders (<2% for both PDE and PED), Lipofectamine controls (30%), and a diblock copolymer without a PEGMA block (8% for DE micelleplexes) in the macrophage model. A similar trend was also seen with these systems in DC2.4 dendritic cells (Figure 28A).[686]

Amphiphilic polymeric micelles are used as drug delivery vehicles due to their ability to solubilize hydrophobic drugs in their cores.[650,651] This property has also been extrapolated with micelleplexes, where the simultaneous delivery of therapeutic nucleic acids (condensed around the micelle cationic shells) and small-molecule cancer drugs (encapsulated in the micelle core) can display synergistic effects in cancer therapy.[199,654,656,663,669,675,687,688] Figueiras and coworkers recently reviewed the opportunities and challenges for the use of micelleplexes in these types of therapies.[659]

Morphological studies of pDNA-based micelleplexes have shown that these complexes typically contain more than one micelle per complex and that their size and composition are dictated by structural parameters on the nucleic acids and the cationic block copolymers. Several studies have shown the effect of the length of pDNA in the morphology of the micelleplexes.[670,685] Complexes containing long pDNA (~2000 bp or more) show a beads-on-a-string structure with DNA "threads" wrapped around the micelles via a beads-on-a-string structure resembling chromatin (Figure 28C). Complexes formed with shorter DNAs form spheroidal structures, in which more than one micelle per complex is observed (Figure 28D). In terms of the block copolymer structure, Reineke and coworkers explored the influence of the PEG block length on the morphology and composition of micelleplexes formed between a 2442 bp pDNA and PEG-*b*-PDMAEMA-*b*-PnBMA triblock copolymers.[682] Triblock copolymers with larger PEG blocks (M_n = 10 kDa) formed micelleplexes that, on average, contain fewer micelles and DNA molecules per complex, when compared with micelleplexes formed with triblock copolymers with shorter PEG blocks (M_n = 2 and 5 kDa) or with PDMAEMA-*b*-PnBMA diblock copolymers where the PEG block was absent.

A distinction should be made between the micelleplexes discussed in this section and the polyion complex micelles introduced in Section 3.1.1. PIC micelles assemble during the mixing of double-hydrophilic polycationic block copolymers and nucleic acids, while micelleplexes are formed using pre-assembled polycationic micelles (Figure 29A). Several studies have systematically contrasted the efficiency of micelleplexes, polyion micelle complexes, and polyplexes.[662,666,676] Won and

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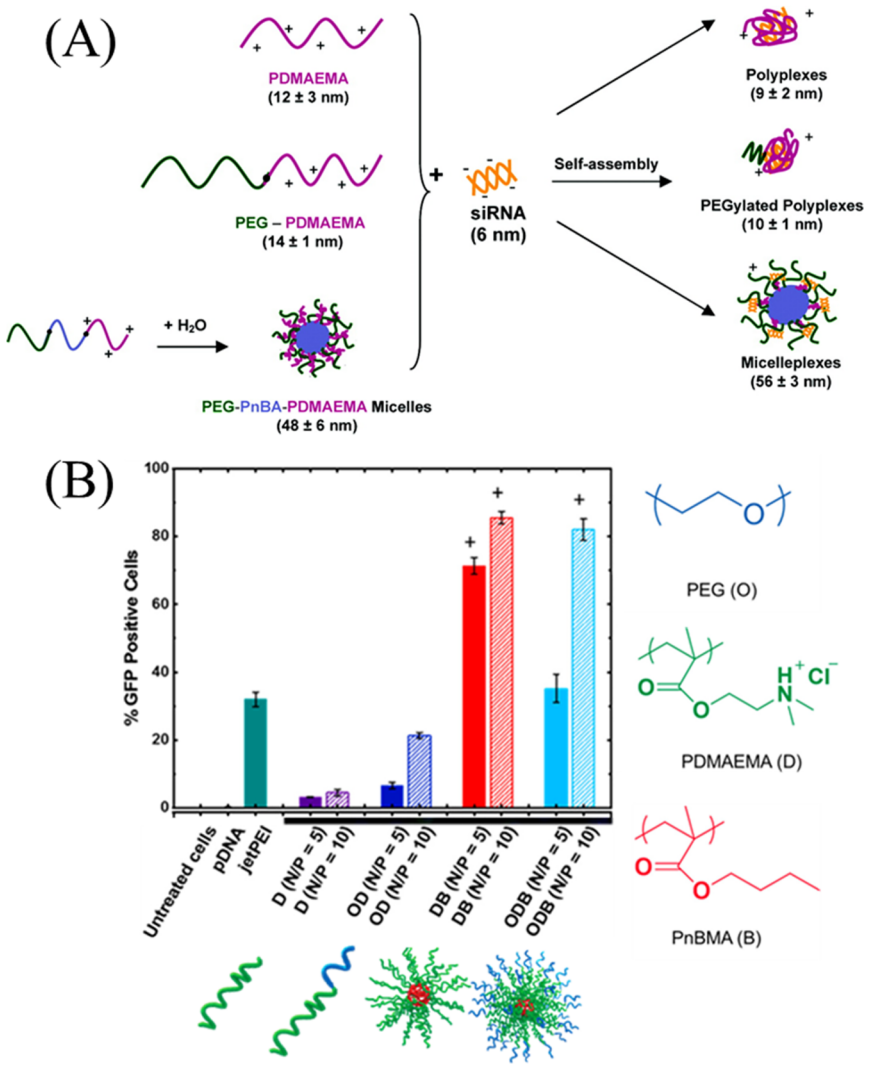

Figure 29. (A) Schematic representation of the formation process of polyplexes, PEGylated polyplexes (PIC micelles), and micelleplexes. Adapted from ref 676. (B) Micelleplexes (DB and ODB) displayed higher pDNA efficiency (% GFP+ cells) than polyplexes (D and OD) and jetPEI® controls for the transfection of HEK293 cells. Reprinted with permission from ref 666. Copyright 2019 American Chemical Society.

coworkers contrasted the efficiency of a PDMAEMA homopolymer (polyplexes), a double-hydrophilic PEG-*b*-PDMAEMA diblock copolymer (PIC micelles), and an amphiphilic PEG-*b*-PnBA-*b*-PDMAEMA triblock copolymer (micelleplexes) (Figure 29A) as either a DNA[662] or siRNA[676] delivery vehicle for the in vivo transfection of HeLa cells. DNA complexes with all polymeric systems exhibited a low transfection performance (<1% GFP positive cells), with the micelleplex formulations having a slightly lower performance than the other two systems. For transfections with micelleplexes containing siRNA, the micelleplexes outperformed the other two systems (23% of GAPDH mRNA silencing vs 14% for the polyplexes and 8% for PIC micelles), although their efficiency was low compared to control Lipofectamine formulations (74% GAPDH mRNA silencing).[676] A recent study from Reineke and coworkers compared the transfection efficiency of HeLa and HEK293T cells with pDNA using either polyplexes (based on either a PDMAEMA homopolymer or a PEG-*b*-PDMAEMA diblock copolymer) or micelleplexes (based on either a PDMAEMA-*b*-PnBMA diblock copolymer or PEG-*b*-PDMAEMA-*b*-PnBMA triblock copolymers).[666] Both micelleplex formulations were shown to outperform the analogous polyplexes (more than fourfold higher % GFP+ cells) (Figure 29B). Micelleplexes displayed higher levels of cell internalization when compared to polyplexes. Additionally, circular dichroism experiments showed that, in micelleplexes, DNA wraps around micelles in a beads-on-a string morphology that preserves the helical DNA native B form, while in tightly bound polyplexes, this structure is distorted, which could contribute to higher levels of GFP expression from the micellplex formulations.

Recently Reineke and coworkers reported micelleplex formulations based on similar micelles (i.e., PDMAEMA-*b*-PnBMA and PEG-*b*-PDMAEMA-*b*-PnBmA) for the gene editing of HEK293T cells with Cas9/guide RNA ribonucleoproteins (RNPs).[667] Because of their negative charge, granted by the guide RNA, RNPs could bind electrostatically to the polycationic micelles to form micelleplexes. Interestingly, the micelleplex formation process was greatly affected by the media in which these complexes were formed. In PBS, small (~30 nm) micelleplexes (containing 14 RNPs per micelleplex) were obtained, while in water, larger (130−160 nm) multimicelleplex particles were obtained with both diblock and triblock copolymer micelles at N/P ratios of 2.5 and 5.

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Micelleplexes formulated in water exhibited a higher gene editing efficiency (40% NHEJ editing) than the PBS formulations (~5%) and a Lipofectamine 2000 control (~22%), which is believed to be due to a faster sedimentation of these larger particles onto the cells.

In summary, pre-assembled polycationic micelles possessing a hydrophobic core are effective vectors for the delivery of nucleic acids, whose use has focused mainly on two areas: (1) the codelivery of therapeutic nucleic acids and small-molecule drugs for cancer gene therapy[659] and (2) the precise characterization of the micelleplex structures to correlate the structure to gene delivery performance often in comparison to polyplexes. The development of micelleplex formulations for gene therapy will continue to exploit concepts from the drug delivery field such as the use of stimuli-responsive and targeting moieties.[689] Ultimately polycationic micelles with highly uniform and reproduceable formulations offer a tunable motif as gene carriers with promising and untapped potential, for instance, in the delivery of new cargos for gene editing.[667]

3.6. Incorporating Stimuli-Responsive Properties

Polyplex formulations experience several environmental changes as they travel through the biological milieu, be it cell culture media or circulatory systems within living organisms. There is a growing recognition that polymers must be engineered to sense changes within the physiological environment and to respond to these changes by rapidly switching between divergent sets of properties. Responsive polyplexes have been designed to respond to a variety of exogenous triggers such as temperature,[690−693] light,[694,695] or ultrasound[696,697] and endogenous signals such as pH,[698−701] reactive oxygen species,[702−707] enzymatic activity,[559,708,709] or changes in redox environments.[710−712] This section is not intended to serve as an exhaustive review of stimuli-responsive polyplexes, and we redirect the readers to more focused reviews.[713−715] Here, we aim to briefly discuss chemical design concepts relevant to pH-responsive, photo-responsive, and redox-responsive polyplexes, with examples selected to reflect our emphasis on chemical synthesis and architectural modifications.

3.6.1. pH-Responsive Polyplexes. Using macromolecules that are pH-responsive is an excellent strategy for designing gene delivery systems to selectively respond to different biological environments. Different organelles and cell types possess a range of pH values, such as standard physiological (7.0−7.4[716]), cytoplasmic (7.4[717]), and endosomal (4.5−6.5[718]). These values can vary significantly depending on the cell type and over the course of an organelle's or cell's life.[719] Moreover, tissues can vary in their extracellular pH values. Tumor tissue has a pH of 6.15−7.4,[716] while gastric pH is 1.7.[720] Systems responsive to changes in pH within these relevant ranges may overcome biological barriers inhibiting effective transgene expression. Typical strategies to create pH responsiveness include (1) incorporation of monomers or functional polymeric backbones whose protonation state is based on pH, (2) cleavable bonds that are common throughout the synthetic literature with some examples highlighted in Section 4.6, including Schiff bases and acetal/ketals, or (3) noncovalent changes in macromolecular structures such as the assembly or disassembly of α-helices or micelles. All of these strategies can be employed to design gene delivery systems that respond rapidly to changes in intracellular pH to effectively deliver nucleic acids. Many examples are described below, but for more comprehensive reviews on pH-responsive nanocarriers for gene delivery we redirect readers to Cho et al.[721] and Park et al.[271] In this section, we will discuss pK_a measurement techniques; then we will focus on pH-responsive strategies to promote endosomal escape and tumor targeting, which represent two key applications of pH-responsive delivery in the literature.

If polymer chemists wish to engineer polymers with pK_a values targeting physiological or endosomal pH, then accurate pK_a measurements of the gene delivery vehicles are essential to determine the protonation state of these polymers in varying cellular environments. The degree of protonation (α) can be determined from the pK_a of an acid and pH of its environment using the Henderson-Hasselbalch equation, $pK_a = pH + \log[\alpha/(1 - \alpha)]$.[722−724] The p$K_a$ of a molecule can be determined through a variety of methods with the most common including acid−base titration and nuclear magnetic resonance (NMR) spectroscopy.[724,725] In acid−base titration, a base is slowly added to a solution of the molecule of interest while monitoring the pH value. Subsequently, the pK_a can be determined using the Henderson-Hasselbalch equation. For pK_a determination using NMR, the change in chemical shifts of nuclei close to the protonation site of the molecule is measured across a range of pH values, and the chemical shifts are compared to the shifts of the fully deprotonated and protonated molecules to determine the pK_a.[725] Titration is often the preferred method due to the simplicity and the ability to do relatively quick pH measurements compared to NMR experiments. Other parameters that can affect pK_a include solvent, solution ionic strength, temperature, and whether the protonatable group is in the form of a monomer or polymer.[724,726] For example, Reineke et al. found that pK_a decreased when a monomer was polymerized, which is due to the unfavorable interactions of charged groups in close proximity to each other.[289] This work highlighted the importance of pK_a measurements that reflect the conditions used in its application. For a more comprehensive perspective on these experiments and other methods for pK_a determination, Reijenga and coworkers have published a review on this topic.[724] In addition to the delivery vehicle pK_a, the intracellular pH is another important factor in gene delivery that can be measured. Intracellular pH can be measured in three ways as outlined in the review by Loiselle et al., including (1) microelectrodes that measure the proton concentration via the electric potential across the probe, (2) NMR measurements that analyze intracellular molecules via pH-dependent NMR shifts, and (3) fluorescence measurements of pH-sensitive fluorophores.[727] The use of pH-sensitive fluorophores is especially useful for understanding intracellular environments and has been widely used in the gene delivery field. Burgess et al. have an excellent review comparing the fluorescent dyes that have been used for intracellular pH measurement.[728]

As previously mentioned in Section 2.4, another widespread strategy for designing polymers to overcome the endosomal escape barrier is to exploit the pH differential between intracellular and endosomal pH or promote interactions between polycations and endosome membranes that result in increased membrane permeability. The former method takes advantage of pH-dependent protonation changes in the polymers that cause osmotic pressure changes and ruptures these vesicles. However, at a physiological pH, the delivery vehicle is protonated to a lower degree, minimizing the cellular

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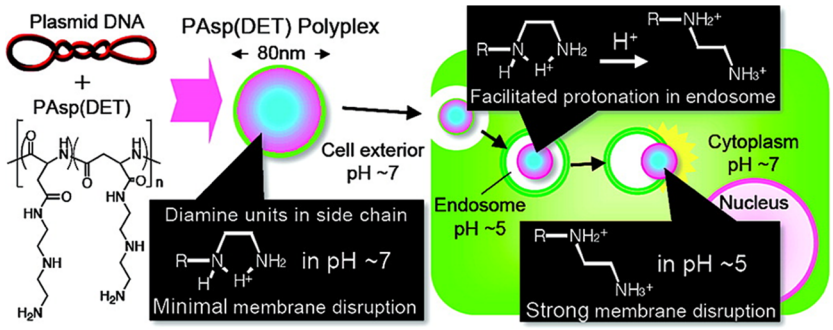

Figure 30. Example of the incorporation of monomers or functional polymeric backbones whose protonation state is based on pH. In this example Asp(DET) is monoprotonated in extracellular conditions but is diprotonated under acidic endosomal conditions causing membrane disruption under these acidic conditions. Reprinted with permission from ref 723. Copyright 2008 American Chemical Society.

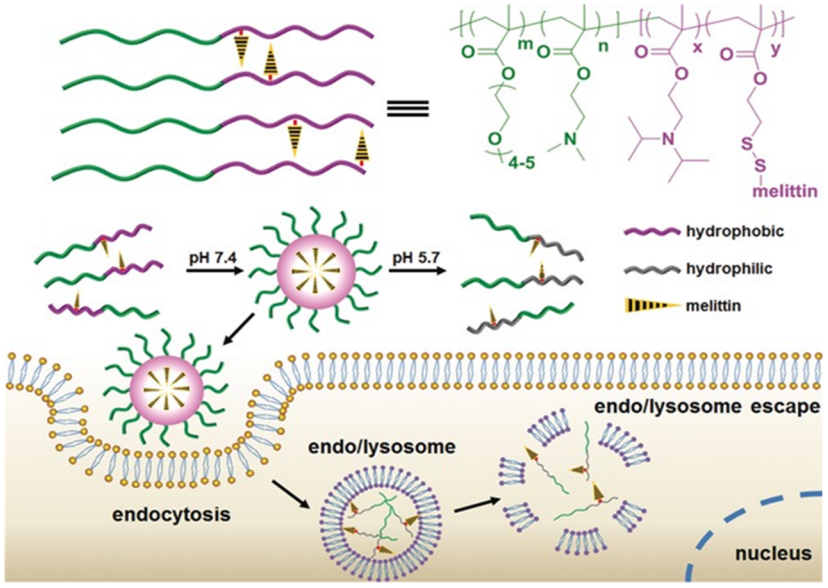

Figure 31. Example of noncovalent changes in macromolecular structures. The micelle dissociates under acidic endosomal conditions revealing melittin, a lytic protein that promotes endosomal release. Reprinted with permission from ref 732. Copyright 2016 John Wiley and Sons.

membrane disruption and toxicity. For example, Kataoka et al. found that, at physiological pH, poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl]aspartamide} (P[Asp(DET)]) was monoprotonated and disrupted the membrane 90% less than at the endosomal pH of 5.5 where it is diprotonated. It was predicted that this membrane disruption, which occurred only under endosomal conditions, led to high transfection efficacy and low toxicity when compared to poly{*N*-[*N*-(3-aminopropyl)-3-aminopropyl]aspartamide} (PAsp-(DPT)), a fully protonated derivative control, and branched polyethylenimine (BPEI) (25 kDa) (Figure 30).[723] This strategy has been used extensively with a wide variety of protonatable delivery systems.[698,729−731]

Another strategy for promoting endosomal escape includes acid-catalyzed degradable polymers that will break down under endosomal conditions. This breakdown is hypothesized to promote endosomal escape, since lower molecular weight polymers bind less strongly to negatively charged nucleic acids[265,733] and tend to be less cytotoxic.[265,462,465] Yin et al. crosslinked low-molecular weight PEI with an acid-sensitive ketal moiety, which would degrade under a reduced endosomal pH.[734] While the crosslinked polymer encapsulated DNA effectively, the degradation of the crosslinks in the endosome allowed for release of the DNA.[734] Many papers have also used this strategy where endosomal conditions degrade the delivery vehicle to promote nucleic acid release.[735,736] In addition, nanoparticles that undergo noncovalent degradation under endosomal conditions can also be used to promote endosomal escape. Bae et al. developed a pH-sensitive diblock that surrounds a PEI/DNA nanoparticle at a physiological pH due to the electrostatic binding between the anionic diblock and cationic PEI/DNA nanoparticle. However, this pH-sensitive diblock detaches from the PEI/DNA nanoparticle once acidified and is neutralized under endosomal conditions allowing the cationic PEI to interact with the membrane for a charge-mediated release.[737]

Responsive peptides, lipids, or micelles can change their macromolecular structure and conformation in a pH-dependent fashion allowing them to interact with endosomal membranes in acidic environments. Pun et al. observed that a virus-inspired polymer for endosomal release (VIPER), improved the GFP expression in HeLa and KB cervical carcinoma cells compared to Lipofectamine and bPEI (Figure 31).[732] Under physiological conditions, the VIPER self-assembled into micelles but dissociated under acidic conditions such as within the endosome. The dissociation also revealed lytic peptides, which could promote delivery to the cytosol. This VIPER system, however, minimally transfected Jurkat and primary T-cells.[738] Pun et al. predicted that VIPER had a poor

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transfection efficiency in T-cells because their endosomes are less acidic than those of HeLa, minimizing micelle dissociation and therefore delivery.[719] Furthermore, pH-responsive fusogenic peptides, typically based on the HA-2 subunit of the influenza virus, are peptides that have the ability to destabilize membranes only at endosomal pH and have been used extensively in drug and gene delivery platforms.[739−741] Hatefi et al. compared some of these pH-responsive peptides for their different properties related to gene delivery.[740] They found that GALA, a peptide comprised of 30 amino acid residues, displayed the highest endosomal membrane disruption and the least cell toxicity.[740] The Szoka lab developed GALA so that, at neutral pH, GALA is water-soluble and a random coil.[742] Because of the glutamic acid residues, under endosomal conditions GALA undergoes a transformation self-assembling into an α-helix with hydrophobic and hydrophilic domains.[742] This α-helix interacts with membranes, destabilizing the membrane, often leading to endosomal escape.[742] Many other examples of GALA being applied to drug and gene delivery systems have been reviewed by Li.[743] Furthermore, we draw attention to recent examples of pH-responsive macromolecules used in gene delivery to improve endosomal escape for a variety of nucleic acid delivery platforms.[744−748]

The dysregulated extracellular environment of a tumor results in an acidic pH and can be used for cancer-targeting gene delivery.[750] Typically, either pH-sensitive protonation or acid-cleavable bonds have been used to effectively target tumor cells by inducing charge-mediated cell membrane disruption only in the acidic tumor cell environment and not under normal physiological conditions. This approach requires delivery vehicles that are sensitive to minute changes between the physiological pH and the intratumoral pH. For example, many of these cleavable bonds are amides that have different neighboring groups to modify the pH at which cleavage is favorable. Guo et al. developed pH-responsive polymer coatings composed of PEI coupled to 1,2-cyclohexanedicarboxylic via amide bonds.[751] These pH-responsive polymers, which were anionic at physiological pH, were used to coat cationic PEI and DNA nanoparticles to minimize cytotoxicity and interactions with healthy cells at physiological pH. Under hypoxic tumor conditions, the polymer would neutralize and detach from the nanoparticles, leaving a cationic nanoparticle, which would then be internalized by tumor cells. Many other groups have also taken advantage of moieties whose charge changes between physiological and extracellular pH for targeted gene delivery with some cited here.[720,752] Li et al. took advantage of a pH-responsive cleavable bond to effectively deliver pDNA to tumor cells. They used an acid-cleavable block polymer of PEG and PAEMA modified with 2,3-dimethylmaleic anhydride (PPD, Figure 32) that contains an amide bond and carboxylic acid groups, which are negatively charged at physiological pH.[749] Under the acidic conditions within a tumor, the authors claim that the amide is cleaved, shedding the carboxylic acid moiety and leaving a positively charged amine. This acid-cleavable polymer, PPD, was coated around their CD-OEI/pDNA polyplex to effectively deliver pDNA. The negatively charged nanoparticle would be able to circumvent the high blood clearance and toxicity of positively charged nanoparticles. After the acid-induced cleavage, the cationic nature of the nanoparticle would enhance charge-mediated cell uptake and endosomal escape for effective and targeted gene delivery (Figure 32). Although this pH-sensitive cleavable bond in PPD has been used

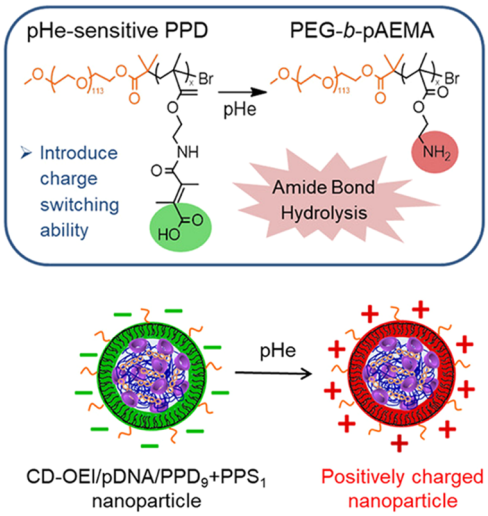

Figure 32. Example of pH-cleavable bonds. Amide bond hydrolysis under acidic extracellular tumor conditions causes the nanoparticle to shift from negatively to positively charged, enhancing tumor cell specificity. Reprinted with permission from ref 749. Copyright 2020 American Chemical Society.

extensively for drug delivery to cancer cells,[653,753−755] there is a great interest in applying this method to gene delivery to either target tumors or to work around the PEGylation dilemma.[561] Similarly, Zhang et al. developed pH-responsive nanoparticles coated with PEI for targeted siRNA delivery to C6 glioma cells.[756] Citraconic anhydride was conjugated to the primary amine groups of PEI, which is acid-cleavable under tumor extracellular pH, causing the charge to shift from neutral to positive. They observed almost no gene silencing at biological pH but greater than 40% gene silencing at the tumor pH (6.2) when dosed with 4 or 8 μg/well of iron in their nanoparticles coated with this pH-responsive PEI.

As we have seen so far, pH-responsive polymers have had a significant impact on both in vitro and in vivo gene delivery thanks to the incorporation of diverse ionizable chemical moieties such as imidazoles, tertiary amines, etc. Apart from spatiotemporal control over the payload release kinetics, these polymers can also be engineered to "sense" the pH physiological environments, thereby serving as a diagnostic aid. To realize the theranostic potential of pH-sensitive polymers, we must work on improving their sensitivity to rapidly detect and respond to minute changes. Finally, most chemists have not considered the "nanobuffering-controlled local pH" wherein polycations display high buffering capacities at close proximities and exert control over the local pH, independently of the global or bulk solution pH.[757] This phenomenon is yet to be exploited in a polymeric gene delivery to improve the sensitivity of pH-responsive vehicles but is expected to improve polyplex delivery performance.[758]

3.6.2. Photoresponsive Polyplexes. Polymers that are responsive to light, typically ultraviolet, near-infrared, or visible light, have been applied for spatially and temporally-targeted delivery. Optical penetration of target tissues is not a hindrance for in vitro applications but presents obstacles during phototherapy, since the light intensity is diminished within a depth of 3.5 mm, depending on the wavelength.[759] Ultraviolet light is typically the most effective in transforming polymer vehicles; however, ultraviolet treatment has a low penetration depth and poses mutagenic concerns.[760] Longer-wavelength,

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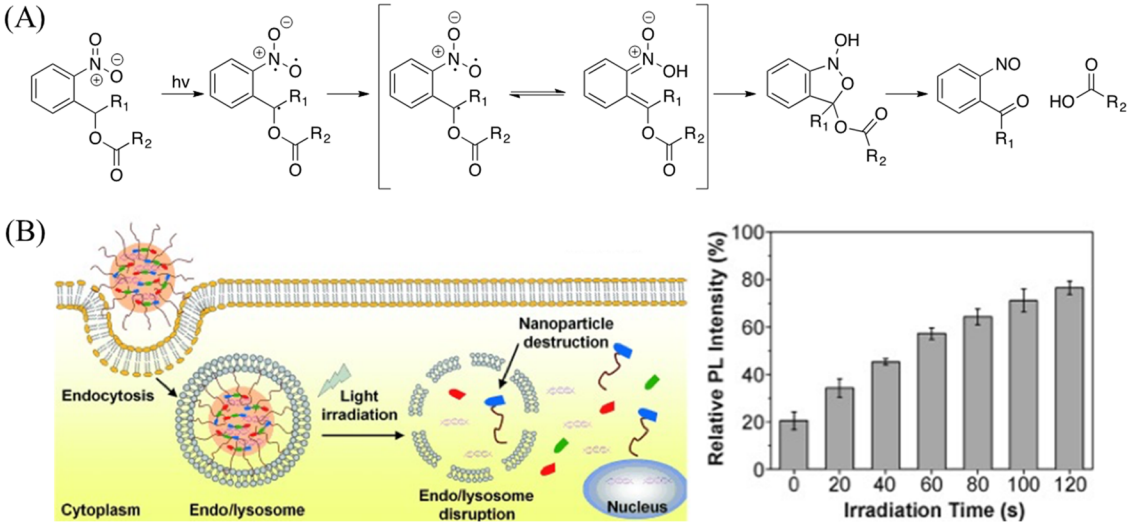

Figure 33. (A) Photoreaction mechanism of *o*-nitrobenzene. Adapted from ref 761. Copyright 2018 John Wiley and Sons. (B) Photoinduced disassembly of polyplex showing DNA release. Dye exclusion assay showing increased release as an increase in irradiation time. Reprinted with permission from ref 762. Copyright 2015 John Wiley and Sons.

lower-energy light like near-infrared and visible light have shown the ability to penetrate human tissue at larger depths; however, they are typically less efficient in triggering responsive motifs. Herein, we describe some examples demonstrating improved delivery of nucleic acids by exploiting the triggered release of payloads in response to ultraviolet, near-infrared, and visible light.

Currently, the most common photolabile linker used for gene delivery is *o*-nitrobenzene (Figure 33A). The research groups of Yin and Chen were able to incorporate *o*-nitrobenzene within the backbone of poly(*β*-amino esters) a system that can be degraded by stimulation with UV light. Both studies showed that both the transfection efficiency and cell viability were higher than their non-degradable control counterparts in mammalian cell types due to the triggered breakdown of the polymer.[763,764] Haag et al. constructed a hyperbranched polyglycerol decorated with an oligoamine pendant using *o*-nitrobenzene linkers. With a 350 nm light induction, they observed a controlled released of DNA (via cleavage of the oligoamine from the polymer backbone).[765] In an in vivo study, Mei et al. demonstrated tumor-targeted delivery in mice via tissue-penetrating near-infrared light. Nanoparticles were decorated with cell-penetrating peptides linked with 4,5-dimethoxy-2-nitrobenzyl groups, which were able to show a tumor-selective accumulation, internalization, and delivery of siRNA by near-infrared light.[766] Epps, Sullivan, and coworkers have shown the development of photo-responsive block polymers for gene delivery by an incorporation of *o*-nitrobenzene on each pendent amine. They are able to form PIC micelles after the complexation of mPEG-*b*-poly(5-(3-(amino)propoxy)-2-nitrobenzyl methacrylate) polymers with nucleic acid cargo.[767] The PEG serves as a stealth corona, while the cationic core contains pendant degradable linkages. Further information on the application of these photo-responsive block copolymers can be found in the focused review by Sullivan et al.[768] The use of *o*-nitrobenzene as a photostimulated degradable backbone or pendent linker cleavage site has shown the benefit of improving release capabilities of cationic polymers in vitro and in vivo.

Visible light provides the inherent advantage of penetrating human tissue while causing less damage than ultraviolet radiation. Hovig et al. co-delivered cationic *β*-cyclodextrin-containing polymers with photosensitizer additives, which initiate photochemical endosomal and lysosomal membrane damage, to enhance the release and delivery of siRNA into osteosarcoma and melanoma cell lines. They found an 80% increase in gene silencing after exposure to 420 nm visible light, attributing siRNA release to endosomal/lysosomal escape.[769] Liu et al. was able to show a similar result by using photosensitizers to increase endosomal/lysosomal escape and DNA unpacking using OEI-based polymers conjugated with an aminoacrylate linker; this system was readily cleaved within the polymer backbone using visible light initiation (Figure 33B).[762] With the use of jetPRIME® as the polymeric delivery vector, Takishima et al. was able to show that a preliminary exposure of cells to blue light inhibited the delivery of pDNA complexes, while unexposed areas still resulted in an uptake in HeLa, HEK293, and HepG2 cell lines. They hypothesized that this technique could facilitate spatioselective delivery by exposing surrounding areas with blue light to destabilize endosomal membranes while leaving the targeted area unexposed.[770] Another technique used a novel silicone-based platform that promoted an increase in surface potential caused by visible light illumination, thus promoting a DNA release and cellular uptake.[771] Although photo-triggered release has been well-documented in the field of drug delivery, this technique is beginning to be implemented for applications in gene therapy with polymers that respond either directly or indirectly with light.[760]

3.6.3. Redox-Responsive Polyplexes. Redox-responsive or "bioreducible" polymers take advantage of the redox gradient existing between the intracellular and extracellular environment.[772] The redox environment is regulated by glutathione, which is a key player in various physiological processes such as shielding against oxidative stress, transporting amino acids, and synthesizing biomacromolecules such as DNA and proteins. Glutathione can exist either in its oxidized disulfide form or its reduced thiol form (GSH), with the latter being more dominant within the cytosol.[773] While

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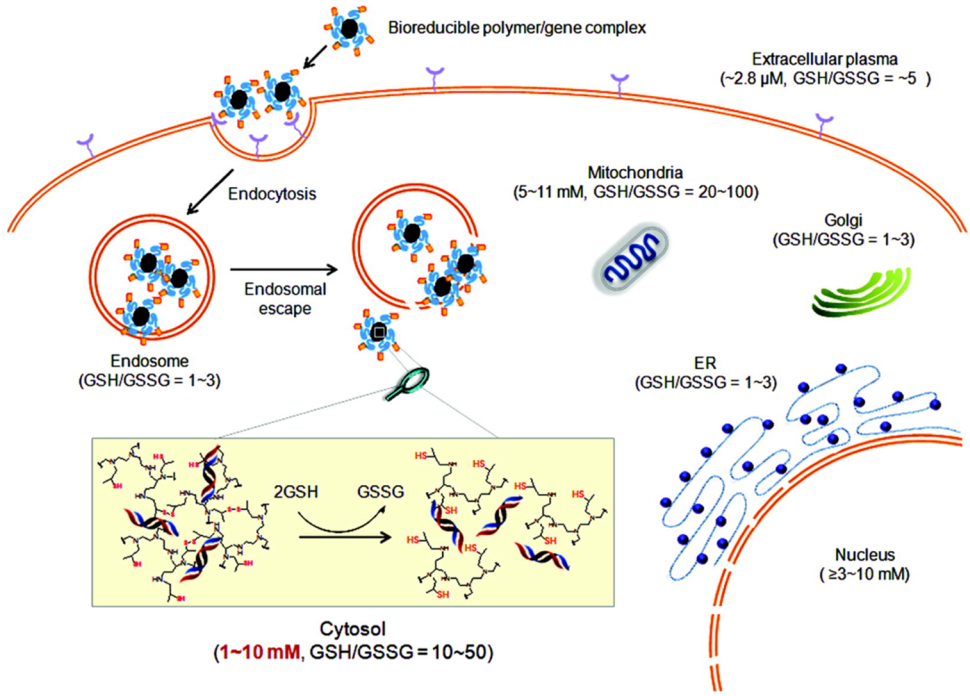

Figure 34. While bioreducible polyplexes are stable in the extracellular environment where glutathione concentration is low (2.8 μM), they undergo rapid degradation within the reducing environment of the cytosol through thiol−disulfide exchanges, which results in nucleic acid unpackaging. Reprinted with permission from ref 775. Copyright 2012 American Chemical Society.

GSH exists in the micromolar concentration range outside the cytosol (Figure 34), cytosolic concentrations range between 1 and 11 mM.[773] This GSH concentration gradient ensures that nucleic acid payloads are strongly bound to polyplexes within the oxidizing extracellular environment.[773] Interestingly, GSH concentration fluctuates widely within various cellular organelles, with the lysosomes and the endoplasmic reticulum offering more oxidative environments and the nucleus offering a more reducing environment than the cytosol. Disulfide chemistry is routinely used to impart bioreducible properties to polymeric vectors. Specifically, when thiol groups within GSH encounter disulfides within polyplexes, thiol−disulfide exchange reactions occur, resulting in new pairs of disulfide and thiol molecules (Figure 34).[774,775] Research on bioreducible polyplexes has disproportionately focused on PAMAs, PEI, PLL, and PDMAEMA, and expanding the scope of disulfide chemistry to other cationic polymers may prove to be interesting.

A thiol−disulfide exchange is inherently biomimetic, since these reactions are critical in protein folding, enzymatic activity, and metabolic processes. There are several advantages in employing disulfide chemistry: (1) it is orthogonal to other bioconjugation schemes, allowing for the incorporation of several other chemical moieties (targeting ligands, pH-responsiveness, PEG, etc.), resulting in multifunctional dual-responsive polyplexes. (2) Covalent bonds are formed in a reversible manner under physiological conditions. Since the reaction kinetics are swift (half-life of 2 h in the cytosol), the polyplex disassembly and payload unpackaging proceed rapidly. (3) Since GSH is a weak acid, free thiols are unavailable even under slightly acidic conditions, effectively inhibiting the reaction in non-cytosolic environments. Apart from being highly pH-specific, the reaction rate can be decelerated by using sterically hindered disulfides[776] and accelerated by using highly charged disulfides. Notwithstanding the numerous advantages of disulfide chemistry, several groups have explored the use of diselenide bonds[777] instead of disulfide bonds since the former are more labile and can be cleaved more readily. A recent paper[778] explored the use of zinc(II) coordinative modules to transform low molecular weight PEI from a weakly binding inefficient vehicle into a bioreducible vector that could transfect challenging cell types efficiently.

We note that disulfide chemistry has become almost ubiquitous in recently published reports on polymeric gene delivery and redirect readers to some excellent in-depth reviews on synthetic methodologies.[773,775,779] We must caution readers that, although disulfide exchange is a universally deployed synthetic strategy, mechanistic studies on the exact role of bioreducible functionalities are rare.[780] Oupicky and coworkers have addressed this knowledge gap by probing the mechanisms through which bioreducible polyplexes outperform non-bioreducible structures. In an interesting report, they discovered that variations in GSH concentration can modulate the efficacy of reducible polyplexes. Importantly, they observed payload-specific effects, with improved transfection of mRNA polyplexes when bioreducible functionalities are incorporated, but no clear benefit for pDNA, oligonucleotides, and siRNA payloads.[781] In a similar study, they varied the degree of disulfide incorporation within PAMAs and noted that, although disulfide-rich polymers promoted DNA transfection levels by enhancing the membrane uptake, there was no difference in the experimentally determined intracellular DNA release rates between reducible and non-reducible formulations.[782] Oupicky and Mao employed atomic force microscopy to capture the depolymerization process through which DNA payloads were released by bioreducible polyamidoamines.[783] Wagner and coworkers synthesized sequence-controlled lipo-oligomers with a controlled placement of redox-responsive functionalities and showed that reducible polyplexes showed

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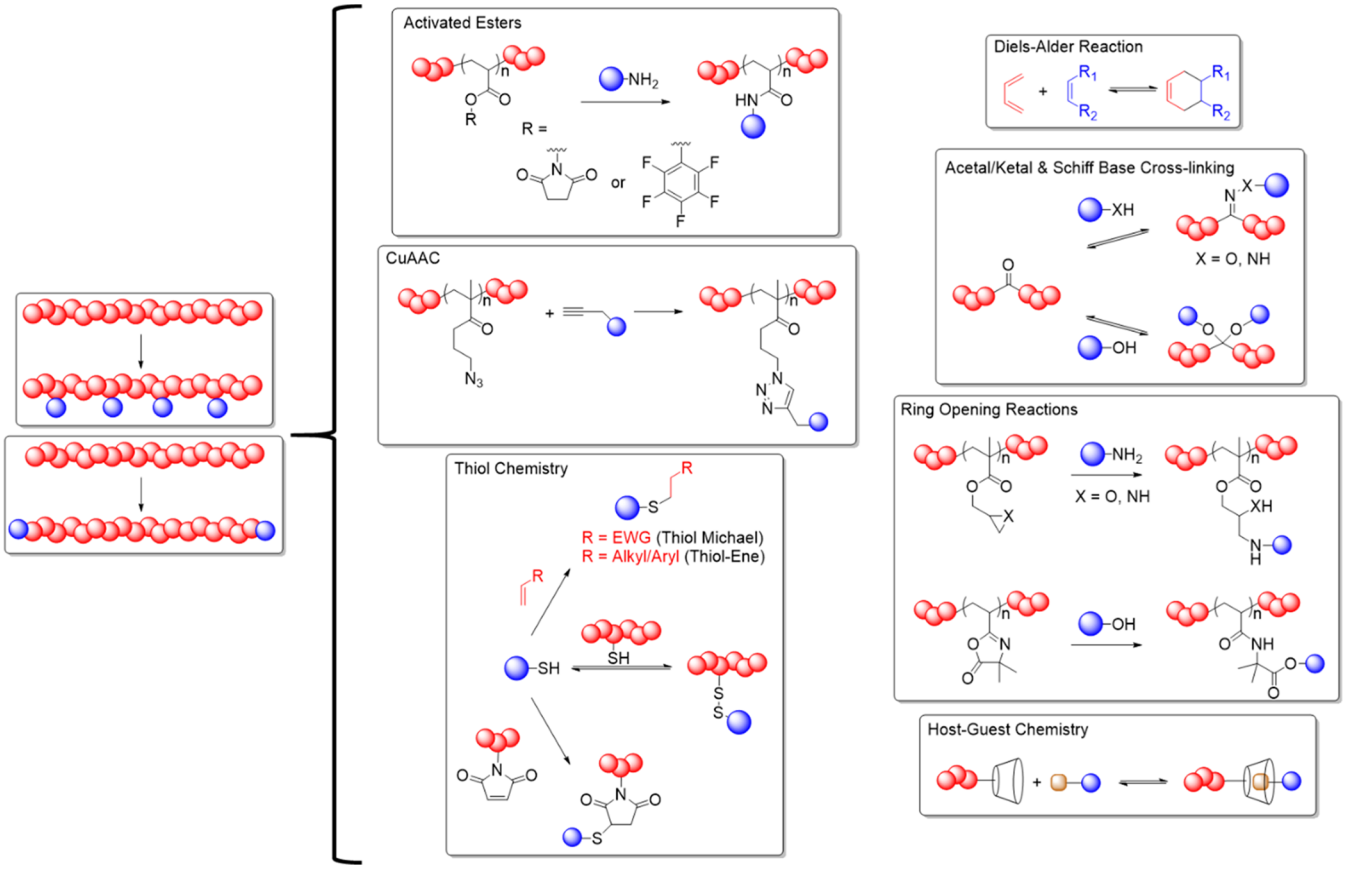

Figure 35. Scheme of main polymer functionalization techniques. Polymers can be functionalized through reactive monomers, end-group modifications, or cross-linking.

more efficient gene silencing than their non-reducing counterparts.[774] Wagner's group also developed a PLL−PEG polymer that incorporated an endosmolytic peptide and covalently conjugated this polymer to siRNA via disulfide bonds, ensuring that a payload disassembly occurred only when both heparin and glutathione were present.[784] Oupicky and coworkers also developed similar conjugated polyplexes, wherein thiol-functionalized siRNA and a polymeric inhibitor (Plerixafor) of the chemokine receptor type 4 were coupled.[785] Narain and coworkers prepared galactose-based hyperbranched polymers by incorporating a disulfide-based monomer and observed that bioreducible polyplexes achieved silencing of the epidermal growth factor receptor that was twice as high as that of Lipofectamine.[786] The combination of fluorination with bioreducibility has also proven to be an effective strategy for imparting serum stability as well as improving cytosolic delivery.[787−789] In these studies, cationic polymers were conjugated to fluorocarbon chains, facilitating the assembly of micelles with a fluorinated core and a polycationic corona. Subsequent DNA condensation was accompanied by increased size and extremely high DNA binding affinities even at N/P ratios as low as 1.[788] In addition to a lowered toxicity, these micelles were able to achieve almost 90% gene silencing in vivo due to the incorporation of bioreducible linkages, in contrast to the 30% silencing achieved by non-fluorinated and non-reducible equivalents.[787] Reversible shielding and PEG shedding can be accomplished by engineering block copolymers incorporating a PEG block as well as cationic polymers linked through disulfide bonds.[790,791] While both stably and reversibly shielded polyplexes exhibited more than 80% cell viability and were demonstrated to be colloidally stable in ionic strengths as high as 150 mM, reversibly shielded polyplexes exhibited 28 times higher pDNA transfection efficacy as compared to stably shielded controls. Collectively, this work shows how glutathione-triggered degradation has been combined with other design elements such as hydrophobicity and PEGylation to improve polyplex properties. Future work should focus on a systematic variation of not only the degree of incorporation of disulfide bonds but also the spatial organization of bioreducible functionalities within the polymer in order to probe the relationship between DNA release rates and transgene expression.

In summary, we have briefly outlined some synthetic pathways for introducing pH-responsive, light-responsive, and redox-responsive functionalities within polyplexes. Inspired by examples from drug delivery, many researchers have creatively combined multiple chemical functionalities to generate dual-/multi-stimuli-responsive polyplexes.[792,793] These design approaches exploit the coexistence of multiple triggers within the physiological environment (e.g., pH and redox gradients are both present within tumors) to further improve delivery performance and circumvent biological barriers. While we do not discuss dual-/multi-stimuli-responsive polyplexes here, we redirect the reader to some recent examples in the gene delivery literature.[729,794−803] Interestingly, many of these studies apply dual-/multi-stimuli-responsive polyplexes to co-deliver dual payloads consisting of drugs and nucleic acids, especially for treating drug-resistant cancers. We believe that these multifunctional design approaches will be more widely applied in the future, further driving the evolution of

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Table 1. Select Examples of Chemical Modifications to Polymers Either through Functional Monomers, Functional Backbones, or Telechelic Modifications (Indicated by "Topology")[a]

chemical modification	polymer	topology	cargo	purpose (s)	refs
Activated Esters	Fluorinated PPI	Reactive Monomers	DNA	Improved Transfection Efficiency Reduced Cytotoxicity	416,814
	Chitosan + Folate	Functionalized Backbone	DNA	Improved Transfection Efficiency Reduced Cytotoxicity	818
	Chitosan + Imidazole	Functionalized Backbone	siRNA	Cell Targeting	816
	Chitosan + Stearic acid	Functionalized Backbone	DNA	Cell Targeting	822
	Chitosan + poly(propyl acrylic acid)	Functionalized Backbone	DNA	Improved Transfection Efficiency Reduced Cytotoxicity	819
	Chitosan + PEG	Functionalized Backbone	DNA	Improved Transfection Efficiency Reduced Cytotoxicity	820
	Chitosan + Lactobionic acid	Functionalized Backbone	DNA	Cell Targeting	815
	Poly(alkyl amines) from Poly(MAS)	Reactive Monomers	DNA	Improved Transfection Efficiency Reduced Cytotoxicity	812
	Peptide-PLL	Functionalized Backbone	DNA	Cell Targeting	840
	PEI−PEG-peptide	Functionalized Backbone	DNA	Cell Targeting	841
	PEI + PEG-NLS	Functionalized Backbone	DNA	Cargo Release Cell Internalization Improved Complexation	803
	Poly(NHSA-*co*-*N*-vinylpyrrolidone)	Reactive Monomers	DNA Oligos	Improved Complexation	842,843
	PPFPMA-*b*-PEGMEMA Nanogels	Reactive Monomers	siRNA	Cell Targeting Cargo Release Reduced Toxicity	828−831
	PEG-*b*-P[Asp(DET)] + Cholesterol	Telechelic Backbone	DNA	Cell Targeting Cargo Release Improved Complexation	844,845
	Functionalized PEI	Reactive Monomers	DNA	Cell Targeting Cargo Release Reduced Toxicity	846
	P(HPMA-*r*-APMA-*b*-DMAPMA)	Reactive Monomers	siRNA	Cell Targeting Improved Complexation	847
	Poly[DMAEMA-*b*-(DMAEMA-*r*-nBMA-*r*-acrylic acid)]	Telechelic Backbone	siRNA	Cell Targeting Improved Complexation	848
	PEG-*b*-P(VBC-*co*-PFPA)	Reactive Monomers	Dye Codelivery	Cell Targeting Theranostics	827
CuAAC	P(HEMA-*co*-HEMA-PPA) + PDMAEMA-N_3	Reactive Monomers	DNA	Reduced toxicity Improved Complexation	438
	Trehalose/CD/Glycofect	Click Polymerization	DNA/ siRNA	Reduced toxicity Improved Complexation	849
	CD-OEI	Click Polymerization	DNA	Reduced toxicity Improved Complexation	627,850
	PDMAEMA "sunflowers"	Click Polymerization	DNA	Reduced toxicity Improved Complexation	851,852
	P[AzEMA-*b*-DMAEMA-*b*-(DMAEMA-*co*-nBMA-*co*-PrAA)] Micelles + Mannose	Reactive Monomers	DNA	Cell Targeting Reduced toxicity	853,854
	CD-OEI	Reactive Monomers	DNA	Improved Transfection Efficiency	855
	PAA-RGD/PEG-RGD Hydrogels	Telechelic Backbone	DNA	Reduced toxicity	856
	γ-(4-propargyloxybenzyl)-L-glutamic acid based *N*-carboxyanhydride	Reactive Monomers	DNA	Cell Penetration Improved Transfection Efficiency	857
	PLGA−PEGMA-Folate	Telechelic Backbones	DNA	Cell Targeting Cargo Release Reduced Toxicity	858
	PHEMA, PDMAEMA	Reactive Monomers	DNA	Cell Internalization Reduced Toxicity	438
	PHPMA + Carbohydrates	Telechelic Backbone	siRNA	Cell Targeting	859
	Cross-linked PEI	Functionalized Backbone	DNA	Cargo Release Reduced Toxicity	860
	Poly(lactide-*co*-TMCC)-*g*-PEG micelles	Telechelic Backbone	Antibody/ siRNA	Theranostics Cargo Release	861
	Ketalized PEG + Galactose	Reactive Monomers	DNA	Cargo Release Cell Targeting Endosomal Escape	862
	TAPP Star-polypeptide	Reactive Monomers	DNA	Endosomal Escape	863
	Fluorinated Poly(Glutamate)	Reactive Monomers	siRNA	Cargo Release Cell Internalization	864
	PLA-*g*-PEG + Peptide	Reactive Monomers, Telechelic Backbone	Dye	Theranostics Cell Internalization	865
Disulfides	PLL-linked protein	Functionalized Backbone	DNA	Cargo Release Cell Targeting	866,867
	PLL-linked peptide	Functionalized, Telechelic Backbone	DNA	Cargo Release Cell Targeting	868

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Table 1. continued

chemical modification	polymer	topology	cargo	purpose (s)	refs
	PEG−PLL Micelles	Telechelic Backbone	DNA	Cargo Release Endosomal Escape	367,368,869
	PLL-linked antibody	Functionalized, Telechelic Backbone	DNA	Cargo Release Cell Targeting	870
	PLL	Functionalized Backbone	DNA	Cell Penetration Reduced Toxicity	871
	Cross-linked PEI	Functionalized Backbone	DNA	Cargo Release Reduced Toxicity	860
	Cross-linked PEI	Functionalized Backbone	DNA	Cargo Release Reduced Toxicity	872,873
	Disulfide-linked siRNA + PEI	Telechelic Backbone	siRNA	Cargo Release Endosomal Escape	874
	PEI-linked siRNA	Functionalized Backbone	siRNA	Cargo Release Endosomal Escape	875
	PEI−PEG-linked peptide	Functionalized, Telechelic Backbone	DNA	Cargo Release Endosomal Escape	791,876,878
	Cross-linked PEI + peptide	Functionalized Backbone	miRNA	Cell Targeting Cargo Release Endosomal Escape	879
	Disulfide-linked PEG−PLA-PEI Micelles	Functionalized Backbone	miRNA	Cargo Release Endosomal Escape	880
	Disulfide linked PEI-siRNA	Functionalized Backbone	siRNA	Cell Penetration Cargo Release Endosomal Escape	881
	Ab-linked PEG−PEI	Functionalized Backbone	DNA	Cargo Release Cell Targeting	882
	VIPER-Melittin	Reactive Monomers	DNA	Endosomal Escape Cargo Release	732,883
	Cross-linked poly(amido amines)	Telechelic backbone	DNA	Improved Transfection Efficiency Reduced Toxicity	884−887
	Cross-linked poly(amido ethylenimines)	Functionalized Backbone	siRNA	Cargo Release Endosomal Escape	888,889
	PGMA-lipoic acid	Functionalized Backbone	ssDNA/ DOX	Cargo Release Cell Targeting Co-delivery	890
	Poly(GMA-lactide) Nanogels	Reactive Monomers	DNA/ siRNA	Cell Penetration Cargo Release Endosomal Escape	891
	Disulfide-linked PCL−PDMA	Functionalized Backbone	ssDNA/ DOX	Cargo Release Cell Targeting Co-delivery	668
	Disulfide-linked PCL-*b*-poly((GMA-tetraethylenepentamine)-*st*-OEGMA))	Functionalized Backbone	DNA	Endosomal Escape Cargo Release	892
	Disulfide-linked PAsp-siRNA	Reactive Monomers	siRNA	Cargo Release Endosomal Escape	893
	Folate-PAsp-PEI-Cysteine	Telechelic Backbone	RNA	Cargo Release Cell Targeting	894
	Cross-linked PDMAEMA	Telechelic Backbone	DNA	Cargo Release Reduced Toxicity	895
	PEG-*b*-PDMAEMA star copolymers	Reactive Monomers	siRNA	Cell Internalization Improved Transfection Efficiency	430
	Disulfide-linked PEG-siRNA	Telechelic Backbone	siRNA	Cargo Release Endosomal Escape	896
	Disulfide-linked PEG-siRNA	Functional Backbone	siRNA	Improved Transfection Efficiency Reduced Toxicity	897−900
	PPEGA-siRNA conjugates	Telechelic Backbone	siRNA	Cargo Release Cell Targeting	901
	Poly(cystamine bisacrylamide-diaminohexane) + peptide	Functionalized Backbone	siRNA	Cargo Release Endosomal Escape	902
	HPMA	Reactive Monomers	DNA Oligos	Cell Internalization	903
	Poly(GlcNAc methacrylate)-siRNA conjugates	Telechelic Backbone	siRNA	Improved Complexation	904
	Gelatin-SH + Polymerized siRNA	Reactive Monomers	siRNA	Cargo Release Cell Targeting Reduced Toxicity	905
	HA	Reactive Monomers	siRNA	Cargo Release Cell Targeting Reduced Toxicity	906
Thiol−Ene	Gal-peptide-PLL	Functionalized Backbone	DNA	Cargo Release Cell Targeting	868
	PEG−PEI	Functionalized/Telechelic Backbone	DNA	Cargo Release Endosomal Escape	907
	Chitosan-protein	Telechelic Backbone	DNA	Cargo Release Reduced Toxicity	908
	PEG-poly lactide + DEAET	Reactive Monomers	DNA	Cargo Release Reduced Toxicity Endosomal Escape	909,910
	PEG-lipopeptide	Functionalized Backbone	DNA	Cargo Release Cell Targeting	911
	PAMAM + PEI + PPI	Functionalized Backbone	DNA	Cargo Release Cell Targeting	912

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Table 1. continued

chemical modification	polymer	topology	cargo	purpose (s)	refs
	PAMAM−PEG + peptide	Functionalized Backbone	DNA	Cargo Release Cell Targeting	913−915
	PMMA, PNIPAM, PHPMA	Reactive Monomers/Telechelic Backbone	DNA	Improved Complexation	916
	PLA + Alkyl Amines	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency	22,909
	PEI−PEG-peptide	Functionalized Backbone	DNA	Cargo Release Cell Targeting	917
	PEI−PEG-peptide	Functionalized Backbone	DNA	Cargo Release Cell Targeting	918
Thiol−Michael	PEG-siRNA Micelles	Telechelic Backbone	siRNA	Cargo Release Cell Targeting Endosomal Escape	384,389
Thiol−Yne	Poly(Tyr-alkyne)-*g*-(2-aminoethanethiol)$_2$	Reactive Monomers	DNA	Cell Penetration Reduced Toxicity	919
	Poly(2-ethyl-2-oxazoline)	Functionalized Backbone	DNA	Improved Complexation Improved Transfection Efficiency	920
	Thioether cationic lipids	Functionalized Backbone	DNA/siRNA	Cell Internalization Improved Complexation	921
Diels−Alder	PEG-*b*-poly(styrene-*alt*-maleic anhydride) Nanogels	Reactive Monomers	DOX	Cargo Release	922
	PEGMEMA-poly(furfuryl methacrylate) Hydrogels	Telechelic Backbone	FITC-BSA	Theranostics Cargo Release	923
	PEG, PPG	Telechelic Backbone	Antibody	Theranostics Cargo Release	924
	Poly(lactide-*co*-TMCC)-*g*-PEG micelles	Telechelic Backbone	Antibody/siRNA	Theranostics Cargo Release	861
Acetals/Ketals	Core-cross-linked star Nanogels	Reactive Monomers	DNA	Endosomal Escape	925
	Polyketal + chloroquine	Functionalized Backbone	siRNA	Endosomal Escape	926
	Ketalized poly(*β*-amino ester)	Functionalized Backbone	DNA/siRNA	Endosomal Escape	927,928
	Dendritic polyglycerol-PEI	Functionalized Backbone	siRNA	Cargo Release Endosomal Escape	929
	Ketalized PEG	Reactive Monomers, Functionalized Backbone	DNA	Cargo Release Endosomal Escape Improved Transfection Efficiency	930,931
	OEI + cross-linked acetals	Functionalized Backbone	DNA	Cargo Release Endosomal Escape	932,933
	P(nBMA-DMAEMA)-PEG	Reactive Monomers	DNA	Cargo Release Endosomal Escape	934
	Ketalized PLL	Reactive Monomers	DNA	Cargo Release Endosomal Escape	935
	Ketalized PEG + Galactose	Reactive Monomers	DNA	Cargo Release Cell Targeting Endosomal Escape	862
	Ketalized PEI	Reactive Monomers	DNA/siRNA	Endosomal Escape	936,937
Hydrazones	Poly(ethylenimine-*b*-EAA-*b*-nBMA) + cationic hydrazone grafting	Reactive Monomers	siRNA	Endosomal Escape	938
	Poly(acryloyl hydrazides)	Reactive Monomers	siRNA	Endosomal Escape	939
	PEG−PEI-peptide + DOX	Reactive Monomers	DNA/DOX	Endosomal Escape	940
	PEI-DOX, Folate	Functionalized backbones, Telechelic backbones	siRNA/DOX	Dual Delivery Endosomal Escape	941
Oximes	PEG−PHLG Star Polymer	Reactive Monomers	siRNA	Endosomal Escape	942
Ring Opening: (Epoxides)	Poly(GMA-oligoamine), poly(GMA-TEPA)-*b*-POEGMA-peptide	Reactive Monomers, Telechelic backbones	DNA	Cell Targeting	443,943
	PEG-P[Asp(DET)] + Ca/PO_4 nanoparticles	Reactive Monomers	siRNA	Endosomal Escape Reduced Toxicity Cargo Release	944
	Poly(lactones)	Reactive Monomers	siRNA	Improved Complexation Improved Transfection Efficiency	945
	Epoxide-derived nanogels	Reactive Monomers	DNA	Imaging Theranostics	946
	Poly(GMA)-*g*-DMEA/DMBA/FITC	Reactive Monomers	DNA/Dye	Theranostics Improved Complexation	947
	PGMA	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency	423
	CD-conjugated PGMA	Reactive Monomers	DNA	Theranostics Improved Transfection Efficiency	948
	Poly(AEA-*b*-styrene) Anionic Nanorods	Reactive Monomers, Telechelic Backbones	siRNA	Theranostics Improved Transfection Efficiency	949

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Table 1. continued

chemical modification	polymer	topology	cargo	purpose (s)	refs
	Disulfide-linked Silica nanoparticles-PGMA	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency	950
	Functionalized PGMA	Reactive Monomers, Telechelic Backbones	DNA	Reduced Toxicity Improved Complexation Improved Transfection Efficiency	951−959
	PGMA-based Glycopolymers	Reactive Monomers	siRNA	Reduced Toxicity Improved Complexation Improved Transfection Efficiency	960
	Pullulan-based PGMA	Reactive Monomers	DNA/ lncRNA	Reduced Toxicity Improved Complexation Improved Transfection Efficiency MRI Imaging	961,962
	PCL−PGMA	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency	963
	Aminated PGMA-*g*-CD + Gd^{3+}	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency MRI Function	964,965
	BIP-terminated PGMA	Reactive Monomers	DNA/CPT	Gene/Drug Co-delivery	966
	PLGA + PLLA	Reactive Monomers	DNA/DOX	Gene/Drug Co-delivery	967
	POSS-derived stars + PDMAEMA/PMPD	Star polymers	DNA/DOX	Gene/Drug Co-delivery Cell Targeting Reduced Toxicity	968,969
	PEG−PEI, peptide	Star polymers	DNA	Cell Targeting Reduced Toxicity	970
Ring Opening: (Azlactones)	Functionalized PVDMA	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency Cargo Release	971−975
Ring Opening: (Thiolactones)	Cationic polymer/lipidoid library	Reactive Monomers	DNA	Reduced Toxicity Improved Complexation Improved Transfection Efficiency	976,977
Host-Guest: Chemistry	PDMAEMA-*b*-*P*(DMAEMA-*r*-BMA-*r*-PAA) + biotin/ avidin micelles	Telechelic Backbone	siRNA	Cell Internalization Improved Transfection Efficiency	978
	Dextran-spermine + β-galactosylated cucurbituril	Reactive Monomers	DNA	Cell Targeting Reduced Toxicity	979
	Hyperbranched polyglycerol + β-CD library	Telechelic Backbone	DNA	Cell Targeting Reduced Toxicity	980
	β-CD + polycations	Telechelic Backbone	DNA	Cell Targeting Reduced Toxicity	525
	PAMAM + β-CD-PEI	Telechelic Backbone	N/A	Improved Biodistribution Cell Targeting Reduced Toxicity	981
	PGMA + β-CD	Telechelic Backbone	N/A	Improved Complexation Improved Transfection Efficiency	955
	PEG-adamantyl + β-CD-MPC	Telechelic Backbone	DNA	Cell Targeting Reduced Toxicity	982
	PGMA-adamantyl + β-CD amine conjugates	Reactive Monomers	DNA	Reduced Toxicity Improved Complexation Improved Transfection Efficiency	983−985
	Hyperbranched PGMA + β-CD	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency	986,987
	PEI + β-CD	Functionalized Backbone	DNA/DOX	Gene/Drug Co-delivery	988
	PEI + β-CD	Functionalized Backbone	shRNA/ PTX	Gene/Drug Co-delivery	989
	PEG-β-CD + ferrocenecarboxaldehyde-PEI-β-CD	Telechelic Backbone	DNA	Cellular internalization Endosomal Escape Cargo Release	990
	β-CD-EDI + adamantyl-CPT	Functionalized Backbone	siRNA/CPT	Gene/Drug Co-delivery	991
	Silica-adamantyl + PGMA-β-CD	Functionalized Backbone	DNA/DOX	Gene/Drug Co-delivery	992
	Aminated PGMA-*g*-β-CD + Gd^{3+}	Reactive Monomers	DNA	Improved Complexation Improved Transfection Efficiency MRI Function	964,965
	PDMA star polymer + β-CD	Functionalized Backbone	DNA/ DOTA-Bd	MRI Imaging/Gene Delivery	993
	Adamantyl-PEG-transferrin + β-CD	Functionalized Backbone	siRNA	Cellular internalization Cell targeting Reduced toxicity	113,118,120,994−996

[a]Each listed example includes the polymer name, delivered cargo, and the general purpose of including these modifications in their scaffolds.

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polyplexes from static unresponsive materials to intelligent, versatile, and adaptive actuators.

4. ENGINEERING MULTIFUNCTIONAL POLYPLEXES THROUGH CHEMICAL MODIFICATIONS

4.1. Synthetic Strategies

The previous examples so far have highlighted polymer structures that inherently possess functionalities to bind, encapsulate, and deliver nucleic acids. However, there have also been many recent synthetic strategies wherein these polymers have been chemically modified to improve their gene delivery function and circumvent the obstacles that plague many nonviral delivery vehicles such as cell targeting, improved colloidal stability, immune system circumvention, and efficient cargo release.[46,804−807] Mauri[27] and Blasco[804] succinctly discuss the methods in which polymers can be functionalized, which include: ester activation to form amides (e.g., through *N*-hydroxysuccinimide or pentafluorophenyl ester activation), click chemistry (copper-catalyzed or copper-free strain-promoted azide−alkyne cycloadditions, CuAAC/SPAAC), thiol chemistry (disulfide exchanges or thiol−ene/-yne), Diels−Alder chemistry, pH-responsive linkages (e.g., imines, oximes, hydrazones, acetals), ring-opening reactions (epoxides, aziridines, azlactones), multicomponent reactions, and host−guest interactions (Figure 35). Each of these chemical modifications serves a specific purpose such as increasing the stability of the polyplexes, attachment of targeting groups for improved cellular recognition, or environmentally responsive elements (e.g., pH, redox, thermal) to improve endosomal escape or unpackaging of the cargo. Additionally, these methods of engineering multifunctional polymers can involve post-polymerization functionalization via reactive polymer intermediates, telechelic polymers via chain transfer agents or initiators, incorporation of noncovalent affinity interactions, or host−guest chemistry. Reactive polymer intermediates are either derived from natural sources or synthesized synthetically via free radical polymerization, ATRP, RAFT polymerization, or peptide chemistry.[807] Each of these methods and their current state of the art for polymer functionalization are discussed below.

4.2. Ester Activation

Ester activation is a nearly ubiquitous strategy that is used to functionalize polymers and nanogels for a panoply of uses including gene delivery.[27,805,808] This strategy has gained momentum in the polymer community, since not only are activated esters stable toward radical polymerization, activated (meth)acrylates are also a facile route to post-polymerization functionalized poly(meth)acrylates and can give rise to a vast library of polymers with diverse side chains not available with conventional (meth)acrylate monomers.[809] Early applications of post-polymerization modifications for therapeutic gene delivery systems incorporate functional monomers via activated esters involved poly(*N*-methacryloxysuccinimide). First synthesized by Ferruti and coworkers in 1972,[810] these reactive electrophilic species enable a facile nucleophilic substitution with primary or secondary acyclo- or cyclo-aliphatic amines, generating a series of chemically diverse derivatives capable of binding DNA for gene delivery. Early advancements in this field have also been reported by the Muller group, who synthesized poly(*N*-methacryloxysuccinimide methacrylate) (PNHSMA) and circumvented the autopolymerization propensity of these monomers. They subsequently successfully conjugated the anticancer drug doxorubicin to the pendant side chains.[811] Since then, Wong and coworkers have synthesized a library of functional polymers from PNHSMA that vary in their pendant groups (both cationic and hydrophobic) and molecular weight owing to the applicability of this chemistry.[812,813] This library enabled a rapid optimization of polymer characteristics for DNA binding and cytotoxicity. As such, these polymers were subsequently evaluated for gene delivery efficacy. They found the imidazole-conjugated species showed the highest levels of transfection efficiency and had a minimal cytotoxicological activity. Alternatively, the Cheng lab has used the idea of an activated ester and sulfonate conjugation to synthesize fluorinated poly(propylenimine) (PPI) dendrimers, which showed an extremely high transfection efficacy (>90%) to both HeLa and HEK293 cells at N/P ratios as low as 1.5.[416,814] Other examples of this specific functionalization strategy include functionalizing PEI and PLL with targeting moieties,[45] chitosan for cell targeting,[815−818] endosomal escape,[819] and polyplex stabilization.[820−822]

Alternatively, pentafluorophenyl (PFP) esters have become the other common method of activating esters for functionalizing polymers. First introduced in 1973,[823] PFP esters did not gain much traction as a tool for polymer functionalization until 2005, when the Théato group synthesized a PFP-modified poly(meth)acrylate.[824] A distinct advantage of using these activated esters was exemplified by Klok and coworkers, where they show a functionalization of linear pentafluorophenyl acrylate (PFPA) polymers with a series of cationic amines/ammonium salts, amino acids, sulfonates, and ethylene glycol proceeded smoothly and were thus able to generate a library of polymers with identical degrees of polymerization yet structurally diverse.[825] This library was shown to lack a substantial toxicity towards EaHy 926 human endothelial cells owing to the utility of this post-polymerization modification. This strategy has also been adopted by other polymer labs to rapidly generate polymer libraries.[826] A similar concept was done by Duong and coworkers, wherein they synthesized micelles that contained a PFPA block, which was used to crosslink the polymers with a diamine, and conjugated with a fluorescein isothiocyanate (FITC) labeling block via disulfide linkages that can then monitor the internalization of the micelles.[827] In a different application, the Zentel lab used pentafluorophenyl methacrylate (PFPMA) monomers to synthesize a series of PPFPMA-*b*-PEGMEMA block polymers that were crosslinked by nucleophilic bis-addition of spermine to produce hydrogels for siRNA delivery.[828−831] These hydrogels formed stable complexes that were successful in gene silencing via the delivery of siRNA; however, it was discovered that only the smaller nanoparticles (40 nm in diameter) were able to produce gene silencing. Other select examples of this chemistry are listed in Table 1.

4.3. Copper-Catalyzed Azide−Alkyne Cycloadditions (CuAAC)

Polymers that are used for gene delivery often are composed of chemically diverse moieties used for various functions such as nucleotide complexation, cellular internalization/targeting, or endosomal escape. These charged, chemically complex molecules can often limit the chemistry that can be used to conjugate sensitive biomolecules to them. Pioneered by the labs of Rostovtsev, Sharpless, and Meldal,[832,833] copper-catalyzed azide−alkyne Huisgen cycloaddition (commonly

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referred to as "click chemistry") has been a substantial breakthrough in the field of chemistry and chemical biology, as it can easily couple biologically relevant molecules together in a biorthogonal fashion. Both copper-catalyzed azide−alkyne click chemistry (CuAAC) and copper-free strain-promoted click chemistry (SPAAC) are highly lucrative for their high yields, great functional group tolerance of substrates, and simple reaction conditions. Novel advancements in this field have provided a panoply of bio-macromolecules related to gene therapy such as synthetic oligonucleotides, polymer nanocomposites, cell engineering, and drug delivery.[806,834−837] Although many researchers have now utilized this chemistry as a tool to synthesize nonviral gene delivery vehicles such as linear polymers,[627] dendrimers,[838] and liposomes,[839] its use in derivatizing polymers will be discussed here.

The landscape of chemical scaffolds employing CuAAC strategies stretches far and wide, as many labs throughout the decade have used this technique to improve the biochemical properties of their delivery vehicles.[997] CuAAC has been used to attach targeting moieties to cationic polymers (such as the ubiquitous PEI and PLL scaffolds), which has been reviewed previously.[45,998] This strategy has also been used to crosslink hydrogels to form networks used for both drug and gene delivery.[856,999,1000] There are also multiple examples of unique incorporations of this chemistry to synthesize polymers for nonviral gene delivery. The Hennink lab synthesized a copolymer of poly(hydroxyethyl methacrylate-*co*-hydroxyethyl methacrylate propargyl alcohol) through ATRP, with a carbonate-functionalized terminal alkyne, and grafted−to a terminally-functionalized PDMAEMA azide.[438] These brush-like polymers were then evaluated for their ability to transfect primate kidney fibroblasts (COS-7) and were shown to improve efficiency in the presence of INF-7, a fusogenic peptide derived from the influenza virus, when compared to linear PDMAEMA and PEI. Similarly, Gao and coworkers have made analogously constructed brushes via CuAAC with an exceedingly high grafting density (1.34 side chains per backbone carbon atom).[1001] However, their utility as gene delivery vehicles has not been reported. Reineke and coworkers have developed a set of linear polymers synthesized by CuAAC employing a trehalose or cyclodextrin (CD) diazide monomer with an oligoamine monomer equipped with terminal alkynes.[627,850,1002] The carbohydrate fixtures on the polymer served to improve the aqueous solubility and biocompatibility, whereas the oligoamines could then complex the DNA payload. Indeed, these oligoamine-carbohydrate copolymers showed a lowered cytotoxicity and improved the transfection efficiency in HeLa and H9C2(2-1) cells when compared to jetPEI. The Pun lab used CuAAC to synthesize a PDMAEMA polymer with a "sunflower" macromolecular structure, along with similar comblike polymers.[852] This was achieved by cyclizing a poly(2-hydroxyethyl methacrylate) (PHEMA) functionalized with both a terminal azide and propargyl ester via CuAAC followed by further tailoring the macrocyclic PHEMA sunflower with DMAEMA to afford the "petals". These polymers were shown to display a greater buffering capacity, strong DNA binding ability, and effective mRNA and DNA transfection efficiency compared to similar noncyclized polymers.[851,883] Yin and coworkers have developed a unique star-shaped helical polypeptide anchored to 5,10,15,20-tetrakis(4-aminophenyl) porphyrin (TAPP), which is functionalized with a cationic guanidyl side chain via CuAAC.[863] These polymers were then complexed with a DNA cargo and utilized for transfection studies. The incorporation of TAPP into this polymer not only enables multivalent cationic guanidinium side chains per polymer for increased cellular uptake, but also the inherent properties of TAPP as a photosensitizer enabled spatiotemporal control of nearly complete endosomal escape upon irradiation with light (661 nm) and consequently led to an improved cellular transfection efficiency in HeLa, B16F10, and RAW 264.7 cells.

CuAAC and other biorthogonal chemistries can be used as powerful tools to decorate micellar structures with small molecules for enhanced cell-specific targeting groups. One common concern in using this chemistry is the use of copper(I) in these reactions, which can lead to undesired cytotoxicity due to the residual copper content.[1003] Although SPAAC is a great alternative to circumvent this problem, the cyclo-octynes used in this chemistry can be expensive or difficult to synthesize. The Giorgio lab has made polymeric micelles out of triblock polymers, wherein the end group is decorated with an azide handle used to link alkyne-functionalized mannose-targeting moieties to deliver siRNA to murine macrophages.[853] Interestingly, they have also directly addressed these cytotoxicological concerns by rigorously studying the CuAAC-mediated conjugation efficiency and residual copper content of their micelles.[854] They found an optimal window of conjugation efficiency, which balances both transfection efficiency and cytotoxicity. This was measured primarily by the concentration of copper sulfate used during the click reaction, as copper sulfate concentrations between 0.25 and 0.75 mM showed a reduced cytotoxicity compared to higher (1 mM) and lower (0.1 mM) concentrations. For their azide-functionalized micelles, if azides are inadequately conjugated with mannose, there would not only be minimal cell recognition but the exposed unreacted azides themselves caused cytotoxicity. Additionally, using excess copper (1 mM) to fully functionalize these micelles results in an appreciable copper-related cytotoxicity. This study does not discourage CuAAC but, rather, vehemently argues that reaction optimization should be of paramount importance for in vivo applications.

4.4. Thiol Chemistry

Thiol chemistry, which includes the hydrothiolation of alkene/alkyne bonds, nucleophilic Michael addition, disulfide exchanges, and thiolactone modifications, has been a staple in the field of bioactive materials and polymer chemistry for several years, and it has been reviewed extensively.[805,1004−1007] The ubiquity for these purposes is primarily due to the inherent benefits associated with these reactions such as high yields, rapid reaction rates, robust reagents insensitive to oxygen or water, and minimal side products. However, some of the benefits—such as high reactivity—can be simultaneously disadvantageous, as thiols are prone to react via radical- or base-catalyzed processes under mild conditions with many types of substrates. This challenge requires knowing the specific purpose and functional groups required for the construction of the polymers of interest and applications thereof. These thiol−ene click reactions have attracted much attention in the gene delivery field in the synthesis of peptide−polymer conjugates due to the high yields and fast rates of reaction, which can be applied to targeting delivery systems.[1008] The most common thiol−ene conjugation reaction is the thiol−maleimide click reaction, as it has been shown to be a very efficient and facile method to conjugate

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large biomolecules together.[1009] This reaction has been use to couple targeting biomolecules to cationic polymers to formulate multifunctional polyplexes. For example, Lu et al. conjugated both a maleimide-terminal PEG and maleimide-terminal bombesin peptide designed to target the neuromedin B receptor of tumor cells to their synthetic 1-aminoethyl iminobis[*N*-(oleicylcysteinylhistinyl-1-aminoethyl) propionamide] multifunctional carrier that showed enhanced siRNA delivery in mice.[911] The Jiang lab introduced a bacterial-derived peptide to PLL polymers in order to facilitate blood brain barrier penetration for an enhanced DNA delivery to gliomas using the same thiol-maleimide chemistry.[915,917] With the same chemistry, the Wagner lab has made extensive libraries of polymer-targeting moiety conjugates that exhibit 10- to 100-fold more efficient gene delivery than their nonfunctionalized counterparts,[918] and it has catalogued various other chemistries that conjugate targeting moieties to polymers.[45] In another application of thiol−maleimide chemistry, Talvitie and coworkers functionalized chitosan-derived nanoparticles decorated with maleimides with a TrkB binding peptide for a two- to fourfold increase in successful pDNA delivery to murine macrophages compared to polymers functionalized with a control peptide.[908] Using a similar thiol Michael addition chemistry, Kataoka and coworkers directly linked lactose to siRNA for RNAi-mediated gene editing to synthesize a pH-responsive conjugate that can release the siRNA after endocytosis allowing for a rapid gene silencing of luciferase acitivity.[384,389] In an interesting application of bis-maleimide crosslinkers, the Kim lab directly linked both sense siRNA and antisense siRNA, forming stable and efficient multimeric polyelectrolyte complexes that exhibited a nearly complete gene silencing effect of their siRNA complex.[874]

A key characteristic of introducing thiols to polymers for functionalization is not only for their utility as nucleophiles but, as highlighted previously, their ability to form covalent and bioreducible disulfide bridges that can crosslink polymers to form hydrogels or attach functional handles for a controlled release of cargo. Several well-characterized polymeric scaffolds used for gene delivery have been modified with disulfide bonds such as poly(amido amines),[722,884−886,888] PLLs,[367,368,868−870,1010] PEIs,[860,872,889,907] and PDMAEMA.[895] The propensity of a polyplex to deliver DNA to cells based on the network of disulfide linkages was examined by the Goepferich group wherein the transfection efficiency of their PEI-based siRNA delivery system is affected by the degree of PEI branching; not only does increasing the branching of PEI improve cellular uptake but increasing the disulfide bridges also requires a careful balance between the two parameters for efficient gene delivery.[873] Indeed, Nam et al. used thiolated PEIs as nanogels for a successful siRNA delivery.[881] Ko and coworkers synthesized a redox-sensitive diblock copolymer for the co-delivery of doxorubicin and single-stranded DNA (ssDNA).[930] The two blocks of this polymer were linked via disulfide bonds, and the assembled polyplex with doxorubicin and ssDNA was transfected with HeLa cells, showing a high efficacy of DNA and drug delivery. Many other examples are presented in Table 1.

The Xu lab introduced α-lipoic acid, a naturally occurring antioxidant, to ethylene diamine-functionalized PGMA polymers to form a bioreducible nanogel with a disulfide core from the α-lipoic acid, which, upon cellular internalization, successfully released the siRNA cargo for gene silencing and showed a threefold increase in eGFP-positive HepG2 cells compared to the unfunctionalized PGMA polymers.[891] Zhu et al. designed triblock copolymer micelles, wherein the three blocks—PEG, PLA, and PEI—were each linked via disulfide bonds and reinforced with hydrogen bonds.[880] These micelles showed an improved efficiency of the cellular uptake of miRNA cargo for a potential gastric cancer therapy. Both the Kataoka and Park groups have also used polyacrylates and PEG-derived polymers decorated with sulfides to directly tether—through disulfide bonds (cleavable linkage) or thiol-maleimide coupling (non-cleavable linkage)—siRNA for controlled release.[896,1011] The Kim lab uses both bioreducible disulfide linkages and thiol-maleimide coupling chemistry to build thiolated branched PEI networks that are conjugated to peptides for tumor targeting via thiol−maleimide coupling, as well as reduceable once endocytosed to release the DNA efficiently.[791,876−879] Overall, these functionalized PEI-based polymers showed improved transfection efficiency and uptake compared to their unfunctionalized counterparts.

Thiol-alkyne chemistry has been briefly explored in the field of gene delivery, as the Cheng lab used it to "click" 2-aminoethane thiol to a polyester, which was a propargyl-functionalized tyrosine mimic.[919] These polymers showed excellent gene delivery properties and could be a novel non-nucleophilic method to incorporate cationic amines into polymers whose backbones are sensitive to nucleophiles. Cook and coworkers synthesized a small library of hyperbranched poly(ethylenimine-*co*-2-ethyl-2-oxazoline) copolymers using propargyl tosylate as the initiator and potassium ethyl xanthate as the nucleophilic end-capping agent.[920] Aminolysis of the xanthate group followed by subsequent photopolymerization provided the hyperbranched thiol−yne functionalization. The abundance of amine groups on the hyperbranched PEI derivatives synthesized through this route showed an improved buffering capacity compared to commercial PEI. Furthermore, these polymers have improved transfection efficiencies and were found to be less toxic, which emphasized the critical role of polymer architecture on gene deliverability. Other applications of thiol−yne chemistry can be found in the design of hydrogels, but their utility for gene delivery has not been explored.[1012]

4.5. Diels−Alder Reaction

The Diels−Alder reaction has been an integral part of polymer functionalization for multiple purposes such as polymer-drug conjugates, nanomaterial assembly, attachment of targeting moieties, and hydrogel synthesis.[1013,1014] Akin to disulfides, these reactions are reversible, albeit thermally. Therefore, the design and implementation of these functionalities can provide another crosslinking/de-crosslinking platform orthogonal to pH or redox-responsive linkers. This technology has been used for both drug and protein delivery via hydrogels crosslinked via the Diels−Alder reaction.[922−924] However, there are few examples in the literature that explore the utility of Diels−Alder chemistry as it relates specifically to polymer functionalization in gene delivery. Brust and coworkers successfully attached DNA to silica-coated gold nanoparticles via the Diels−Alder reaction by attaching a maleimide group to the end of a siloxane and reacting this to one of two different dienes linked to the phosphate group of an oligonucleotide (synthesized directly from a modified phosphoramidite and subjected to an automated DNA synthesis).[1015] The Shoichet group directly linked siRNA oligonucleotides to both poly(lactide-*co*-2-methyl-2-carboxytrimethylene carbonate)-*g*-PEG

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cationic polymer via CuAAC and to trastuzumab, a monoclonal antibody, via a maleimide/furan Diels−Alder coupling.[861] These structures showed improved gene silencing and toxicity when compared to commercial transfection reagents. The Hayes lab used the thermal degradability of a retro-Diels−Alder reaction to release a tethered RNA maleimide from both furan and pyrrole-based linkages to silver nanoparticles.[1016] This system can then be applied to promote osteogenesis in human adipose stem cells by the precise temporal photothermal release of siRNA using the retro-Diels−Alder system.[1017]

4.6. Schiff Bases and Ketals

Schiff base chemistry, including imine and oxime linkages, allows for degradable polymers due to the instability of the hemiaminal intermediate generated under acidic aqueous conditions. These modifications can be tailored to the specific aim of the polymer, such as a pH-dependent release of cargo or hydrolytically stable linkages. Many polymers that are functionalized with these linkages for gene delivery are pH-responsive hydrogels used to release their cargo upon lysosomal or endosomal acidification post-endocytosis.[808,1018] These oxime- or imine-linked hydrogels have been used as both drug and gene delivery vehicles and will be discussed below with extra selected examples depicted in Table 1.[925,1019−1021]

Similarly, another example of chemically reactive species is hydrazide-functionalized polymers, which can readily react with aldehydes to form the corresponding acyl hydrazones and are sufficiently stable under most physiologically relevant conditions.[939] Montenegro and coworkers have recently reported the efficient functionalization of poly(acryloyl hydrazide) with a cationic aldehyde or a hydrophobic, aliphatic aldehyde and screened their ability to deliver plasmid DNA, siRNA, and mRNA to HeLa and HEK293 cell lines.[939,1022] Lin and coworkers also used these hydrazone linkers to make comblike polymers for siRNA delivery.[938] These polymers contain a pH-sensitive ethyl acrylic acid block, a hydrophobic butyl or hexyl methacrylate block, and either an *N*-acryloxysuccinimide or *β*-benzyl-L-aspartate *N*-carboxyanhydride block that can be used as a handle to fine tune the grafting density of the cationic block. These comblike polymers showed enhanced gene silencing when complexed to siRNA compared to commercial transfection reagents.

Dong and coworkers developed a dual deliverable polyplex of both doxorubicin and siRNA to cancer cells by designing a complex assembly of folate-conjugated PEI, doxorubicin-conjugated PEI via hydrazine linkages, and siRNA.[941] Both the siRNA and doxorubicin were able to be released selectively upon internalization leading to improved gene silencing and giving credence to systems with tandem drug and gene delivery capabilities. Similarly, the Zhang lab made tandem gene/drug delivery vehicles through functionalizing a tumor-targeting PEI-based polymer with doxorubicin via imine linkages and complexing it to DNA for a synergistic codelivery complex.[940]

Additionally, some alternative pH-responsive functional groups, which do not include Schiff base chemistry, are acetal/ketals, which can either be directly incorporated into the polymer backbone or be found as functionalized side chain(s). For example, the Murthy lab demonstrated that complexing siRNA with 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), chloroquine, and a polyketal enhanced the delivery efficiency of these nanoparticles to macrophages, as they efficiently released cargo under acidification compared to vehicles without the polyketal as evidenced by an increased fluorescence of cells treated with the polyketal.[926] The Kwon group used linearized PEI with acetal-linked side chains to improve both DNA and siRNA delivery to NIH 3T3 cells, showing an improved internalization via confocal microscopy and quantified by an approximately fourfold silencing of eGFP.[927,936,937] Guk and coworkers have also made linear PEI polymers incorporating acetals into the backbone to enhance the delivery of siRNA to macrophages.[928] These polyplexes resulted in a higher RNAi efficiency when compared to linear PEI without acetals incorporated into the polymer, due to a limited cellular unpackaging of these polyplexes without the acetals. Dimde and coworkers have also developed a dual-functional dendritic polyglycerol hydrogel complete with benzacetal groups, terminal amines, and linked to PEI-modified acrylamide via thiol-Michael addition.[929] These acetal-linked polymers also showed a controlled intracellular release of the siRNA and caused a silencing of the GFP expression in HeLa cells.

4.7. Ring-Opening Chemistry

Ring-opening reactions, although omnipresent in polymer chemistry, have only recently been adopted for a macromolecular modification applied to gene delivery. These reactions are thermodynamically driven by ring strain relief facilitated by a nucleophilic attack of an alcohol, thiol, or amine. Three-membered heterocyclic rings (e.g., epoxides and aziridines) offer considerable strain and are often used for these polymer functionalizations.[1023] Like activated esters, these functional groups serve as the foundation for post-polymerization modifications.

GMA remains the most common monomer for the synthesis of epoxide-containing, biologically applicable polymers.[1024] Leroux and coworkers synthesized PGMA linear and star-shaped polymers that were functionalized with different amines, which gave rise to a mini-library of polymers that bound DNA oligonucleotides well and had an improved transfection efficiency compared to linear PEI.[953,1025] Gao went on to make a PGMA-derived multifunctional polymer conjugated with cyclodextrin (CD), ethylenediamine, and guanidine side chains that provided a system that was termed "aggregation-induced emission" to trace whether or not the polyplex formed successfully released the DNA cargo.[948] In a similar study, the Liu lab reported the synthesis of PGMA-derived polymers functionalized with primary and secondary amines, which showed an improved transfection efficiency and a remarkable reduction in cytotoxicity compared to commercial PEIs.[952,1026] Although an analogous post-polymerization functionalization strategy involving aziridine ring-opening has many examples in the literature, there has not been any substantial work done when applied to developing unique polymers for gene delivery.[1027]

However, a different prime example of these monomer-based reactive precursors subject to post-polymerization modifications include azlactone-functionalized polymers. A comprehensive review of these polymers has been reported previously.[972] First utilized by Heilmann and coworkers in 1984, poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) can be functionalized by primary amines via a nucleophilic ring-opening reaction affording a chemically stable amide linkage.[1028] Lynn and coworkers initially used azlactone-derived polymers to develop layer-by-layer (LbL) assemblies[1029] and

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then subsequently utilized this chemistry to make PVDMA-based polymers for gene delivery by incorporating both primary and tertiary amine functionalities into their polymers to make a library of 12 cationic polymers.[971] They discovered that an improved gene delivery was achieved when both shorter carbon chain lengths of pendant amine groups and tertiary amines were used.

4.8. Host-Guest Chemistry

Supramolecular assemblies—formed by noncovalent interactions such as electrostatics, hydrogen binding, π-π stacking, or van der Waals interactions—have attracted interest in their biomedical applications.[1030−1032] The propensity for these structures to self-assemble due to the association strength of these structures provides researchers a foundation to exploit these properties and design nanoparticles for gene delivery.[1032] For example, the interaction between β-cyclodextrin (β-CD) and adamantane (Ad) is a well-documented host-guest interaction.[955] What is unique about this interaction is β-CD possesses a single hydrophobic core—whereby other hydrophobic moieties (such as Ad) can noncovalently interact—as well as a hydrophilic outer surface. This property imparts unique amphiphilicity to β-CD, and thus these structures can form inclusion complexes with other various hydrophobic guest molecules such as Ad.

The Davis lab developed polycationic oligomer libraries containing β-CD in the backbone to delivery siRNA.[113,118,994,995] These nanoparticles were shown to be less than 100 nm in size, and in order to circumvent hepatic clearance in vivo, Ad-PEG and Ad-PEG-transferrin conjugates were appended to the resultant polymer via host-guest interactions. This improved gene delivery efficiency via a tumor-specific targeting in both mouse and cynomolgus primate models and helped avoid the rapid renal clearance of these particles. Additionally, the prime candidate from these experiments (termed CALAA-01) showed enough promise to be taken to clinical trials using an siRNA sequence that blocks expression of the M2 subunit of ribonucleotide reductase. This is the first example of these types of nonviral delivery vehicles taken to clinical trials, giving credence to its ability to condense siRNA, its low cytotoxicological profile, and tumor-specific targeting.

The Xu lab functionalized PGMA polymers with an adamantyl amine, followed by complexing these polymers with an ethyleneamine-functionalized PGMA(PGEA)-β-CD polymer, creating a branched cationic polymer capable of complexing DNA exceedingly well.[983] This system exhibited a better complexation ability than either of the individual polymers themselves and reduced cytotoxicity. They also examined how the topologies of this host-guest chemistry affect gene delivery efficiency, by synthesizing polymers (adamantyl-modified α-CD (Ad-CD) or α-CD-grafted PGEA (CD-PGEA)) with varying amounts of β-CD-cored CD-PGEAs and discovered the α-CD-Ad polymers showed the highest gene delivery ability. All these polymers showed a reduced cytotoxicity when compared to commercial transfection reagents as well.

Another example of host-guest chemistry used in this way is by the Tang lab, whereby PEI polymers were affixed with Ad/β-CD pairs to synthesize intriguing co-delivery vehicles for a cancer treatment.[988,989] This theranostic approach, where an adamantyl prodrug of paclitaxel (PTX) was conjugated to a β-CD-conjugated PEI-based polymer, enabled a simultaneous release of short hairpin RNAs (shRNAs) and a prodrug activation of PTX to provide a synergistic anticancer effect in vivo. Their system downregulated the expression of surviving and Bcl-2 genes while also providing a targeted release of PTX. This synergy proved more effective than either a single dose of PTX or shRNA delivery for ovarian cancer therapy separately. The same lab also developed co-delivery systems for both 5-fluoro-2′-deoxyuridine/DNA and doxorubicin/DNA using Ad/β-CD host-guest chemistry with PEI polymers.[1033,1034] Additionally, Zhao and coworkers developed a system for the codelivery of camptothecin (a topoisomerase inhibitor used for the treatment of cancer) and siRNA for cancer therapy.[991] Again, a prodrug of camptothecin containing an adamantyl group and disulfide linker was conjugated to a β-CD-amino dendrimer to both deliver camptothecin and bind to siRNA, followed by a release of the siRNA and glutathione-mediated disulfide reduction to release camptothecin. These amphiphilic structures formed vesicles in an aqueous solution, which then provided an improved delivery for camptothecin (an otherwise poorly aqueous soluble drug) and simultaneous intracellular imaging, as fluorescence was able to be detected upon camptothecin release. Similarly, Xu and coworkers co-delivered doxorubicin and DNA using coated silica-based nanoparticles.[992] The silica nanoparticles were functionalized with Ad and subsequently conjugated to a β-CD core tailored with two ethanolamine-functionalized PGMA arms. This system showed more evidence of a synergistic gene/drug co-delivery treatment option for cancer.

Another example of host-guest chemistry used to develop polymeric gene delivery systems is the work done by Palanca-Wessels and coworkers, where they synthesized a biotinylated cationic block terpolymer composed of DMAEMA, nBMA, and propylacrylic acid and bound it to a streptavidin-conjugated monoclonal antibody directed against CD22 for gene silencing.[978] Taking complete advantage of the exceedingly tight binding of biotin to streptavidin ($K_d \approx 10^{-14}$ mol/L), the pH-responsive cationic block can not only complex siRNA but easily and selectively associate with the antibody to specifically target DoHH2 cells, a transformed follicular lymphoma cell line. Additionally, HeLa-R cells expressing CD22 were shown to be transduced more effectively than CD-22 negative HeLa-R cells, giving credence to the stability of the biotin−streptavidin linkage. Other examples of host-guest chemistry are also depicted in Table 1.

4.9. Polymeric Topology: Telechelic Backbones

In addition to functional monomers that can act as chemical anchors for functionalization, there are known examples of functional macromolecules that can be conjugated post-polymerization and applied as gene delivery vehicles. Among the several different classes of functional polymers, end-functionalized polymers possess many important structural elements as vehicles for gene delivery. Telechelic polymers are end-functionalized polymers that bear reactive end groups at both chain ends and can either be homotelechelic (same functionality at both chain-ends) or heterotelechelic (differing functionality).[1035] These types of polymers necessitate well-controlled polymerization techniques, such as ATRP or RAFT, to ensure high chain-end fidelity for functionalization. Telechelic polymers can be used as cross-linkers, chain extenders, and precursors for block/graft copolymers. Although these polymer types have been used for a broad range of applications such as drug delivery, peptide/protein

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conjugation, and imaging/sensing and have been reviewed previously,[1035,1036] their utility as gene delivery vehicles will be explored below, and relevant polymers displaying this architecture are detailed in the "topology" column of Table 1.

As discussed previously, functionalizing polymers via activating esters has been long established, and it permits modifications to both the end groups or the polymer backbone. The Kataoka group synthesized a heterotelechelic polymer functionalized on one end with a cholesteryl steroid via carbodiimide-mediated amidation and a cyclic targeting peptide on the other.[844,845] This enabled both an improved colloidal stability of the complexes as well as cell-specific tumor targeting for the genetic material. The Lewis lab synthesized a folic acid end-functionalized PMPC-*b*-PDMAEMA diblock polymer for cell-specific folic acid receptor targeting.[1037,1038] This multifunctional charged polyelectrolyte with a single folic acid end group linked via an amide linkage was found to be colloidally stable and achieved a significant transfection efficiency to cells lines overexpressing folate receptors (MCF-7 and KB cells).[1038] Similarly, Benoit and coworkers synthesized a macro-CTA end-functionalized with folic acid for tumor targeting.[848] This enabled the synthesis of cell-specific PDMAEMA-*b*-P(DMAEMA-*co*-BMA-*co*-propylacrylic acid) diblock copolymers for efficient siRNA delivery and provided a useful synthetic strategy to apply this CTA to other polymers as well. Saeed and coworkers also synthesized a similar system with a homotelechelic folic acid functionality, with an additional disulfide linkage incorporated in the backbone that tethered the hydrophobic poly(lactic-*co*-glycolic acid) (PLGA) and hydrophilic PEGMA blocks.[858] Xu and coworkers successfully synthesized a heterotelechelic PHPMA homopolymer and attached a tetra-antennary mannose dendrimeric end group via pyridyl disulfide-mediated attachment and a covalently linked thiol-modified siRNA oligonucleotide via disulfide bonds, which would release the siRNA cargo upon intracellular exposure to glutathione.[859] Homo- or heterotelechelic polymers are also building blocks for the development of functional hydrogels. Networks formed with these can undergo de-cross-linking via Diels-Alder chemistry,[923] redox-responsive chemistry,[881] or pH-responsive chemistry.[856] Table 1 shows multiple examples of hydrogels and nanogels synthesized with a chemoselective release of their nucleic acid cargo. These strategies for modifying hydrogels and nanogels were recently explored extensively in various reviews.[27,1018]

From the diverse chemistry to functionalize polymers presented in this section, it is evident that each modification has been used to fulfill a certain biological purpose: aiding in endosomal escape, facilitating cargo release, cellular targeting, or improving polyplex stability. Although many of these reactions are robust, versatile, and possess both a broad substrate scope and a plethora of potential applications, a universal reaction for functionalizing all polymers to fulfill every biological need does not exist. While researchers can choose from several synthetic pathways when they impart functionalities to polymers, they are also bound by the limitations of each chemical method that is available. The constant innovation of efficient and bioorthogonal bioconjugation techniques will lead to exciting ways of functionalizing polymeric gene delivery vehicles that can be tailored to meet precisely formulated therapeutic goals.

5. POLYPLEX PHYSICAL PROPERTIES AND THEIR IMPACT

Although chemical composition and polymer architecture are powerful design parameters that can direct polyplex fate, the impact of physical attributes such as size, shape, and charge density cannot be ignored. Bio-interfacial phenomena that govern whether polyplexes can cross biological barriers are extremely sensitive to physical aspects of polyplex design. In this section, we discuss the roles played by polyplex size distribution, morphology, and surface charge. We also briefly mention recent studies demonstrating the efficacy of decationized or "neutral" polyplexes that question long-held assumptions about the necessity of a net positive surface charge. We then examine the effect of mechanical stimuli on transfection outcomes and finalize this section describing common and novel physicochemical characterization techniques used to shed light into the polyplex formation process and the properties of the resulting polyplexes.

5.1. Size

Size has long been recognized as a critical design attribute in nonviral gene delivery,[1039,1040] as the biological interactions of engineered nanoparticles with its physiological milieu are highly sensitive to particle size distribution.[1041] To navigate multiple extracellular and intracellular delivery barriers, we must pay attention to how polyplex size distribution influences bio-interfacial interactions pertinent to in vivo as well as in vitro administration. At an organism level, size has been implicated in margination and other vascular transport phenomena,[1042] biodistribution and pharmacokinetics,[1042,1043] protein corona formation,[507,1044] and subsequent interrogation by immune cells such as macrophages.[1045] At the cellular level, there is strong evidence that membrane association, internalization via a variety of pathways, and finally intracellular trafficking events are all size-dependent.[159] In this section we will first describe the size specifications targeted for different therapeutic applications, summarize synthetic strategies used to control polyplex size distribution, and summarize research focused on elucidating polyplex size effects on transfection efficiency, toxicity, and inter-organelle transport.

When they encounter the plasma membrane of targeted cells, moieties larger than 1 kDa are prevented from permeating through the membrane and are instead processed via endocytotic pathways.[1046] Larger particles ranging from 500 nm to 5 microns in size are rapidly cleared via phagocytosis, while those smaller than 200 nm typically elicit responses similar to those of viral vectors.[1039] Whether these sub-200 nm nanoparticles undergo macropinocytosis, caveolar-mediated, or clathrin-dependent pathways is undoubtedly cell-type dependent, because the composition of anionic proteoglycans and lipid domains on the plasma membrane can play a significant role. However, if polyplexes are designed to avoid clathrin-dependent modes of cellular entry, instead seeking pathways exploiting lipid rafts, they stand a higher chance of mimicking viral voyages within the cell, bypassing endosomal acidification and lysosomal degradation and directly handing over nucleic acid cargo to the endoplasmic reticulum.[159] In this context, Hoekstra and coworkers have described an elegant example of employing size as a lever of control to manipulate intracellular routing and promote specific organelle targeting.[248] They concluded that clathrin-mediated pathways are preferred by nanoparticles smaller than 200 nm in diameter, whereas larger particles (up to 500 nm in

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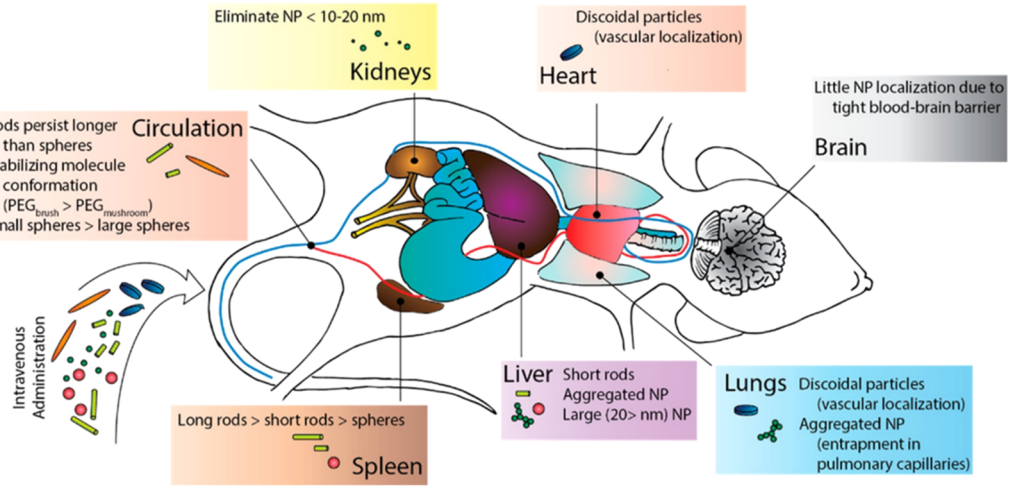

Figure 36. Schematic summarizing the impact of aspect ratio, morphology, and particle size on preferential accumulation in various organs of therapeutic interest. Reprinted with permission from ref 1049. Copyright 2017 American Chemical Society.

diameter) were internalized predominantly via caveolar channels, allowing them to evade lysosomal processing. Using chemical inhibitors of endocytotic pathways,[1047] they also observed prominent differences between lipoplexes and polyplexes, with the former almost exclusively being transported via clathrin pits and polyplexes adopting a combination of caveolar and clathrin-mediated routes.[1048]

In addition to intracellular routing, polyplex size specifications must also take into account the wide-ranging size constraints presented by diverse biological barriers, especially when systemic administration and long circulation lifetimes are desired (Figure 36). For instance, while particles smaller than the renal membrane pores (6 nm) are rapidly cleared via the kidneys, particles larger than 200 nm will be quickly cleared from circulation via the spleen and other RES mechanisms.[1039] Brain-targeted delivery requires passage through the tight junctions of the blood-brain barrier through receptor-mediated transport, transcytosis, or through carrier-mediated transport since passive diffusion allows only lipophilic molecules smaller than 400 Da.[1050] It is widely agreed that an inverse correlation exists between nanoparticle size[1051] and blood-brain barrier transport, necessitating the design of ultrasmall polyplexes that are functionalized with ligands such as glucose, transferrin, or synthetic peptides.[390,1051,1052] Another recent study highlighted the difficulty of precise size control to cross the lung periciliary layer (20−40 nm) to deliver anti-sense oligonucleotides for lung cancer treatment.[1053] Particles larger than 100 nm in diameter would be vulnerable to alveolar macrophage capture (particles), while excessively small particles would accumulate in the kidney. Therapeutics for cancer generally are said to exploit the enhanced permeability and retention (EPR) effect, wherein solid tumors are perfused by a leaky vasculature and dysfunctional lymphatic vessels, allowing nanomedicines to accumulate selectively in cancerous tissues. This "passive" EPR-based targeting strategy has been a strong motivation for designing nanoparticles possessing sub-100 nm diameters; however, a recent report by Chan and coworkers offers compelling evidence that the EPR concept is not wholly accurate.[1054] Using a three-dimensional (3D) imaging of patient-derived models of cancer tissue, they discovered that gaps in cancerous vasculature are extremely rare and that active trans-endothelial pathways are the preferred mechanism for nanoparticles to extravasate into tumors. This could explain the discrepancy observed between nanoparticle size specifications for cancer therapy and the actual dimensions of vascular gaps; although leaky blood channels supplying tumors can range in size from 380 to 880 nm, particles larger than 100 nm do not penetrate tumor tissue as effectively as sub-100 nm particles do.[1055−1057] Further, the EPR effect is much more pronounced in mouse models, which possess a much denser vasculature than humans.[1058] As a result, an EPR-based accumulation of nanocarriers in solid tumors may not be viable when translated from mouse studies to large animals. Andersen et al.[1059] reported highly heterogeneous trends in the accumulation of nanocarriers between tumors implanted in 11 dogs, suggesting that patient-to-patient variability and the stage of tumor growth are critical variables.[1060] Choi and coworkers detailed the size dependence of the EPR effect by studying the trade-off between circulation lifetime and non-specific tissue uptake of nanoparticles of varying sizes in tumor-bearing mice.[1061] Hepatic gene silencing is also size-sensitive, since Kupffer cells, parenchymal, and non-parenchymal cells within the liver selectively uptake particles of different size ranges upon intraportal administration.[1062]

The use of gold or other metallic nanoparticles as templates enables facile modulation of particle size, allowing for systematic examination of particle size effects. In addition to the ease of fabrication and tunability of size and shape, gold nanoparticles can be readily functionalized with thiol-based molecules containing cationic moieties allowing nucleic acid payloads such as siRNA and mRNA to be incorporated.[1063−1066] Among PEI-decorated gold nanoparticles, the transfection efficiency was found to be much higher among sub-10 nm populations compared to sub-100 nm particles, a difference that the authors attributed to the endosomal escape efficiency displayed by ultrasmall gold nanovectors.[1067] In contrast, Narain and coworkers systematically examined the effects of size distribution among three subsets of gold nanoparticles conjugated to glycocationic polymers (10, 40, 100 nm). They discovered that intermediate sizes had the highest transfection efficiency and that the smallest particles bound too tightly to their DNA payloads, hindering a cytosolic release.[1068] They observed that, although larger particles exhibited a higher cell uptake, these uptake pathways were associated with significant cytotoxicity. Another study concluded that sub-10 nm particles alone could permeate

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nuclear pores to deliver ODN cargoes,[1069] although other reports suggest that nuclear entry by the nanoparticle vehicle may not be required for effective nuclear entry of the payload. Gold nanoparticles are well-suited for studying the effects of carrier size on biodistribution, since the gold content can be easily evaluated via inductively coupled plasma-mass spectrometry, without the need for fluorescent or radioactive labels. A recent study found that, while larger particles (42.5 and 61.2 nm) accumulated mainly in the liver and the spleen, smaller particles (6.2 and 24.3 nm) were broadly distributed all over the body, including therapeutically relevant organ targets such as the heart and the lung.[1043] Silica nanoparticles are also attractive scaffolds for studying particle size effects, since their diameters can be tuned by modulating process parameters during nanoprecipitation as well as via microfabrication.[1042,1070] Moreover, the incorporation of aminosilanes during silica nanosphere preparation as well as the large surface areas afforded by its mesoporous architecture[1071] enables high DNA loading and control over charge density. It was discovered that, as silica nanosphere diameter increases, the DNA binding capacity is diminished even while cell uptake was improved many-fold (ostensibly due to the higher sedimentation velocity of larger particles). Because of this trade-off between the uptake and DNA binding, nanospheres of intermediate diameters (330 nm) were identified as the best-performing vectors.[1072]

Sequence-defined cationic polypeptides have also been proven to form ultrasmall and monodisperse polyplexes (<10 nm), making them an effective vehicle for targeted tumoral delivery, especially with the addition folate tags.[1073] Merely changing the sequence, composition, and degree of polymerization of the polypeptides allows the realization of different polyplex size regimes. In a study that focused on understanding the role played by the peptide/pDNA complex size, although large complexes worked best during transfection, small complexes (400 nm) were internalized more efficiently.[1074] Segura and coworkers have employed bovine serum albumin (BSA) as the nanoparticle core, wherein native BSA molecules were functionalized with ATRP initiator groups, following which cationic PDMAEMA brushes were grafted from the BSA core via SI-ATRP.[1075] Polyplex diameter was tuned by modifying the length of the PDMAEMA chains during ATRP; however, no discernible size effects were found, possibly due to the narrow range of sizes accessed (5−15 nm). Nevertheless, this synthetic strategy can be applied to protein cores of various sizes and shapes to create polyplexes of diverse morphologies and size regimes.

Unlike with inorganic nanoparticle cores and polypeptides, precisely controlling the size distributions of nucleic acid assemblies formed using synthetic polymers presents greater challenges. Multivalent polymer architectures such as dendrimers[208] and star polymers[1076,1077] rely on the molecular weight modulation of polymeric arms to achieve desired size distributions, whereas, in the case of linear polymers, the relationship between molecular weight and polyplex size may be non-monotonic due to differences in polymer-DNA binding strength.[1078,1079] A common observation across these studies is that, even though larger polyplexes may enjoy inherent advantages of higher settling velocities and enhanced cellular contact, smaller polyplexes internalize in a more efficient fashion and are able to travel more rapidly through the crowded cytosolic environment to reach the nuclear periphery.[251,1080] Polyplexes of intermediate size ranges are perhaps best positioned to balance cell uptake, payload release, and intracellular dynamics.[1081] Although larger particles performed better in vitro, intermediate-sized systems worked best in balancing circulation stability with cell uptake. Zentel and coworkers engineered nanogels constituted from well-defined cationic triblock polymeric micelles that were cross-linked to preserve their size and morphology even after siRNA complexation, allowing precise adjustment of nanogel size distributions to tune gene-silencing outcomes.[828−830] Using this platform, they demonstrated that size could be used to manipulate the intracellular polyplex distribution, with smaller nanogels found to evade endosomal capture at higher rates than their larger-sized counterparts.

Tuning self-assembly conditions as well as block copolymer compositions to generate micellar architectures of targeted size ranges would be a powerful way to resolve the trade-off between prolonged circulation and cellular uptake.[662,1082,1083] However, the synthesis and processing of micelles with well-defined size distributions is not only experimentally challenging but also requires careful physical characterization. Although polymeric micelles are a promising platform for engineering size-controlled polyplexes, their excessive reliance on PEG blocks of varying lengths and architectures to prevent undesired aggregation is problematic since PEG does not always guarantee colloidal stability. For instance, Reineke and coworkers reported that, when started from a uniform population of PEGylated micelles, micelleplexes formed by complexing micelles with ribonucleoproteins (RNPs) in water were severely aggregated and their diameters were found to be 5 times larger than those complexed in PBS.[667] On the other hand, when pDNA payloads were used instead of RNPs, the same micelleplex delivery system formed well-defined populations with narrow size distributions in both PBS and in water,[666] underscoring the numerous experimental subtleties inherent to the use of micelles as gene delivery vectors.

We also draw attention to the creative applications of nanoparticulate systems in gene delivery, particularly nanocarriers engineered from inorganic materials such as gold and other metallic nanoparticles,[1084,1085] silica-based nanoparticles,[1086,1087] quantum dots,[1088,1089] recombinant proteins,[1090,1091] and carbon nanotubes[1089] as well as organic-inorganic hybrid systems.[1092] These approaches allow us to directly control polyplex size by engineering particle cores of desired morphologies. We redirect readers to more focused reviews summarizing these developments.[86,1092−1094] While measuring polyplex size distributions, most researchers employ DLS by default, although these readings do not accurately represent the actual polyplex size distribution within serum-rich biological environments. Flow cytometry,[1095] nanoparticle tracking analysis,[1096] and Taylor dispersion analysis[1097] could be incorporated into polyplex characterization workflows to complement DLS. Further, most studies focused on examining polyplex size effects tend to be observational in nature rather than deliberately designed. Thoroughly understanding the contribution of polyplex size to pharmacokinetic and toxicity profiles of gene therapeutics relies on adopting highly controlled polyplex formulation methods that enable us to "dial in" precise polyplex size distributions. Examples of such approaches, such as microfluidics-assisted assembly and confined impingement jet mixing, are discussed in Sections 6.4 and 6.5. Transitioning from "a posteriori" to "a priori" frameworks of studying polyplex size will lead to safer and more effective polymeric vehicles.

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5.2. Shape

The recognition of particle shape as a key design parameter has been growing steadily in the biomaterials community, largely due to the recent spurt in fabrication methods being innovated to create complex non-spherical morphologies. The challenge inherent to accessing exotic non-spherical shapes is that spherical conformation is most energetically favorable to nanoparticles since it possesses the least surface area to volume ratio. Unlike with engineered nanoparticles, non-spherical shapes are abundant in nature, with bacteria, viruses, and pollen employing particle geometry as a key design motif to accomplish their biological functions. The fact that viral pathogens exist in a wide range of shapes, from spherical, wormlike, rods, and ellipsoids, is thought to be a contributing factor to tissue-specificity or viral tropism. Theoretical models of vascular transport often favor nanoparticles possessing non-spherical morphologies since the rolling or tumbling motions of high-aspect-ratio particles could align them with the blood flow, imparting favorable vascular transport characteristics and enhancing margination (Figure 37).[1098,1099] Long circulation

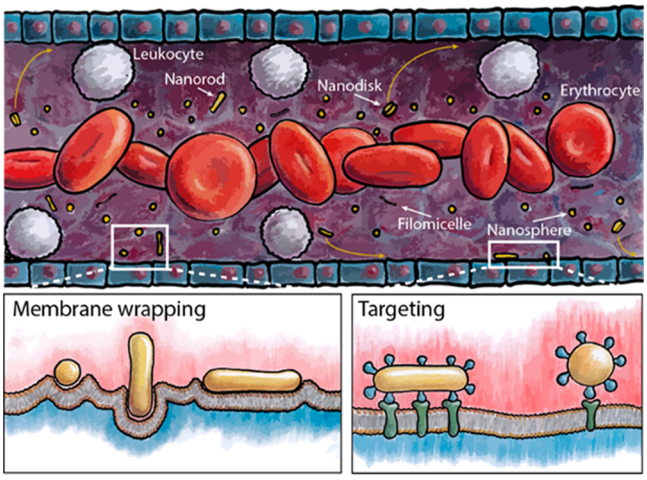

Figure 37. Effect of particle shape on margination and hydrodynamics within blood vessels. The particle shape and anisotropy can also be exploited to enhance cellular targeting and uptake. Reprinted with permission from ref 1049. Copyright 2017 American Chemical Society.

times, immune evasion, biodistribution profiles that avoid first-pass organs such as the liver, kidneys, and spleen, and enhanced cellular uptake are some of the benefits of engineering nanoparticles with controlled geometries.[1100−1107] While the role of nanoparticle shape in cell uptake,[1108] organelle distribution, and in vivo transport has been extensively studied by the drug delivery community,[1090,1091] particle shape and particle orientation at the time of endocytosis has largely been unexplored in polymeric gene delivery.[1049,1109−1112]

Shape control of polyion complex micelles has been demonstrated in several studies. Mao and coworkers assembled PEG-*b*-polyphosphoramidate (PPA) polymers with plasmid DNA payloads in solvents of varying polarity to achieve morphologies ranging from spherical to rings, flexible worms, and rigid rod-shaped micellar structures.[1113] Subsequently, Mao's group moved away from block copolymers to explore the roles of PEGylation length and graft density in PPA-*g*-PEG polymers to effect shape control.[1114] Shape variation was achieved without resorting to organic solvents during micelleplex assembly, and computation calculations aided in a systematic exploration of design parameters of graft polymers such as charge density, PEGylation length and graft density. The "DNA compaction factor" summarized how the competition between PEG steric repulsion and electrostatically driven DNA condensation influenced morphology as well as transfection outcomes. PEGylation has been a convenient lever of control to engineer morphological transformation[519] with cleavable PEG coronas mediating rapid changes in shape. While wormlike micelles had superior colloidal stability and longer circulation lifetimes, PEG shedding induced transformation to spherical micelles[1115] that exhibited superior transfection performance.[1116] Modulating PEG brush density and length in multi-arm structures to vary "crowdedness" has been shown to control polyplex aspect ratios and, with higher PEG loadings, promote rod formation and impart structural rigidity.[1117] Other studies focused on optimizing the polyplex aspect ratio through PEGylation control have pointed out that, for aspherical polyplexes, moderate aspect ratios must be employed, with cell uptake hindered when extremely elongated polyplexes were generated.[1118] PEG-alternatives such as zwitterionic molecules[446,1119] and poly(2-ethyl-2-oxazoline)[588] (Section 3.4) have also proven to be effective in obtaining polyplexes of desired aspect ratios. Brush polymers are highly versatile scaffolds for the shape control of polyplexes since charge density, backbone lengths, arm lengths, and brush density can be independently controlled to yield rods and cylinders of varying aspect ratios and rigidities.[176]

Since engineering non-spherical nanocomplex shapes through polymeric self-assembly processes is challenging, several researchers have turned instead to inorganic particle templates of varying morphologies. A modular approach combining gold,[1120,1121] graphene,[1122] carbon nanotubes,[1123] silica,[1124] or magnetite[1125] nanoparticles possessing unique geometries such as peapods,[1125] rods, or ellipsoids[1124] with surface modification tools such as ATRP has generally been effective. For instance, nanostructured microrods were prepared by using filtration membrane pores as templates, and LbL coatings consisting of PEI vehicles and plasmid DNA cargo were applied subsequently to enhance phagocytosis by alveolar macrophages.[1126] When mesoporous rod-shaped silica particles were exposed to human serum and plasma, they not only acquired a much larger quantity of coated proteins than their spherical counterparts but also displayed distinct shape-dependent adsorption patterns when the composition of the protein corona was analyzed.[1127] The Steinmetz group has developed a unique approach to accessing non-spherical morphologies, wherein plant viruses such as the Tobacco Mosaic Virus are PEGylated and used as delivery vehicles. Since the Tobacco Mosaic Virus can be engineered to be rod-shaped or spherical, they offer a means to systematically study the contribution of particle geometry on biodistribution and pharmacokinetic profiles.[1128] Similarly, virus-mimetic "nano-berries" have exploited supramolecular assembly and aspect ratio engineering to recapitulate the pH-sensitive disassembly of viruses within host cells.[1129] Desimone and coworkers have employed particle replication in non-wetting templates to encapsulate siRNA within PLGA particles (80 × 320 nm in size, Figure 38) with up to 50% encapsulation efficiency.[1130] Subsequent to soft lithographic processing, these particles were coated with lipids and silenced genes associated with prostate cancer. The Desimone lab has also engineered bioreducible

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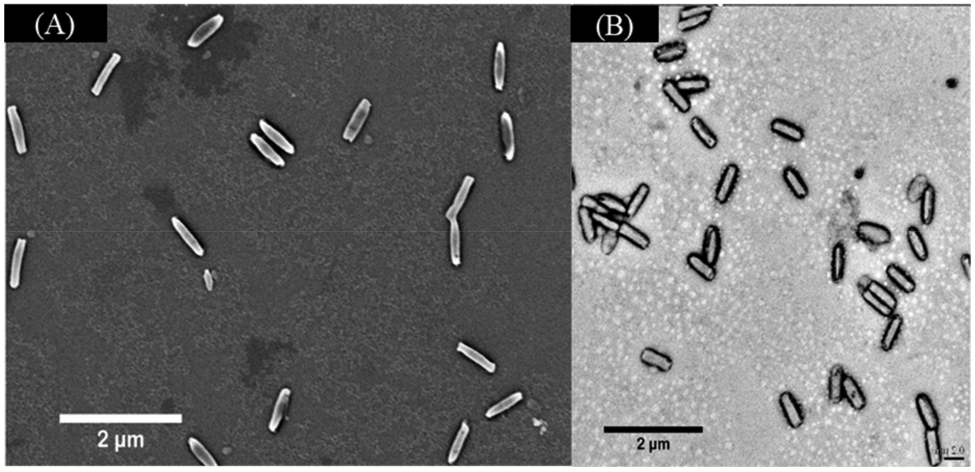

Figure 38. (A) Scanning electron microscopy (SEM) and (B) TEM images of lipid-coated PLGA nanoparticles encapsulating siRNA. Atypical aspect ratios and rod-shaped complexes could be achieved using soft lithography. Reprinted with permission from ref 1130. Copyright 2012 American Chemical Society.

hydrogel carriers of siRNA of controlled morphologies[1131] using this templated particle fabrication method and has demonstrated protein particle templates for an RNA replicon-based vaccination.[1132]

Similar to the diversity of viruses, nonviral gene delivery research must take polyplex shape into serious consideration; indeed, the development of non-spherical polyplexes that possess suitable nucleic acid condensation and delivery capabilities can dramatically transform transfection outcomes. In particular, polyplexes wherein sizes are jointly optimized with aspect ratios promise to overcome multiple intracellular and extracellular barriers faced by spherical particles.[1133]

5.3. Surface Charge

The zeta potential is a commonly used estimate of polymer charge density and is typically calculated from electrophoretic mobility measurements in capillary cells, under the assumptions of the Helmholtz-Smoluchowski model. In addition to the electrokinetic characteristics of the uncomplexed polymer, polyplex zeta potentials are also an important part of the characterization workflow and are usually studied as a function of charge ratios or N/P ratios. Electrokinetic characterization is motivated by three reasons: (1) In conjunction with gel migration assays, zeta potential values help researchers determine the optimal N/P ratio to achieve complete payload encapsulation and protection. (2) Polyplex zeta potential has frequently been touted as a strong predictor of transfection efficiency as well as cytotoxicity stemming from membrane disruption and rupture. (3) Polyplex colloidal stability, protein corona composition, and complement activation are intricately linked to the charge density of the polymeric vectors. In this section we will discuss the modulation of zeta potential to optimize cellular uptake, whether high transfection can be achieved even at lowered charge densities, and charge-switchable polyplexes.

Early studies pointed out the necessity of a net positive charge for polymers to condense nucleic acids into tightly packed toroidal structures, to prevent nuclease entry,[1134] and more importantly, mediate non-specific endocytosis by exploiting electrostatic attractions with the negatively charged cell membranes.[1135] However, polyplexes possessing a positive zeta potential were neutralized through the adsorption of negatively charged proteins, explaining why transfection is frequently inhibited in serum-rich media.[1136] Given the critical biophysical role played by a polyplex charge within each mechanistic step of the nucleic acid delivery process, right from uptake to endosmolytic escape, several groups embarked on systematic experimental efforts to delineate the effects of charge density and molecular architecture. Anderson and coworkers developed a library of nearly 500 PBAEs and concluded that the top-performing polymers shared a common structural motif characterized by a high charge density.[464] High surface charge was identified as prerequisite for effective nucleic acid delivery in multiple studies,[1137] spanning diverse cell types such as macrophages,[1138] pulmonary epithelial cells,[1139] and even in mouse xenograft models of cancer.[1140] Instead of assuming a linear monotonic relationship between surface charge and transfection performance, several groups adopted to a more nuanced approach to optimizing surface charge, recognizing that electrostatic interactions are influenced by polymer architecture,[1141,1142] molecular weight,[1140] and environmental parameters such as solvent pH and counterion valency.[1143] Architectural tuning of cationic polymers by adjusting the proximity between charged groups in complex multivalent architectures such as comb polymers, brushes, dendrimers, and hyperbranched polymers can have a profound impact on the charge density and rigidity of the polymeric vehicles even when identical cationic functional groups with the same pK_a are utilized.[454] A combined experimental and theoretical study of ionenes revealed that the complexation mechanism between the polymer and its payload is dictated by the interplay between molecular weight and charge density.[1144] A statistical design of experiments (DoE) that aided an investigation of a library of poly(2-ethyl-2-oxazoline)/PEI copolymers revealed that the optimal combination of molecular weight and charge density was payload-dependent and that the sweet spot was much narrower for RNA payloads compared to plasmid DNA.[1145] Borrós and coworkers synthesized PBAE polymers incorporating different mixtures of oligopeptides of anionic/cationic charge residues with the objective of tuning a polyplex zeta potential.[1146] Surprisingly, they found that polyplex zeta potential did not follow the expected trend in accordance with the charge density of the cationic/anionic oligopeptides used; instead, the charge borne by polyplex surfaces was shaped by the packing

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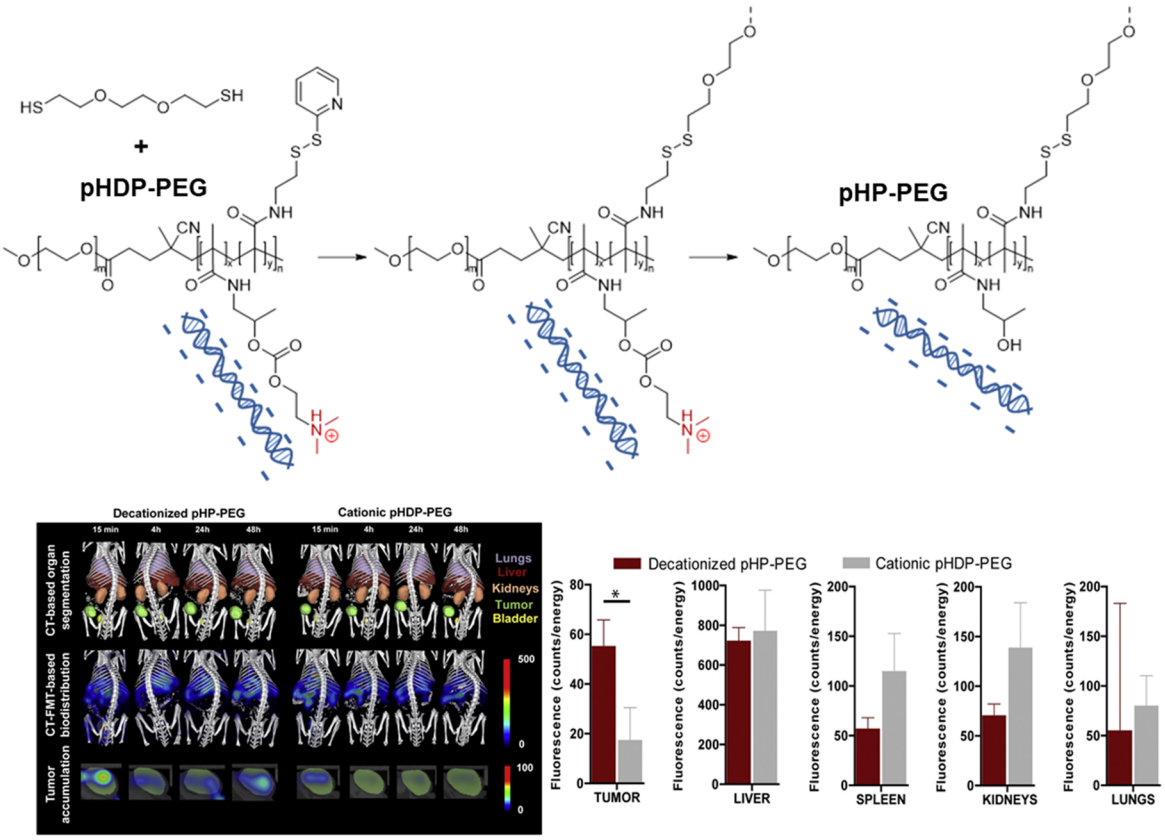

Figure 39. Hennink et al. reported the synthesis of cross-linkable and decationizable PHDP-PEG polymeric vectors. Decationized polymer displayed improved biodistribution when compared to their cationic counterpart during in vivo experiments with systemic administration. Reprinted with permissions from refs 1157 and 1159. Copyright 2013 and 2014 Elsevier (respectively).

distribution of the polymers and the nature of cationic functional groups employed. Ultimately, the zeta potential must be treated with caution, as it is not a simple additive quantity that can be "dialed in" by stoichiometrically balancing cationic and anionic moieties. It is an approximate global measurement of surface charge that does not capture the inherent heterogeneity of charge distributions and binding states. While zeta potential measurements may be useful in comparing formulations from multiparametric libraries and deriving structure-activity correlations, they may not always be predictive of cellular interactions and transfection outcomes.[1147]

Several reports describe formulations that exhibited efficient transfection despite low charge densities and sometimes net negative zeta potential values, questioning the validity of the overly simplistic "positive surface potential" heuristic. Enhancing hydrophobic interactions between nucleic acids and polymers through the incorporation of lipophilic functional groups seems to be a common design strategy to increase the effective charge density.[492] Incorporation of fluoroalkyl groups[101,788] resulted in effective DNA condensation properties at N/P ratios as low as 1, and micellization through the formation of hydrophobic core[599,644,645] has generally been able to prolong colloidal stability even in high ionic strength environments, unlike electrostatically assembled polyplexes. Imparting hydrophobic modifications to the polymer backbone,[634,1148,1149] end groups, or pendant chains[267,1150] has yielded polyplexes with extremely low charge densities, thereby resolving the toxicity-efficiency trade-off. Architectural tailoring that results in multivalent topologies such as brushes can also eliminate the need to employ high charge densities and N/P ratios to engineer efficient polymeric vectors.[1151]

While hydrophilic motifs such as PEG and zwitterionic functionalities are frequently incorporated to prolong circulation time and provide stealth properties, they inevitably lead to screening of positive charges,[1152] possibly preventing electrostatically mediated non-specific endocytosis. Unlike neutral and negatively charged polyplexes, positively charged polyplexes are prone to the formation of a protein corona, which marks them out as targets for immune clearance. For highly specific cellular delivery, a highly positive surface potential can be detrimental since the protein corona may interfere with biorecognition processes driving targeted cellular uptake. Further, for intra-dermal and intro-muscular delivery routes, which are relevant to DNA vaccine delivery, cationic polyplexes tend to get sequestered or trapped by oppositely charged extracellular proteins instead of activating T-cells.[1152] As described previously, several groups have engineered pH-responsive polyplexes that dynamically "shed" their hydrophilic stealth layer in tumoral environments where the extracellular pH is lower, thereby "unmasking" the positive charge and allowing polyplexes to enter tumor cells.[1153−1156] Considerable synthetic ingenuity is involved in ensuring pH-dictated targeting of distinct cellular phenotypes (healthy vs cancerous). These systems must be carefully engineered to a narrow pH range, since the pH ranges from 7.4 in normal physiological environments to values ranging between 6.5 and 7.2 wherever tumoral acidosis is present.[749] Additional strategies to reduce excess surface charge include the deliberate inclusion of polyanions to form ternary complexes and the decationization

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and cross-linking of polyplexes following electrostatically mediated DNA condensation.[1157]

In the face of ambiguous experimental studies, simulation and molecular modeling studies can help us arrive at a clear understanding of the role of charge density on nucleic acid compaction and release, interactions with serum proteins, cell membrane, and endosomal vesicles. Researchers also need to deploy a battery of characterization techniques to understand the chemical heterogeneity of polyplex surfaces in biological media and measuring charge distributions.[1127]

5.3.1. Decationized Polyplexes. Polyplexes with a net positive charge tend to complex nucleic acids effectively and deliver them to cells in vitro with high efficiencies, and yet they frequently underperform when used for in vivo applications.[1158] Depending on their physicochemical characteristics, many polyplexes with a positive charge display undesired biodistribution, high toxicity, and poor serum stability and may exhibit the inability to release the nucleic acid intracellularly. In this section we summarize a synthetic approach that allows for initial DNA complexation through positively charged polymers but subsequently neutralizes this positive charge through the incorporation of degradable linkages and the eventual loss of ionizable functional groups. By rendering the polyplexes neutral after DNA compaction, the drawbacks of a net positive charge (Figure 39) can be circumvented while ensuring payload protection.

In 2013 Hennink et al.[1157] reported a synthetic approach to prepare neutral polyplexes, by cleaving the positive pendant groups of the polycation used to complex pDNA after polyplex formation and crosslinking. This process results in the formation of either neutral polyplexes or polyplexes with slightly negative charge densities. For this goal, the authors employed *N*-[2-(2-pyridyldithio)]ethyl methacrylamide (PDTEMA) to synthesize a PEG-*b*-P((HPMA-DMAE)-*co*-P(PDTEMA)) terpolymer polycation. The HPMA-DMAE (carbonic acid 2-dimethylamino-ethyl ester 1-methyl-2-(2-methacryloylamino)-ethyl ester) repeating units contain tertiary amines (used for electrostatic complexation of DNA) linked to the polymer backbone via a carbonate ester group suitable for cleaving via hydrolysis. The PDTEMA repeat units include pyridyldithio units that undergo efficient disulfide exchange under mild conditions, providing a mechanism for crosslinking the polyplexes prior to the decationization process.

After decationization the polyplexes are stable in HEPES-buffered saline, and no release of DNA was observed in gel electrophoresis experiments. Exposing the decationized polyplexes to 1,4-dithiothreitol, a thiol reagent used to simulate the reductive intracellular environment, causes DNA release, which was not observed with the cationic polyplexes. The decationized polyplexes show more than 50-fold lower cell uptake into HeLa cells when compared to the cationic counterparts as well as to ExGen-500, a linear PEI control. The low non-specific cell uptake of the decationized polyplexes provided an opportunity to combine the stealth properties of these polyplexes with targeting strategies to achieve cell-specific uptake. This concept was demonstrated by introducing folate targeting moieties into the decationized polyplexes by linking folic acid to the PEG macroinitiator prior to polymerization to display it on the polymer end groups.[1160] Folate-containing decationized polyplexes displayed a higher cellular uptake (three- to fourfold higher) in vitro in OVCAR-3 and HeLa cells, two cell lines that overexpress folate receptors, when compared to polyplexes that lack the folate targeting. It was also found that this trend in cell uptake was reversed in A549 cells, a folate receptor negative cell line. The folate-containing PEG-*b*-P(HPMA-DMAE)-*co*-P(PDTEMA) terpolymer was also optimized to form stable decationized polyplexes with another payload type, siRNA.[1161] Optimized polyplexes were developed through tailoring of the molar ratio of the PDTEMA crosslinkable units in the statistical cationic block as well as the chemistry of the dithiol crosslinker. Even at higher PDTEMA contents the siRNA polyplexes remain degradable in the presence of extra 1,4-dithiothreitol after decationization. Moreover, folate-containing decationized siRNA polyplexes displayed gene knockdown, even in the presence of serum, in Skov3-luc cells, a cell line where folate receptors are overexpressed. In vivo[1159] studies in zebrafish models resulted in lower toxicity and teratogenicity when compared to cationic polyplexes. Fluorescent labeling of the decationized polyplexes revealed superior colloidal stability in plasma, longer circulation times, and higher tumor accumulation than their cationic counterparts.

Overall, decationization is a polymer design principle that could be strategically incorporated in therapeutic delivery vehicles, to prevent non-specific uptake and encourage specific cellular targeting.

5.4. Mechanical Properties

Cells are extremely sensitive to microenvironmental cues, particularly mechanical properties such as rigidity, elasticity, and compressibility. This is true of both cells cultured in lab settings as well as those in their native physiological niches. Mechanical cues from the environment are transduced into biochemical signals that have cascading effects on cell adhesion, migration, and differentiation. Therefore, mechanical properties of the cell culture substrate have long been a critical focus of the tissue engineering community but are severely under-investigated in the context of nonviral gene delivery.[1162] Mooney and coworkers reported that cell proliferation and apoptosis were regulated by the elastic modulus of the culture substrate, with stiffer substrates promoting both polyplex dissociation as well as transgene expression.[1163] However, these early studies were performed in two-dimensional (2D) cell culture formats, which do not accurately recapitulate the physiological environment. Segura and coworkers employed extracellular matrix-mimetic 3D hydrogels based on hyaluronic acid to study the interplay between the adhesive ligand presentation and elastic modulus.[1164] They tested hydrogels varying in compliance from soft to stiff and concluded that transgene expression can be modulated through mechanical manipulation of cell culture scaffolds. In contrast to earlier studies that favored high stiffness, they concluded that intermediate values of elastic modulus were optimal for maximizing transfection efficiency.[1165] This discrepancy is not unexpected since the regulation of endocytotic pathways by substrate mechanics was found to be a complex function of cell type, properties of the nanomaterial tested, and the time points chosen for measurements.[1166] Indeed, a follow-up study[1167] by Mooney's research group found that, unlike with DNA payloads, siRNA delivery remained unaffected by changes in substrate modulus.

Other studies compared 2D and 3D cell cultures during polyplex-mediated gene delivery and concluded that, while endocytic pathways differed significantly, cytoskeletal dynamics and molecular signals driving high transfection were quite similar.[1168] Apart from engineering hydrogels to match the

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stiffness of different tissue types (e.g., bone (>10^9 Pa) or muscle (10^3 to 10^4 Pa)), the scaffold architecture and porosity can also be modified to enhance cell spreading, thereby promoting transfection.[1169] Instead of employing polyplexes formulated from commercial PEI-based reagents Yang et al. identified a biodegradable PBAE through systematic synthesis and screening and tested hydrogel scaffolds varying in moduli from 2 to 175 kPa.[1170] Hydrogels with moderate degrees of stiffness (28 kPa) demonstrated the best transfection performance when employed in concert with the polymer lead structure. This study suggests that a mechanical modulation of cell culture platforms must be accompanied by careful optimization of synthetic vector properties through polymer chemistry approaches. In general, the gap between tissue engineering platforms such as PEG and hyaluronic acid and polymer synthetic tools must be bridged by a co-development of the cellular microenvironment as well as the delivery vehicle to exploit synergies. Similarly, mechanoresponsive polyplexes can be engineered to sense mechanical contrasts between healthy tissue and diseased tissues and release their nucleic acids upon application of a mechanical trigger in vivo. These "smart" polyplexes will be enormously useful to induce the production of therapeutic proteins or growth factors in conditions such as atherosclerosis, where healthy arteries are supple and diseased arteries are stiff.[1018]

Several avenues of research exist to combine particle cores of varying stiffness, using a vast palette of particle engineering tools at our disposal and subsequently incorporating polycationic surface chemistries via surface-initiated polymerization. Orthogonal control over particle mechanics and chemical functionality would be a powerful step forward in understanding the interwoven effects of stiffness and chemically driven interactions between cells, nucleic acids, and vectors.

Investigating the roles played by physical design parameters such as size, shape, charge, and mechanical stimulation is very important to progress in polymer-mediated nucleic acid delivery. Although these parameters have been shown to modulate organ distribution, membrane interactions, and cellular uptake, a systematic exploration of the physical design space is lacking. Polymeric gene delivery must exploit advances in particle fabrication techniques to control physical properties and improve delivery outcomes to exploit the full tunable parameter space in this area.

5.5. Physicochemical Characterization of Polyplexes and Their Formation

Since physicochemical characteristics of polyplex formulations such as size, shape, and surface charge are influential in determining the fate of polymeric gene delivery vehicles, characterization techniques used to quantify these properties assume a vital role in polymer development. In addition, we note the importance of the molecular organization of polyplex assemblies, particularly polyplex composition, quantification of unbound polymers and nucleic acids, binding affinities, binding configurations, nucleic acid helicities within assemblies, and other structural descriptors. In this sub-section, we draw attention to several physical and chemical analytical tools: NMR spectroscopy, isothermal calorimetry (ITC), surface plasmon resonance (SPR), Fourier-transformed infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), DLS, static light scattering (SLS), small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), TEM, turbidimetric titration, electrophoretic light scattering (ELS), circular dichroism spectroscopy (CD), ultracentrifugation (UCF), fluorescence correlation spectroscopy (FCS), and atomic force microscopy (AFM).

In Table 2 we briefly describe how these techniques improve our understanding of the solution properties of polymers and polyplexes, the thermodynamics of nucleic acid-polymer binding, and the molecular understanding of polyplex architectures. Some of these techniques (such as DLS, NMR, and zeta potential measurements) have been developed as turnkey platforms that are inexpensive, facile, and highly accessible to non-experts. On the other hand, some other techniques (such as SAXS) require considerable expertise during data acquisition and interpretation. Despite the analytical challenges involved, we posit that the mechanistic insights provided by these powerful methods are irreplaceable in identifying and understanding the intermolecular forces implicated in polyplex formation. We note that a few of these methods (such as SANS and cryoTEM) require dedicated infrastructure and deep analytical expertise, which emphasizes the importance of close collaborations between polymer chemists, characterization facilities, and biophysics experts. The development of cutting-edge physicochemical characterization tools provides fundamental insights on polymer-nucleic acid interactions, the uniformity and reproducibility in formulation, and ultimately provides mechanistic understanding that is essential for clinical translation.

6. EXPERIMENTAL CHALLENGES ASSOCIATED WITH POLYPLEX FORMULATION: SOLUTION PARAMETERS AND TRANSPORT LIMITATIONS

Like many interfacial phenomena in nanoscience, polyplex formation is shaped by the competition between thermodynamics and kinetics. While thermodynamic limits tend to favor highly aggregated and hydrophobic equilibrium structures, researchers circumvent these challenges by kinetically trapping polyplexes in potential metastable non-equilibrium structures with attractive properties such as narrow size distributions. Kinetic trapping exploits the fact that, even though initial interactions between nucleic acid and polymers are extremely rapid, occurring in less than 50 ms, the subsequent rearrangement of polyplexes and the eventual aggregation are much slower processes, taking place over a time scale of hours. While a thorough theoretical treatment of polyplex formation physics is outside the scope of this Account, we emphasize that electrostatic interactions are not the sole intermolecular forces driving polyplex formation. When polyplexes are formed, this process is typically accompanied by the release of counterions, the loss of the hydration layer bound to the phosphodiester backbone, and hydrophobic aggregation as well as the formation of hydrogen bonds.

Recognizing that polyplex properties are impacted by the manner in which the nucleic acids and their polymeric binders come into contact with one another, the primary goal during polyplex assembly is to ensure predictable and reproducible experimental conditions that promote a consistent production of polyplexes of the desired sizes, morphologies, and compositions. While polymer structure and composition have been exhaustively examined, the manipulation of polyplex properties through systematic optimization of assembly conditions is an under-investigated and sometimes-overlooked approach to improving the biological outcomes of polymeric gene delivery. In this section, we review (1) traditional

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Table 2. Summary of Methods Used to Measure Physicochemical Properties of Polyplexes

method	measurements	vector	cargo	purpose(s)	refs
NMR	Changes in broadness and intensity of NMR signals (^{1}H, ^{19}F, ^{31}P ^{13}C−^{1}H HSQC) upon complexation. Relaxation dynamics of polymers and DNA upon binding (CPMG-, DOSY- and PFG-NMR) Polymer-nucleic acid spatial proximity (NOESY-NMR)	Lactosylated-PEG-*b*-poly (silamine)-*b*-PDMAEMA	pDNA	Follow PIC complexation	369
		PEI	pDNA	Measure free and bound polycation	1171
		PEG−PAMAM dendrimer	20-mer DNA	Study polymer/DNA binding, polyplex size and composition	222
		PEG−PAMAM dendrimer	29-mer TAR-RNA	Study polymer/RNA binding, polyplex size and composition	223
		Eu^{3+}, Gd^{3+}-chelating Oligoethyleneamines	pDNA	Proof-of-concept for developing polyplexes with intrinsic MRI detection	1172
		Fluorinated PEI	DNA	Determine polymer stability	1173
		Phospholipids	pDNA	Self-assembly of phospholipids	1174
		Phospholipids	siRNA	Self-assembly phase (lamellar or hexagonal)	1175
		Phospholipids	ODN	ODN encapsulation efficacy	1176
		PEI- αCD, PEI- phenylboronic acid	siRNA	Confirm supramolecular assembly of delivery vehicle	1177
		Mannose-CD and adamantane-PEI	saRNA	Confirm supramolecular assembly of delivery vehicle	1178
		PEI-CD-Cholesterol micelle	siRNA	Determine drug in core of micelle with siRNA surrounding	1179
		PEG−PAMAM dendrimer	DNA	Monitor binding between minor groove of DNA and dendrimer	1180
		PAMAM	siRNA	Determine polyplex size	1181
		PEG-*b*-PDMAEMA-*b*-PnBMA PDMAEMA	pDNA	Determine polyplex composition	666
ITC	Equilibrium binding constants. Thermodynamics of binding	Branched-cationic tripeptides (CPPs)	pDNA	Compare binding thermodynamics between two CPPs	1182
		Poly(glycoamidoamine) derivatives	pDNA	Role of hydroxyl groups and amide bond spacing in determining pDNA binding affinity.	1183
		PEG-*b*-PGBA, PEG-*b*-PLL	mRNA	Determine effect of polymer rigidity on binding	1184
		Cationic liposomes	pDNA	Differentiate between exothermic electrostatically driven and endothermic lipoplex rearrangement.	1185
		PAMAM	pDNA	Effect of dendrimer generation on binding	1186
		DOTAP and derivatives	pDNA	Separated thermodynamics of protonation and binding	1187
		PEI	pDNA	Determine equilibrium constants, stoichiometric number of binding and enthalpy	1171
		Chitosan oligosaccharides	siRNA	Separation of ion-pairing and aggregation binding thermodynamics	1188
		Agmatine- maltotriose-PEG-OCH_3	dsDNA	Thermodynamic dependence on complexation methods and N/P ratios	1188
		PEG-AEMA stars	dsDNA	Determine stoichiometry for optimal nanoparticle formation	1077
		Poly(glycoamidoamine)	pDNA	Detect different binding events: electrostatic complexation, aggregation, protonation-based	1189
		Chitosan	pDNA	Effect of pH, buffer, and deacytalation of polymer	1190
		PAMAM	siRNA	Understand thermodynamics of polyplex assembly	1191
		Chitosan	siRNA		1192
		PAEMA, PMAG-*b*-PAEMA	pDNA, DNA	Effect of hydrophilic block on polyplex binding	1193

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Table 2. continued

method	measurements	vector	cargo	purpose(s)	refs
		Chitosan	pDNA, DNA	Effect of chitosan length on binding	1194
SPR	Binding of polymer to nucleic acids/proteins	PAMAM dendrimers with folate and riboflavin targeting groups.	dsDNA	Effect of targeting moieties on polyplex formation and protein binding	1195
		Acylated Chitosan derivatives	miRNA	Effect of chitosan acylation on binding	1196
		PEI or PDMAEMA	DNA	Understand polyplex interactions with glycoaminoglycans	1197
		PEI−PEG-cetuximab PEI−PEG-trastuzumab	pDNA, siRNA	Understand polyplex binding to cell surfaces antibodies	1198
		PAMAM-Cholesterol	siRNA	Confirm formation of core-shell nanoparticles	1199
FTIR	Peak shifts and changes	PAMAM	DNA	Discriminate between bound and free DNA molecules within polyplexes	1200
		PAMAM	pDNA	Monitor polymer/DNA binding, and changes in DNA secondary structure	1186
		DOTAP, DOPE	pDNA	Monitor lipid/DNA binding, and changes DNA secondary structure	1201
		DOTAP, DMPC	pDNA		1202
		RALA peptide	pDNA	Confirm pDNA encapsulation	1203
		Poly(glycoamidoamine)	pDNA	Understand non-electrostatic driving forces for polyplex binding	1189
Turbidimetric titrations	Turbidity changes during DNA (polymer) titration with polycation (DNA)	PDMAEMA-*b*-PnBMA	DNA, PSS	Correlate binding and micelleplex stability to polyanion flexibility	1204
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Correlate binding and polyplex stability to PEG-block length	682
		PDMAEMA-*b*-PnBMA	DNA	Correlate binding and polyplex stability to DNA shape and size and ionic strength	685
CD	Circular dichroism (CD) spectra of nucleic acids and. Monitor changes on spectra maxima wavelengths and molar ellipticities.	P(HPMA-*co*-APMA)-*b*-PDMAPMA	siRNA DNA	Evaluate protection from RNAse degradation by block copolymers	1205
		PDMAEMA-*b*-PnBMA	dsDNA	Study dependence of DNA helicity on polyplex/micelleplex architecture and establish histone-mimetic binding configurations employed by micelleplexes	1204
		PEG−PAMAM dendrimers	DNA	Study polyplex binding	1180
		PGAAs	pDNA	Monitor changes in DNA structure during binding	1189
		mPEG−PAMAMA dendrimers	DNA	Monitor DNA structure during complexation as a function of polycation complexation	1200
		PDMAEMA, PTMAEMA, PLL	pDNA	Monitor changes in DNA structure when complexes to different polycations	1206
		Peptide-functionalized PLL	pDNA	Monitor changes in DNA structure in polyplexes before and after lyophilization/reconstitution	1207
		PEG-*g*-PEI, PLA coating	pDNA	Monitor DNA stability during polyplex encapsulation into PLA nanoparticles	1208
		PEI, Alkylated-PEIs	DNA	Monitor differences in binding due to PEI alkylation	1209
		PAMAM dendrimers	DNA	Monitor DNA structure in complexes as a function of dendrimer generation (G2-G9)	1186
		PDMAEMA-*b*-PnBMA	dsDNA	Monitor changes on DNA secondary structure upon complexation	1204
		PEG-*b*-PDMAEMA-*b*-PnBMA PEG-*b*-PDMAEMA PDMAEMA-*b*-PnBMA	DNA	Correlate changes on DNA secondary structure inside polyplexes to the structure of the polycationic vector (linear vs micelles)	666

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Table 2. continued

method	measurements	vector	cargo	purpose(s)	refs
		PEI, PEG−PEI	siRNA	Correlate changes in siRNA secondary structure with polyplex PEGylation and N/P ratios	1210
		Linear and branched PEI	siRNA	Correlate changes in siRNA secondary with differences in binding due to polycation architecture	873
		PAEMA POEGMA-*b*-PAEMA PMAG-*b*-PAEMA	pDNA	Compare changes in DNA structure when complex with polycations with different hydrophilic blocks	623
UCF	Sedimentation coefficients, quantification of unbound polymer content	PEI	siRNA	Quantitate polyplex composition	1211
		Alkylated-PVP	pDNA		1212
		PEG-*b*-P[Asp(DET)]	pDNA		1213
		PEI	pDNA	Examine DNA conformations in solution, hydrodynamic properties of polyplexes as a function of N/P ratio	220
		PEG-*b*-P[Asp(DET)]	pDNA	Quantify the associating number of PEGylated block copolymers within micelleplexes as a function of PEG architecture	845
		PEG-poly(aspartamide)	siRNA	Quantitate polyplex composition	375
		PEG-*b*-P[Asp(DET)]	mRNA		1214
		P(HPMA-*co*-APMA)-*b*-PDMAPMA	siRNA DNA	Correlate cationic charge density, siRNA binding affinities, and transfection levels.	1205
		PEI and Chitosan	ODN	Calculate the porosity of complexes	1215
		Quaternized PVP	pDNA	Quantitate polyplex composition	1216
		PEG-*b*-P[Asp(DET)]	pDNA	Monitor complexation and releasing of DNA	1217
		PEG-*b*-P[Asp(DET)]-*b*-PLL	pDNA	Determining micelle composition	1218
		PEG-*b*-P[Asp(DET)]-cholesteryl	pDNA	Correlate toxicity with proportion of free polymers	844
FCS	Size and diffusion coefficient of fluorescently labelled polycations and nucleic acids	PEG-poly(aspartamide)	siRNA	Determine polyplex composition	375
		PEG-*b*-P[Asp(DET)]	pDNA	Determine polyplex composition as a function of pH	1213
		DMAEMA, PEI, DAB	ODN	Characterize ODN-polymer complexation as a function of formulation ratios	1219
		Lipids	siRNA	Lipoplex stability in the presence of serum	1220
		PEI	mRNA	Payload assembly/disassembly	1221
		PEG−PLL-Au-NPs	siRNA	Determine polyplex composition	1222
		Cationic oligomers	siRNA	Evaluate complex stability in the presence of serum	1096
		PEI	pDNA	Monitor intracellular fate of PEI	1223
		PEI	pDNA	Purify polyplexes from free polymers and monitor heparin-triggered disassembly	219
		Stearic acid-P[Asp(DET)]	siRNA	Evaluate stabilizing effects of hydrophobic moieties	1224
		cRGD-PEG-*b*-PLL	Cholesterol-siRNA	Correlate colloidal stability to polymer and nucleic acid functionalization	370
		PEG-*b*-PAPNBMA	siRNA	Monitor siRNA intercellular trafficking	1225
		P[Asp(DET)]	siRNA	Prove that siRNA-polymer conjugation improves serum stability through diffusion coefficient determination	1011
		PHPMA-*b*-PEG P(HPMA-*co*-DTEMA)-*b*-PEG	siRNA, pDNA	Monitor payload release	1161
ELS	Zeta potential	PEI-*g*-PEtOx	DNA	Correlate polyplex zeta potential to N/P ratio, and temperature	588

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Table 2. continued

method	measurements	vector	cargo	purpose(s)	refs
		PEI/HA	DNA	Monitor zeta potential changes upon addition of hyaluronic acid	1226
		PEI-*b*-PLL-*b*-PLG	DNA	Polyplex zeta potential as a function of pH	720
		PGAA	pDNA	Monitor zeta potential changes upon addition of glycosaminoglycans	1227
		P(HPMA-*co*-APMA)-*b*-PDMAPMA	siRNA DNA	Effect of charge density on electrostatic binding strength and gene knockdown	1205
		PDMAEMA-Cholesterol	DNA	Correlate zeta potential to cholesterol content	1228
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Correlate zeta potential to PEG block length	682
		PEG-*b*-P[Asp(DET)]	pDNA	Correlate zeta potential to N/P ratio	1217
XPS	Atomic composition and chemical states	PLGA, PDADMAC, PAA nanofibers, PAMAM dendrimer	pDNA	Confirm grafting of cationic dendrimers to electrospun PLGA nanofibers	1229
		Hyperbranched PAMA, PEI	pDNA	Confirm immobilization of polymers	1230
		PLGA nanocapsule,	siRNA	Confirm protein conjugation	1231
		PEI−PEG-coated manganese oxide nanoparticles	siRNA	Confirm surface functionalization	1232
		PLL-coated mesoporous silica nanoparticles	ODN		1233
		PDMAEA-coated mesoporous silica nanoparticles	siRNA	Confirm PDMAEA attachment	1234
		Alginate-sulfate nanoparticles	pDNA	Nanoparticle composition and interaction strength between pDNA and polymer	1235
		Polysaccharide hyaluronan-sulfate	siRNA	Confirm ternary complex formation between siRNA, polysaccharide, and calcium ion bridges	1236
DLS	Size of polyplexes upon complexation and over time	Lipids	mRNA	High throughput characterization of lipoplexes	1237
		PDMAEMA-*b*-PnBMA	dsDNA	Measure long-term stability of complexes	1204
		PDMAEMA-*b*-P(DMAEMA-*co*-PAA-*co*-BMA)	siRNA	Monitor polymer/siRNA binding	1238
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Study stabilization due to PEG-block length	682
		PDMAEMA-Cholesterol	DNA	Monitor effect of DNA addition rate on polyplex size	1228
		PEG-*b*-PAEM	pDNA	Polyplex stability in presence of heparin	625
		PEI-*g*-PEtOx	DNA	Correlate polyplex size and stability to N/P ratio and temperature	588
		PGAA	pDNA	Polyplex size evolution in the presence of glycosaminoglycans	1227
		PDMAEMA-*b*-BMA	DNA	Micelleplex size and stability	685
		PEI	siRNA	Correlate polyplex size and stability to N/P ratio	1211
		Cationic oligomers	siRNA	Study the limitations of DLS vis-à-vis AFM, FCS and NTA during size determination of heterogeneous populations	1096
SLS	Polyplex molecular weights. Internal structure of polyplexes and micelleplexes	PDMAEMA-Cholesterol	DNA	Determine polyplex size and composition	1228
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Correlate micelleplex size and composition to PEG-length	682
		PDMAEMA-*b*-PnBMA	DNA	Measure micelleplexes size and composition and correlate to DNA topology	685
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Micelle core radius was shown to be independent PEG corona block length	682

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Table 2. continued

method	measurements	vector	cargo	purpose(s)	refs
		PDMAEMA-Cholesterol	DNA	R_g measurements for polyplexes with non-spherical morphology (unsuitable for SLS based measurements), probe hydrophobic interactions on polyplex structure.	1228
		PS-*b*-P4VPQ	ODN	Effect of DNA concentration on micelleplex aggregation. Structural description of DNA binding to micelle corona and location of DNA within aggregates	1239
		DEAE/dextran	mRNA	Quantify compactness, polymer content, and RNA encapsulation as a function of charge ratio	1240
		PMPC-*b*-PVBTMA PEG-*b*-PVBTMA PEG-*b*-PLK	Sodium acrylate	Quantify PIC morphology (sizes of core and corona) as well as stability in various ionic strengths	1241
		PLL,PEG-*b*-PLL, PVBTMA, PEG-*b*-PVBTMA	DNA	Effect of DNA stranded-ness (ssDNa vs dsDNA) on coaxial stacking of DNA helices within polyplexes	1242
SANS	Probe-free analysis of the structures of multicomponent systems	Lipids	DNA	Lipoplex formation kinetics and geometry of complexation intermediates	1243
CryoTEM	Imaging of polyplex size and morphology in hydrated stated.	PDMAEMA-*b*-PnBMA	dsDNA	Effect of cationic polymer architecture and polyanion flexibility on PIC morphologies	1204
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Visualize beads-on-a-string micelleplex morphology attained using triblock copolymers of optimized block lengths	682
		PEG-*b*- PAEM	pDNA	Correlate polyplex morphology to DNA amount and type	625
		PEG-*b*-PDMAEMA-*b*-PnBMA	pDNA	Effect of block order and hydrophobicity on micelleplex morphology	662
		PDMAEMA-*b*-PnBMA	DNA	Effect of block copolymer architecture and pDNA size on micelleplex morphology	685
TEM	Imaging of polyplex size and morphology. Tracking cell internalization of labelled particles	Coumarin-PLGA	BSA protein	Track internalization of labeled nanoparticles	1244
		P4VPQ	ODN	Visualize micelleplex morphology	1239
		PEI-*g*-PEtOx	DNA	Visualize polyplex morphology	588
		PEI/HA	DNA	Polyplex stability and shape	1226
		PGAA	pDNA	Study glycosaminoglycan-polyplex interactions	1227
		PEI-*b*-PLL-*b*-PLG	DNA	Correlate polyplex morphology to pH	720
		PEG-*b*-P[Asp(DET)]-*b*-PLL	pDNA	Measure DNA packaging inside polyplexes	1218
		PEG-*b*-P[Asp(DET)]	pDNA	Image DNA packaging and conformation changes	1217
AFM	Size and morphology of polyplexes	Cationic oligomers	siRNA	Monitor polyplex size and shape	1096
		Poly(lysine-co-histidine)	pDNA		1245
		DMAEMA-MPC	pDNA		1246
		Spermine-modified dextran and pullulan	siRNA		1247
		Cyclodextran	pDNA		990
		PEI	pDNA	Identify correlations between polyplex morphology and polycation architecture	273
		Hyperbranched PAMAM	siRNA	Monitor assembly/disassembly of bio-responsive polyplexes	1248
		PLL multilayer films	ODN	Study coating morphology and rigidities	1249
		PLL multilayer films	DNA	Study coating morphology	1250
		PAMAM, PEI	DNA	Polyplex assembly/disassembly	1251

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Table 2. continued

method	measurements	vector	cargo	purpose(s)	refs
		DMAEMA multilayer films	DNA	Film rigidity and assembly/disassembly	1252
		PEI-siRNA multilayer films	siRNA	Measure polyplex sizes as a function of N/P ratio	1253
		PDMAEMA-Cholesterol	DNA	Monitor polyplex size and shape	1228
		PHPMA−PLL	DNA		1254

methods to modulate polyplex properties via the optimization of formulation parameters such as solvent environment and N/P ratios, (2) hydrodynamic methods that overcome transport limitations by achieving rapid and controllable mixing of polymer and payload streams, and (3) the encapsulation of polyplexes within polymeric particles and fibers via emulsion methods, electrospinning, and electrospraying.

6.1. Exploring the Roles of Formulation Parameters during Polyplex Assembly

Several research groups have tried to remediate the intrinsic lack of reproducibility and standardization associated with the polyplex assembly process by understanding and controlling the underlying formulation parameters. Through a stepwise exhaustive exploration of the vast experimental space within polyplex assembly, Candiani and coworkers identified the best-performing complexation conditions for four commonly employed polycationic vectors, namely, PEI, branched PEI, PLL, and PAMAM dendrimers.[1255] They discovered that optimal limits for experimental factors such as plasmid dose, incubation time after polyplex formation, polyplex dilution, polymer molecular weight, the N/P ratio (the ratio of ionizable nitrogen groups to phosphate groups within nucleic acids), buffer composition, sequence of addition, and volume ratios had strong effects on both the transfection efficiency as well as the cytotoxicity. Importantly, their study suggests that each of these formulation parameters had to be separately optimized for different polymeric reagents, underlining the high variability observed in polyplex assembly conditions across different studies. Even though this study was extensive, they focused on optimizing one variable at a time, while keeping other variables constant, an experimental design strategy that is tedious, uneconomical, and inadequate in capturing strong second-order interactions between two or more experimental factors. In contrast, studies that employ a statistical design of experiment methodologies[1256−1258] to optimize formulation parameters tend to produce more clear-cut conclusions, since multiple experimental factors are varied simultaneously to discover hidden interactions.

To develop a robust protocol for formulating polyplex particles from PBAEs, Green and coworkers investigated the roles of buffer composition, pH, polymer storage conditions, and polyplex mixing in detail. Surprisingly, they discovered that mixing volume ratios between polymer solutions and payloads had no impact, while the polyplex incubation time post-mixing proved to be consequential, with both short and long incubation times proving detrimental to transfection efficacy.[1260] The incubation time is a particularly challenging experimental factor to optimize since it has ramifications for polymer degradation (and loss of delivery efficacy for degradable polymers) as well as polyplex size distributions. Wide discrepancies have been observed while ascertaining whether the sequence of addition (e.g., polymer or nucleic acid added first) is of significance. The order of formulation steps has been shown to impact polyplex formation, which has been described in detail by Kwon and coworkers;[1259] they reported the formation of several smaller polyplexes (60 nm) when plasmid DNA or siRNA was added to PEI. When the reverse sequence was employed, a smaller number of aggregated polyplexes (200 nm) was formed instead, resulting in both an improved transgene expression and a higher cytotoxicity (Figure 40). Another study[1261] compared dropwise addition of polymer reagents and vigorous pipette mixing and

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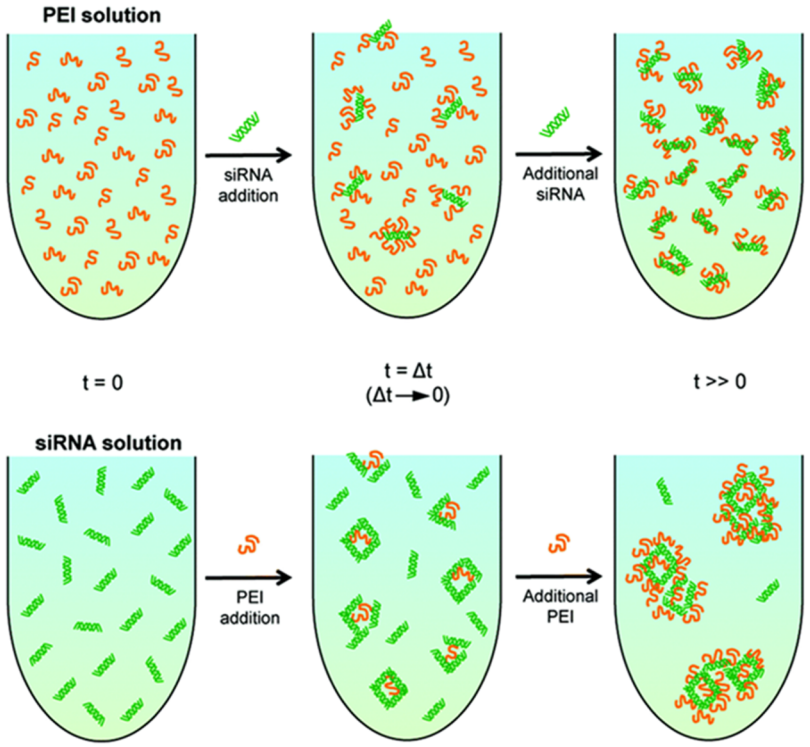

Figure 40. Effect of sequence of addition on polyplex size and composition. Addition of PEI to siRNA results in aggregation, while the reverse order leads to uniform, well-dispersed populations. Reprinted with permission from ref 1259. Copyright 2015 Royal Society of Chemistry.

concluded that the former resulted in larger polyplexes (~400 nm for dropwise addition as opposed to ~150 nm for mixing), which enhanced transfection in serum-free media through gravitational settling. In serum-supplemented transfection media, however, these size differences were completely neutralized by severe polyplex aggregation in both samples.

The polyplex stability and polymer-nucleic acid binding efficacy are both highly sensitive to environmental pH[1262] as well as ionic strength and identity.[1111] To ensure reproducibility, most groups choose to formulate polyplexes in standard buffers such as HEPES and PBS, instead of water, although divalent cations seem to improve the delivery efficiency.[1263] Lowering the pH during a polyplex assembly promotes strong binding between cationic polymers (charged groups are generally below their pK_a increasing protonation) and their payloads,[1257,1258] whereas polyplexes are also more prone to aggregation in high-ionic-strength buffers due to charge screening.[1264] This suggests that systematic investigations of ionic environment must be performed every time a novel polymeric delivery system is developed, since conclusions cannot be generalized from one experimental condition to another.

6.2. Ternary Complexes

Coatings prepared from biopolymers such as heparin sulfate,[302,1265] hyaluronic acid,[1263,1266] gelatin,[1267] and basic fibroblast growth factor[1268] have been shown to enhance the biological performance of polyplex formulations in diverse contexts. For instance, in applications requiring the controlled release of drugs or growth factors, Hammond and coworkers were among the first to demonstrate the benefits of incorporating biological derived polyanions such as heparin sulfate, chondroitin sulfate, and basic fibroblast growth factor to improve the performance of polyelectrolyte complexes.[1269−1271] Reineke and coworkers demonstrated that membrane association, cellular internalization, and transfection efficiency could be significantly improved manyfold by heparin-coating trehalose-based polyplexes.[302] The biological enhancements effected by this glycosaminoglycan (GAG) additive was not only found to be dose-dependent but also composition-dependent. While polytrehalose vehicles exhibited improvements in transfection efficiency, transfection was completely suppressed in PEI-based vectors upon the addition of heparan sulfate. Combining glycopolycationic vehicles with heparan sulfate seems to be an effective approach to transfecting challenging cell types such as primary fibroblasts and pluripotent stem cells. Hyaluronic acid (HA) is another GAG additive that has been widely used in cancer therapy owing to the overexpression of HA-binding CD44 receptors by tumor-forming cells.[1272] An HA coating seems to impart colloidal stability in biological media in a molecular weight-dependent manner,[1273] modify cell uptake kinetics,[1268] and reduce toxicity.[1274] Similarly, gelatin-coated polyplexes were found to be stable for up to 24 h in serum-rich media while still retaining their transfection efficiency, a result that contrasts with traditional PEGylation approaches. This study suggests that gelatin, which is ubiquitously used in the food and pharmaceuticals industries, could be a plausible steric stabilization alternative to resolve the PEGylation dilemma. Poly(glutamic acid) (PGA) peptide coatings were found to alter biodistribution profiles and impart tissue specificity to polyplexes depending on the quantity of PGA used.[1275] At low concentrations, large micron-sized particles were formed and mostly localized within the liver, while higher PGA concentrations imparted serum stability and reduced polyplex sizes, promoting spleen and bone marrow targeting. These approaches demonstrate the potential of applying biopolymer coatings to polyplexes through physisorption to modulate

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cellular internalization, receptor targeting, and achieve stealth properties. However, the coating process must be engineered to achieve precise surface densities and reproducible results so that fully defined polyplex nanoparticles are produced to fulfill diverse therapeutic niches.

6.3. The Importance of Formulation Ratio or Charge Ratio (N/P)

The dilemma confronting polymeric gene delivery is that efficient intracellular delivery is frequently accompanied by high levels of cytotoxicity.[1276] Apart from molecular weight, polymer architecture and composition, the N/P ratio, or the charge ratio between nitrogen atoms in polymers to phosphates in nucleic acid cargoes, is the single most influential experimental variable used to resolve the efficacy-toxicity conundrum. It is generally agreed that excess polymer is required for the formation of colloidally stable polyplexes owing to the net positive surface charge resulting from the surfeit of cationic polymers.[1277] However, the implications of using excess polymer are both complex and consequential due to the existence of intertwined relationships between polyplex formulation ratios and downstream biological events. On the one hand, adverse effects range from serum protein-induced aggregation and altered biodistribution profiles,[116] possibly provoked by enhanced interactions between polymers and extracellular proteins,[1247] and cellular membrane disruption caused by the induction of nanoscale pores and membrane leakage.[1278] On the other hand, excess polymer and high N/P ratios have also been shown to promote endosomal escape,[215,1279] disrupt the nuclear envelope,[137] ensure payload protection from degradation, and prevent aggregation. Typically, the role of charge ratio on key biological responses such as transfection efficiency, toxicity, hemocompatibility, and payload binding has been studied in isolation. Most of these studies have noted a strong N/P dependence of both cell viability and transfection efficiency and generated trade-off curves at the intersection of which the optimal N/P ratio can be identified to maximize viability in an efficacy-constrained manner.[1280] However, we believe that the best approach is to co-investigate the role of the N/P ratio in tandem with other attributes such as PEGylation,[844,1281] hydrophobicity,[599] and molecular weight.[849] We also draw attention to the creative use of physical characterization tools (Section 5.5) such as whole cell patch clamp measurements of membrane currents,[1282] scattering techniques such as SAXS and SANS,[1240] AFM, and NMR[222,223,1171] as well as polyplex purification approaches such as ultrafiltration,[1283] asymmetric fractional flow fractionation, and Taylor dispersion.[1097] The above tools allow us to thoroughly probe the binding state of polymers within polyplexes, visualize the dynamic equilibrium between bound and unbound states, and understand the role played by free polymers during transfection. These studies will also help researchers to answer several pressing questions in this field. Is the membrane porosity caused by free polymers merely an undesirable side effect or an indispensable cellular entry pathway? Are excess polymers essential to prevent the development of late endolysosomal vesicles and facilitate rapid intracellular payload release or do they merely activate cellular defense mechanisms such as cytosolic nucleases[1282] that depress transfection? It is difficult to draw conclusions on the effects of N/P ratios, since we cannot compare across divergent experimental setups, polymer compositions and architectures. We speculate that the question of whether the "burden of transfection" is mostly borne by free polymers or polyplex-bound polymers must be explored on a case-by-case basis, making the optimization of the N/P ratio a vital development exercise. We also note that the decationization of polyplexes (Section 5.3.1)[1161] and the development of polymeric vehicles that do not rely on electrostatic interactions for polyplex assembly will minimize the need to carefully optimize the N/P ratio.

6.4. Directing Polyplex Assembly through Microfluidics

Microfluidic systems are miniaturized flow chambers wherein at least one dimension of the flow channel is less than a millimeter. Because of these small dimensions, it becomes easier to achieve a highly predictable flow regime termed laminar flow, defined by a region where the Reynolds number is less than 2100. Low Reynolds number flows have special properties, since complexation processes behave quite differently at such small dimensions compared to bulk mixing conditions. Microfluidic tools, therefore, have been harnessed to control the mixing and assembly conditions during polyplex formulation, offering a powerful way to manipulate the physical properties of polyplexes, notably size distribution and composition. Polyplex preparation "on a chip" was initially explored as a means of improving the properties of commercial PEI-based reagents.[1284] Despite the extensive optimization of addition sequence, concentrations, and mixing speeds, it was recognized that standard pipette mixing and vortexing procedures were unable to prevent polyplex aggregation and the formation of heterogeneous populations.[1285] In contrast to bulk mixing, polyplexes assembled in a microfluidic device not only had smaller diameters and narrower dispersites but also retained payload integrity and compaction, resulted in superior transfection efficiency, and lowered toxicity.[1284] When the assembly process was confined within picoliter-sized droplets through emulsion formation,[1286] the quantity of cationic polymer and plasmid within each polyplex can be tailored precisely while droplet dispersion within a buffer can ensure that aggregation does not occur.[1287] This microfluidics-assisted confinement approach was able to produce homogeneous polyplex populations that were resistant to aggregation and resulted in lowered cytotoxicity compared to standard mixing procedures (Figure 41). Further improvements to the

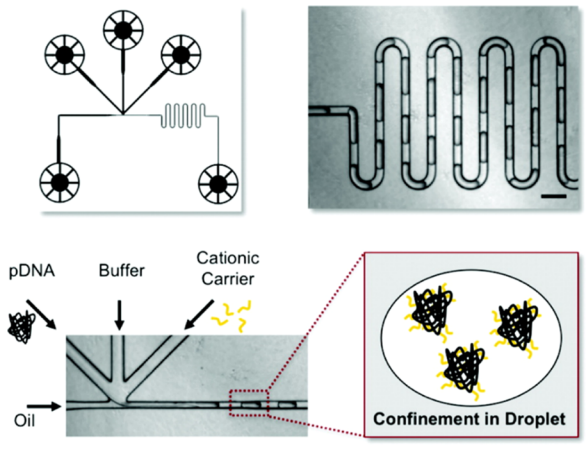

Figure 41. Microfluidics-assisted confinement was applied to generate picoliter droplets, thereby controlling polyplex size distribution and composition through confinement. Reprinted with permission from ref 1287. Copyright 2011 American Chemical Society.

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microfluidics-assisted confinement approach were effected by hydrodynamic focusing of flows at the intersections between multiple microchannels.[1288] This modification shrinks the diffusion length scales, allowing for faster mixing and more uniform polyplex particles. 2D hydrodynamic focusing can be restricted to a single plane,[1289] but greater confinement and improved mixing profiles can be achieved using 3D hydrodynamic focusing.[1290,1291] The integration of a dielectrophoretic separation step within these droplet microfluidic tools can enable an in situ screening to sort polyplexes based on size specifications, improving polyplex properties even further.[1292] While hydrodynamic and droplet-based methods are subject to diffusion limitations and rely on passive mixing, acoustic waves can be used to accelerate mass transport, thereby increasing the mixing efficiency by reducing the length scale over which diffusion occurs.[1293] These "acoustofluidic" methods[1294,1295] have been shown to produce even narrower polyplex sizes compared to traditional microfluidic tools.[1290] Overall, we conclude that batch mixing techniques such as pipette mixing for the most part do not ensure reproducible results with many systems, which is important for scale-up, clinical testing, or animal studies; indeed, continuous-flow approaches and microfluidic platforms for polyplex formation may resolve this issue.[1296−1298]

Microfluidic technologies can be a valuable tool to screen a multitude of polymer designs and formulation variables by integrating cell culture, transfection, and microscopy modules within the same microfluidic chip.[1299,1300] A microfluidics-based high-throughput screening strategy minimizes the quantity of biological reagents consumed, ensures experimental consistency, and enables a rapid discovery of hit polymers for diverse payloads and therapeutic applications (Figure 42).

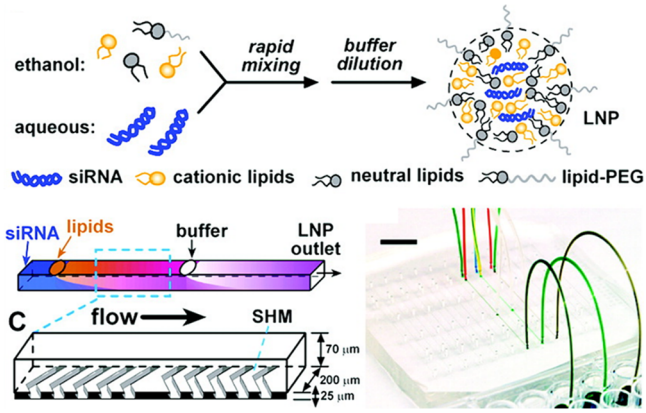

Figure 42. Formation of lipoplexes for high-throughput screening enabled by microfluidic formulation. Reprinted with permission from ref 1301. Copyright 2012 American Chemical Society.

Since small volumes (<10 μL) can be reliably and rapidly mixed within these devices, numerous combinations of polymer and payload concentration mixing conditions, N/P ratios, and flow rates can be screened rapidly,[1301] if these high-throughput microfluidic platforms are integrated with in-line tools to monitor the evolution of polyplex sizes as well as the binding interactions between the polymers and nucleic acids.[1302]

Powerful as microfluidic tools may be, they still require access to dedicated cleanroom facilities to fabricate intricate microdevices based on polydimethylsiloxane as well as specialized know-how. Further, to achieve scale-up for clinical translation, multiple units must be operated in parallel to produce the requisite quantities of polyplexes. Millifluidic devices such as confined impinging jet mixers can be fabricated more easily using standard machining tools. Unlike microfluidic devices that are based on laminar flow, confined impinging jet mixers operate in the turbulent flow regime, where the characteristic mixing time can be reduced to tens of milliseconds.[1303] This time scale compares well to the 50 ms time span that was observed for spontaneous electrostatically driven assembly to occur between cationic polymers and nucleic acid payloads. Rapid turbulent mixing narrows the temporal window for polyplex aggregation, creating well-defined polyplex formulations characterized by tailored nucleic acid loadings, tunable diameters, and narrow dispersities. Drawing inspiration from the pioneering work of Johnson & Prudhomme, who first described the role played by turbulent mixing during flash nanoprecipitation to achieve narrow crystal size distributions for pharmaceutical manufacturing, Mao et al. used confined impinging jet mixers to engineer polyplexes with improved physical properties and transfection.[1304]

6.5. Kinetic Control of Polyplex Assembly through Turbulent Mixing

The term "flash nanocomplexation" was coined to describe this assembly process wherein a fluid stream comprising the cationic polymer solution comprising linear PEI would meet an opposing fluid stream containing plasmid DNA at extremely high velocities to create highly controlled assemblies through a confined impingement of these jets. By tuning the channel diameters, the volume of the mixing chamber, concentrations, and flow rates, different mixing times can be achieved, thereby varying the kinetic regimes for a polyplex assembly.[1306] The most recent report of a confined impinging jet mixer-mediated polyplex formation[1305] illustrated how careful modulation of the characteristic mixing time could be used to obtain polyplexes with any desired number of plasmids per nanoparticle (between 1 and 21) as well as diameters as low as 35 nm (Figure 43). Kinetic control over polyplex mixing served to reduce the mixing time relative to the characteristic assembly time and yielded polyplexes with altered biodistribution profiles and minimized the formation of necrotic tissue in the liver during in vivo delivery. In addition to demonstrating in vivo efficacy, this study thoroughly characterized polyplex size and composition through DLS and SLS, underlining the intimate relationship between polyplex physical properties and biological behavior. A potential risk of this confined impinging jet mixer-mediated flash nanocomplexation is that only robust payloads like pDNA can be used, which do not undergo chain breakage or scission during turbulent mixing. Flash nanocomplexation is yet to be explored for RNA and protein-based payloads, but it is expected that subtle modifications to the flow geometry and a slight reduction of flow rate could prevent payload damage while retaining a turbulent flow and high energy dissipation rates. Overall, confined impinging jet mixers are highly promising tools that can be explored to alleviate polyplex aggregation and modulate payload dosing within polyplexes.

6.6. Electrohydrodynamic Processing of Polyplexes

The encapsulation of polyplexes within polymeric nanofibers via electrospinning[1307] is a powerful way to prolong release kinetics, which is particularly critical while delivering nucleic acids for wound healing and tissue regeneration.[1308] Electro-

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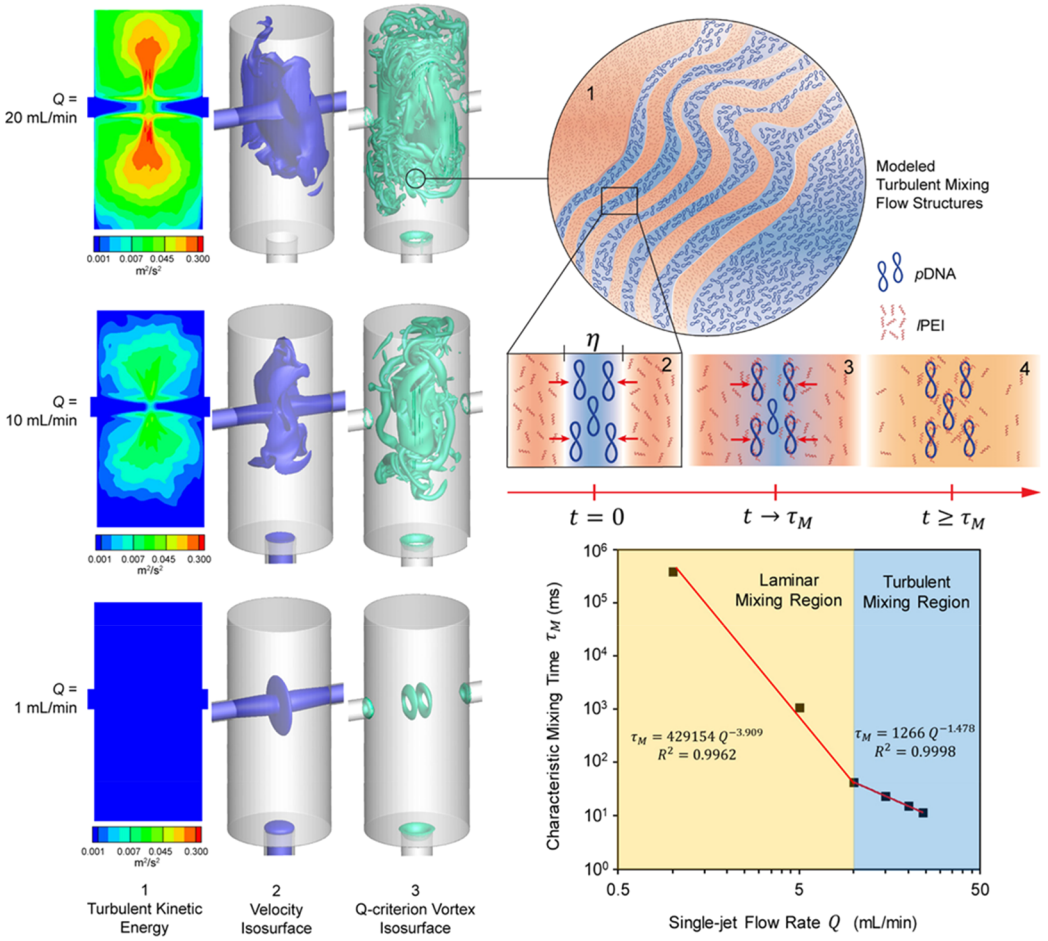

Figure 43. Mao and coworkers used confined impingement jet mixers to engineer uniform polyplex populations via flash nanocomplexation. Turbulent mixing was exploited to reduce the characteristic time of mixing, preventing undesired aggregation. Reprinted with permission from ref 1305. Copyright 2019 American Chemical Society.

spun polymer mats are applied as extracellular matrix (ECM) mimics due to their high surface area-to-volume ratio, conformal adherence to cells, porous architecture, and tunable mechanical properties. In contrast to substrate-mediated gene delivery of naked DNA from the outer surface of electrospun fibers, several groups have developed inventive methods to incorporate DNA within the core of the fibers, where they are likely to be more stabilized. By condensing nucleic acids payloads with chitosan[1309,1310] or PEI-based carriers[1311,1312] prior to embedding these polyplexes within fibers via electrospinning, it is possible to prolong release lifetimes of polyplexes to up to a month instead of obtaining burst release within a few hours. However, some groups have also reported prolonged release profiles and efficient gene silencing mediated by naked siRNA embedded within nanofibers, despite the lack of a complexation pre-step,[1313,1314] suggesting that different techniques may need to be adopted for diverse payload types. An interesting formulation approach is the physical entrapment of pDNA/PEI polyplexes within degradable PLGA microspheres via emulsion techniques.[1208] This method can be adapted to electro-hydrodynamic processing through a coaxial electrospraying of pDNA within a sheath of PEI, resulting in the preservation of pDNA integrity without sustaining any damage due to high electric fields.[1315] Although the N/P ratio could be modulated by tuning flow rates during electrospraying, polyplex size distribution was found to be highly variable across different processing conditions. Though electrohydrodynamic polyplex formation outperformed bulk mixing, it needs to be further optimized to expand the application to other polymeric vehicles beyond PEI. A particularly interesting capability afforded by electrohydrodynamic processing of polyplexes is the compartmentalization of imaging modalities and pH-sensing functionalities within distinct hemispheres of bicompartmental microparticles.[1315] While the hydrophobic PLGA compartment facilitated incorporation of fluorescent molecules for microparticle visualization, the cross-linked PEI compartment induced endosmotic swelling and bursting, promoting siRNA release and gene silencing. The authors argued that synergistic effects that result from compartmentalization cannot arise from using mixtures of individual particles. While electro-hydrodynamic formulation of polyplexes is a creative way to control morphology, composition, and internal architecture, further research is essential to obtain narrower formulation size distributions and fine-tune release kinetics.

Overall, we have presented an overview of diverse approaches to polyplex formulation that go beyond manual methods and exploration of solution parameters such as pH, ionic strength, or polymer dose. We expect that the application of microfluidic, electro-hydrodynamic, and millifluidic methods in gene delivery will continue to grow, accessing interesting

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material properties and enabling tight control over size distribution and nucleic acid dosing.

7. ALTERNATIVE BIOMATERIAL PLATFORMS FOR TRANSFECTION

In contrast to polymeric vehicles obtained via controlled radical polymerization or post-polymerization modification, some biomaterial platforms rely on polymer processing methods rather than chemical synthesis to obtain desired material properties. Examples include substrate-mediated gene transfer from protein-coated planar substrates, hydrogel-mediated gene transfer in 3D cell culture environments, and core-shell nanoparticles where polycationic coronas are grafted from inorganic nanoparticle templates. Although these atypical biomaterial platforms lack chemical sophistication and do not require complex chemical synthesis procedures, they are simple yet powerful tools to probe physical design parameters in polymer-mediated gene delivery.

7.1. Substrate-Mediated Transfection in 2D and 3D Cell Culture Environments

Tissue engineering seeks to reprogram cellular behavior with the goal of controlling proliferation, differentiation, migration, or the induction of desired cellular phenotypes. Bioengineers achieve these goals by impregnating tissue engineering scaffolds with growth factors, trophic factors, and transcription factors to manipulate cellular responses. However, engineering sustained release of these protein-based cargoes is crippled by the instability of these large and complex biomolecules, which have half-lives as low as 2 min in physiological milieus.[1316] Further, delivery strategies and processing conditions must be individually optimized for each bioactive cargo, making allowances for the size, charge, surface chemistry, and stability of each protein. Despite advances in protein delivery, large doses and repeated injections are a frequent necessity. To counter the cost and stability constraints imposed by protein delivery, genetic cargoes encoding for the desired protein or protein fragment were explored as alternatives to protein depots. Gene delivery presents several advantages over protein delivery: its universality is attractive since delivery platforms need not be redesigned for different DNA sequences, it lowers costs, and does away with the need for repeated and large doses, since transfected cells function as "biofactories" secreting the protein or growth factor of interest in a sustained fashion, maximizing its bioavailability. However, gene delivery is associated with a significant time lag that can span several days, which contrasts with the immediate availability of bioactive cues guaranteed by protein delivery.

A common gene delivery approach employed by tissue engineers is the immobilization of DNA to the substrate on which cells are cultured, thereby placing the genetic cargo within an immediate proximity of its cellular target (Figure 44). It has been argued that substrate-mediated gene delivery is inherently biomimetic in its design, since both viral vectors as well as endogenously produced growth factors exploit interactions with the extracellular matrix to mediate cellular internalization.[1317] Cells growing on nucleic acid-immobilized substrates can either endocytose the DNA directly or ensure DNA release from the substrate by disrupting chemical or physical associations between the nucleic acid and the substrate. By engineering concomitant delivery of multiple nucleic acid payloads or by co-delivering genes with proteins,

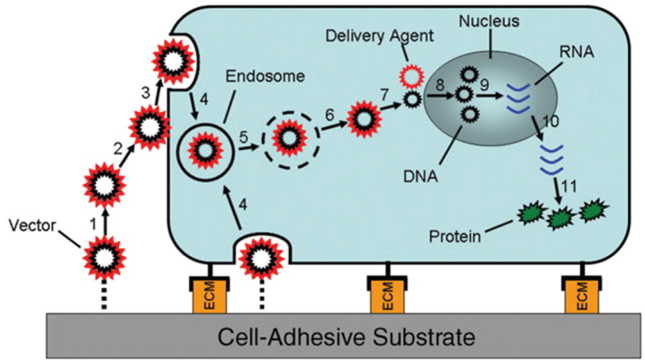

Figure 44. Substrate-mediated gene delivery from 2D substrates wherein naked nucleic acids of polyplexes, either specifically or non-specifically immobilized to the substrate, are transferred to adherent cells. Mechanism of substrate-mediated gene delivery: (1) vector release, (2) membrane association, (3) endocytosis, (4) early endosome, (5) late endosome, (6) escape from endosome, (7) nuclear translocation, (8) nuclear entry, (9) transcription into RNA, (10) transport of RNA to cytoplasm, and (11) translation of RNA into protein. Reprinted with permission from ref 1319. Copyright 2005 Materials Research Society.

we can engineer complex tissue architectures where multiple cell types are organized in a hierarchical fashion.[1318]

Polymeric biomaterials intended to deliver therapeutic nucleic acids typically focus on optimization of material properties with systemic routes such as intravenous, oral, intradermal, and intramuscular administration in mind. However, precise targeting of disease sites such as tumors or specific tissue types is almost impossible with systemic delivery approaches. The need to engineer targeting modalities can be obviated by employing local delivery or substrate-mediated delivery.[1320] Substrate-mediated gene delivery platforms can also be designed to mimic the extracellular matrix, wherein cellular targets can infiltrate the matrix, eventually leading to local cellular uptake of DNA embedded within the matrix.[1321] Substrate-mediated gene delivery seeks to alleviate several shortcomings associated with "bolus transfection". First, the local concentration of nucleic acids at the cell−polymer interface is much higher for substrate-mediated delivery compared to bolus methods, ensuring that transfection is not bottlenecked by transport limitations, such as a slow diffusion of polyplexes and diameter-dependent settling velocities, ultimately minimizing serum-induced degradation en route to cells and promoting opportunities for polyplex-cell contact. Second, the high local concentrations achieved by substrate-mediated methods eliminates the need to use high loadings of nucleic acids as well as polymeric vectors, minimizing cost as well as cellular toxicity. Finally, by sustaining a therapeutically-relevant release rate of nucleic acids for prolonged durations, substrate-mediated delivery can dramatically improve the delivery efficiency by mediating repeated transfection events. While synthetic vectors for gene delivery are typically evaluated on standard 2D tissue culture polystyrene plates, cellular responses to polyplexes in a 2D culture are not necessarily predictive of in vivo outcomes. In general, 3D polymeric scaffolds or matrices are considered to be more realistic models that simulate the native physiological milieu of living tissues. Moreover, cellular phenotypic expression varies dramatically between 2D and 3D environments, and loss of key phenotypes has been observed for cells cultured for long durations in 2D cultures. In the context of gene delivery, "dimensionality" has

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Table 3. Summary of 2D Platforms for Substrate-Mediated Transfection

substrate	DNA complexation agent	summary	refs
Tissue Culture Polystyrene	Naked	Chitosan and hyaluronic acid coatings employed to immobilize DNA	1350
		Lipid coatings employed to immobilize DNA	1351
	PEI	Col I-coated surfaces employed to immobilize DNA	1346
		Examined the effects of ECM coating composition and density on cytoskeletal dynamics.	1348
			1347
			1326
	Lipoplexes	Enhanced substrate-mediated lipofection through peptide incorporation	1352
	PEI/Lipoplexes	Compared recombinant and full-length fibronectin coatings	1344
	Lipoplex/Polyplex	Screened a library of ECM-mimetic substrates	1349
PLGA	Fibronectin	Guided neurite extension using NGF-patterns	1341
	PEI	Neurite extension using NGF-patterns	1341
		Covalent binding of PEI polyplexes to PLGA through EDC/NHS	1332
	Lipoplexes	Investigated ECM coating composition to heal spinal injury	1340
	Lipoplexes/PEI	Effect of serum deposition on DNA loading and transfection	1334
Polydopamine	Protamine	Inducing rapid endothelialization of implanted vascular devices	1353
	Naked	Studied cell spreading, morphology and membrane perturbation induced by silicon nanowires	1354
Avidin/Neutravidin	PLL	Effect of polyplex immobilization density	1355
	PLL/PEI		1336
	Chitosan	N/P ratio AND biotinylation degree regulated gene delivery	1356
Coculture models	Lipoplexes/PEI	Neuronal archi*t*ecture controlled by engineering gradients of growth factors secreted by transfected cells in a co-culture model	1357
	Lipoplexes		1358
Chitosan	Naked	Reprogramming of human fibroblasts into neural crest stem-like cells	1359
		Directed differentiation of HGFs along neural pathways	1360
SAM	Lipoplexes	Surface chemistry, hydrophobicity, charge density studied on SAM libraries	1329
	PEI	Effect of PEG incorporation on polyplex size distribution and stability.	1330
	His-PEI	Histidine-NTA linkages immobilize polyplexes on SAMS of Ni/Au	1331
Silicon-based nanosheets/ nanowires	Lipoplexes	Silica network architecture used to modulate transfection outcomes	1361
	PAMAM/Ad +PEI/Cd	Specific binding of polyplexes on Silicon nanowires	1327
Steel/Titanium	PEI	Polymer brushes functionalized with adhesion ligands (RGD)	1362
Polyallylamine bisphosphonate	Anti-DNA antibody	Gene-eluting stents engineered using covalent immobilization	1333
Polyurethane	Naked	Impact of nanotopography on cellular motility and spreading	1363
Intestinal sub mucosal	PEI	Non-specific immobilization of polyplexes on biological substrates	1339

repeatedly been demonstrated to have profound effects on endocytosis pathways, cytoskeletal dynamics, mechanisms of gene transfer, and cellular signaling pathways. Nevertheless, 2D studies have several advantages: simplicity, throughput, and homogeneous access to nutrients in the cell culture media. In this section, we will briefly summarize substrate-mediated gene delivery approaches attempted in both 2D and 3D cell culture environments and outline directions for future studies. The reader is redirected to several review articles[1317−1320,1322−1325] on this topic, where they will find a more biologically focused discussion of substrate-mediated transfection. We will restrict our focus to material design and synthetic considerations.

7.1.1. Substrate-Mediated Transfection in 2D Cell Culture Environments. The surface immobilization of nucleic acids is performed by depositing either naked DNA or pre-complexed polyplexes or lipoplexes on tissue culture polystyrene substrates that are pre-coated with gelatin, chitosan, PLL, or poly(lactic-*co*-glycolic acid) (PLGA) that promote both cell adhesion as well as DNA entrapment. While embedding uncomplexed or naked DNA within coated cell culture substrates is facile and allows for rapid payload internalization, it requires high DNA loadings to facilitate transfection. In contrast, preformed polyplexes or lipoplexes offer better protection to DNA from serum nucleases and mediate efficient transfection even at low nucleic acid doses. A potential disadvantage of using synthetic materials to complex DNA is that the vector properties can drastically alter the size distribution and surface charge, and aggregation-prone materials such as PEI could adversely affect transgene expression. Another design parameter in substrate-mediated gene delivery is the choice between specific and non-specific immobilization approaches.[1326] Specific approaches such as avidin-functionalized substrates to bind to biotinylated polyplexes, adamantane-cyclodextrin interactions,[1327] self-assembled monolayers,[1325,1328−1331] covalent chemistries,[1332] or antibody-antigen binding[1333] offer greater control over the transfection process since the immobilization density[1326,1334] and nucleic acid dosing[1335−1337] can be precisely tuned. Moreover, immobilization approaches can employ cleavable peptide sequences as covalent tethers such that polyplexes are released from the substrate through cellular degradation processes mediated by matrix metalloproteinase (MMP).[1338] Substrates can be functionalized with an optimized mixture of biochemical cues driving cellular adhesion and matrix, thereby prolonging nucleic acid release and maximizing transgene expression. A less elegant, albeit highly effective, approach to nucleic acid immobilization is through non-specific interactions such as electrostatic forces[1339] or mere physical

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Table 4. Summary of 3D Platforms for Substrate-Mediated Transfection

scaffold material	DNA complexation	summary	refs
PLGA	Naked	Platelet-derived growth factor delivered from porous PLGA for angiogenesis	1387
		Vascular morphogenesis through delivery of Del-1 from injectable implant	1343
		Spinal cord repair by delivering plasmids over extended durations	1388
		Layered design of porous and non-porous PLGA scaffold	1388
		Subcutaneous implantation of DNA-loaded scaffold	1389
	PEI	BMP-4 delivery to heal critical bone defect	1342
	PEI/PAA/PDA/PLL	Initial plasmid dose, choice of promoter and vector composition studied	1335
	PLGA	Probe effects of pore architecture on DNA stability and release kinetics	1337
Fibrin	Naked	Full thickness wounds healed by delivering EGF to keratinocytes	1390
		Fibrin encapsulation did not enhance VEGF plasmid delivery	1391
	Peptide lipoplexes	Therapeutic angiogenesis through delivery of transcription factor HIF-1alpha	1392
	Lipoplex	Spatial control of transfection through fibrin microarrays	1383
		Comparing cell encapsulation vs "seeding onto" approach	1375
Alginate	PEI	Hydrogel-mediated VEGF delivery outperformed bolus delivery	1393
	Naked	Role of RGD density and hydrogel stiffness during siRNA delivery	1167
Atellocollagen	Naked	Gene silencing for inhibiting tumoral growth	1394
		Intramuscular gene delivery	1395
Chitosan	Naked	Peripheral nerve regeneration through BDNF delivery to MSCs	1396
Collagen	Naked	Platelet-derived growth factor to heal chronic wounds	1397
		Inhibit collagen deposition through anti-sense delivery	1374
	Lipoplex	Dual delivery of VEGF and BMP-2 for healing critical bone defects	1398
Gelatin	Naked	Intramuscular delivery of FGF-4 for ischemia	1399
	PEI	Bone growth through dual delivery of bFGF and BMP-2	1400
Hyaluronic acid	Naked, PEI	Interactions between PEI and hyaluronic acid modulated transgene expression	1378
	PEI	Effect of polyplex diameter gene delivery	1345
		RGD density and matrix stiffness evaluated conjointly	1165
		Effect of pore architecture on vascularization	1376
		Extended DNA release over 30 days mediated by multiple transfection events	1401
	PEI/Lipoplex	Caged nanoparticle encapsulation sued to prevent polyplex aggregation	1377
PEG	PEI	MMP-degradable peptides incorporated via Michael addition chemistry for MSC transfection	1379
		Bioinstructive hydrogels created using RGD gradients to guide cell migration	1382
		Electrospun mats functionalized with MMP-responsive peptides for diabetic wound healing	1382
		Cellular infiltration is key to obtaining extended DNA release in MMP-responsive hydrogels	1380
	Transfast	Affinity peptides enhanced polyplex retention to improve transfection	1402
	Lipoplexes	Tuning RGD density to control cell migration to balance hydrogel degradation rates	1381

entrapment within a polymeric matrix (PLGA is typically used).[1332,1340−1343] While the release kinetics cannot be controlled, these substrate-mediated approaches are easy to implement and facilitate a rapid release of DNA from the substrate. Another effective non-specific approach is the use of extracellular matrix coatings such as collagen, fibronectin, or laminin on planar substrates. The groups of Pannier, Shea, and Segura have devoted extensive efforts to studying the interplay between the cellular microenvironment and transfection mechanisms (Table 3). Shea and coworkers noted that transgene expression could be amplified on serum-coated substrates compared to uncoated ones and systematically probed the role of protein density and identity on gene delivery.[1326,1334,1340,1344,1345] Segura and coworkers concluded that fibronectin coatings promote polyplex internalization and uptake by guiding polyplexes through a more favorable clathrin-mediated endocytosis, in contrast to collagen coatings, which tend to favor the less-effective caveolar routes.[1346] They have also probed the roles of RhoGTPases in modulating substrate-mediated gene transfer to mesenchymal stem cells on fibronectin-coated surfaces.[1347] Mixtures of recombinant ECM proteins were deployed to understand the effects or surface chemistry on cell morphology, spreading, and integrin expression and their downstream impacts on polyplex internalization.[1348] A recent study from Pannier and coworkers extended this idea further to elegantly demonstrate the impact of cellular microenvironment on substrate-mediated transfection. Combinatorially-designed binary and ternary mixtures of glycosaminoglycans such as heparin sulfate and adhesion peptides such as RGD were deposited to generate a library of 20 ECM-mimetic cell culture substrates.[1349] These combinatorially-generated substrates resulted in a 2- to 20-fold higher transgene expression than homogeneous protein coatings. The authors hypothesized that cell adhesion, spreading, and polyplex internalization could be maximized by screening for the most suitable ECM substrates within this library.

In addition to biologically-derived cell culture substrates, graphene and graphene oxide substrates can either be covalently or non-covalently modified with cationic polymers such as PEI to immobilize DNA on its surface.[1364,1365] These graphene oxide-based platforms are highly promising to modulate cell proliferation, differentiation, and survival through the use of nanotopographical cues, spatial patterns, and a dual release of drugs and nucleic acids.[1366−1369] Microcontact printing of self-assembled monolayers can be used to create microarrays of transfected cells,[1370−1373] creating a high-throughput screening platform for probing correlations between gene expression and cellular responses to

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environmental cues.[1329] Further, microcontact printing and similar patterning techniques are of use in tissue-engineering applications such as neurite guidance, which require well-defined patterns of gene expression promoting neural growth factor secretion along delineated areas.[1341,1358] Finally, 2D platforms for substrate-mediated delivery can be valuable tools to understand the impact of nanotopography and surface chemistry on cellular responses such as integrin signaling, cytoskeletal activation, cell migration, and adhesion, with the ultimate goal of improving the "transfectability" of challenging cell types through environmental modulation.[1363]

7.1.2. Substrate-Mediated Transfection in 3D Culture Environments. The earliest attempts at a scaffold-mediated transfection utilized simple polymer matrices such as PLGA or poly(vinyl acetate), generally described as "gene activating matrices".[1374] The release rate of nucleic acids could be tuned by modifying the pore architecture of PLGA through appropriate modifications to polymer processing conditions. Although these were initial 3D model systems for matrix-mediated gene delivery, PLGA-based systems provided valuable insight on the mechanistic differences between substrate-mediated and bolus delivery approaches. These systems were also probed to evaluate whether specific nucleic acid immobilization could affect improvements in delivery efficiency, over the non-specific impregnation of PLGA with DNA.

Among 3D cell culture substrates (Table 4), hydrogels are more widely used than PLGA or other polymer matrices, since the former combine a rich aqueous environment with a structural support for cells to adhere to and proliferate, while the latter provide structural support alone. By providing large open spaces for cellular migration and infiltration, hydrogels have recapitulated the extracellular matrix of native cellular environments. Cells can either be seeded onto the hydrogel surface, from where they can subsequently infiltrate the porous matrix, or they can be encapsulated within the hydrogel by mixing cellular suspensions with hydrogel precursors prior to cross-linking.[1375] The beauty of hydrogel formation is that extremely mild chemical cross-linking procedures can be implemented, minimizing damage to cellular processes. It has been shown that the "seeding onto" approach is superior to cellular encapsulation since cell migration and infiltration are necessary to degrade the hydrogel matrix and release genetic payloads for uptake. Similarly, polyplexes or lipoplexes can be introduced into the hydrogel either through encapsulation or through surface immobilization (through biotin-avidin interactions,[1345] electrostatic interactions, or covalent bonds). The former approach was found to be ineffective even when the hydrogel mesh size was much smaller than the polyplex, since polyplex diffusion is hindered and the hydrogel must degrade to release complexes.[1376] Other challenges include the optimization of hydrogel-vector interactions such that polyplexes are retained long enough on hydrogels to sustain prolonged DNA release for the duration of cell migration. However, extremely strong interactions between polyplexes and the matrix can hinder cellular uptake and DNA release. Polyplex aggregation can also drastically alter transgene expression profiles since smaller polyplexes tend to transfect a larger number of cells during substrate-mediated transfection. Segura and coworkers developed a "caged nanoencapsulation" approach to prevent polyplex aggregation and enhance transgene expression efficiency.[1376] Balancing hydrogel degradation rates to match the rate of cell migration is especially tricky. Any attempt to tune degradation kinetics would inevitably be accompanied by changes in hydrogel composition, adhesion ligand density crosslinking density, swelling ratio, and mechanical properties, all of which are critical to achieving efficient transfection.[1377]

An independent control of all these key material properties (Figure 45) is relevant to a hydrogel-mediated gene delivery,

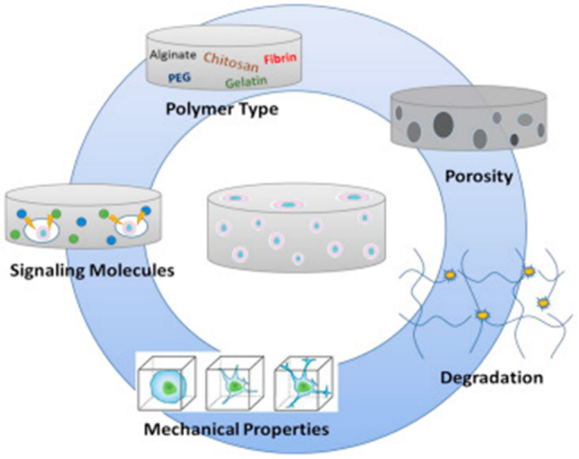

Figure 45. Design considerations for 3D hydrogels. Reprinted with permission from ref 1318. Copyright 2018 Elsevier.

which in turn requires a creative use of synthetic chemistry and expanding the toolbox of hydrogel building blocks beyond PEG. Although PEG is synthetically convenient and ensures structural integrity, it does not interact specifically with cellular receptors, unlike biologically derived polysaccharides and ECM-mimetic materials such as hyaluronic acid.[1378] Blending PEG with ECM-based materials such as HA, collagen, or fibrin is a common strategy to enhance hydrogel biofunctionality. Further, materials such as hyaluronic acid and fibrin can be degraded by cell-secreted enzymes, unlike PEG hydrogels, which require the enzymatic action of matrix metalloproteinases, a family of zinc-based endopeptidases. Cross-linked hydrogels can incorporate matrix metalloprotease-responsive peptides, and DNA release rates can be readily tuned within these enzymatically degradable hydrogels to prolong transgene expression.[1165,1379,1380] These studies suggest that degradability allows for cell infiltration and migration and is therefore a key requirement for matrix-mediated gene delivery to be effective.

RGD peptide motifs are frequently incorporated within cross-linked hydrogels to facilitate cellular adhesion and proliferation.[1377,1380] It is important to note that the spatial presentation of RGD motifs, rather than the surface density alone, is critical in determining cell proliferation and migration behaviors that ultimately impact transfection. In 2D cultures, it was demonstrated that a clustered presentation of RGD was more effective than uniform spatial distributions. Several studies have noted the strong effects of RGD density[1381] within hydrogels, with some studies observing a non-monotonic relationship between RGD concentration and transgene efficiency.[1165] Mooney and coworkers systematically varied the RGD density in conjunction with alginate hydrogel modulus and observed that RGD density was a much more influential design parameter than the hydrogel stiffness in shaping gene silencing efficiency.[1167] To engineer vascularized

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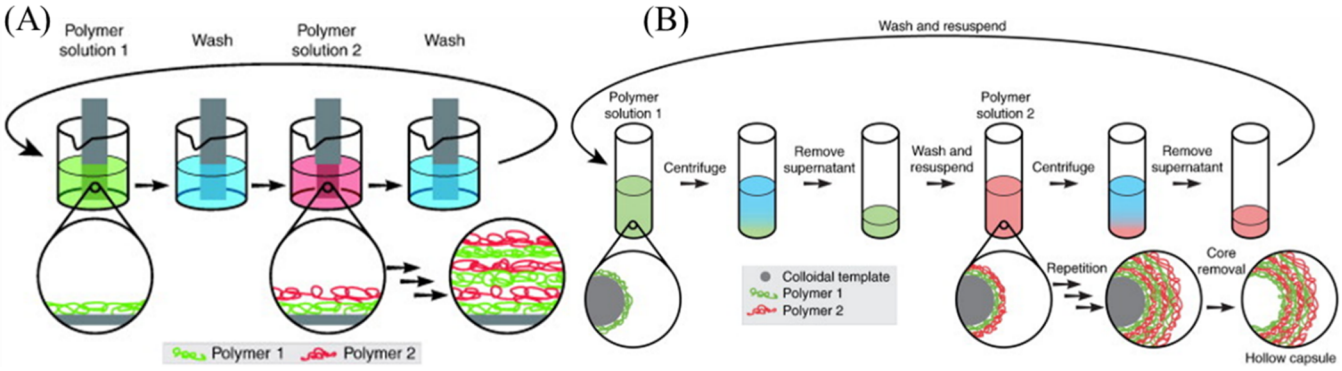

Figure 46. Schematic of an LbL assembly of polyelectrolyte multilayer films on (A) planar substrates and (B) nanoparticles. Reprinted with permission from ref 1444. Copyright 2012 Elsevier.

tissues or to guide the extension of neural conduits, it's necessary to ensure the spatial localization of genetic payloads along well-defined regions within hydrogels. Shea and coworkers generated cross-linked hydrogels bearing spatial patterns of immobilized polyplexes through biotin−streptavidin interactions.[1345] They note that specific immobilization strategies are essential to pattern hydrogels and that the spatial organization of cell-adhesive cues such as RGD is critical in determining transgene expression. The placement of bioactive cues such as RGD can be a useful lever of control to modulate transfection outcomes. For instance, cellular migration has been shown to promote cell-polyplex contact, but this requires the creation of gradients of bioactive signals through microfluidic synthesis of hydrogels.[1382] Spatially-patterned hydrogels can also lead to the creation of cellular microarrays for screening studies. Lipoplexes encapsulated by fibrin hydrogels could be spotted as a microarray allowing for discrete hydrogel spots allowing pDNA densities, fibrinogen concentration, and cell densities to be varied independently.[1383]

Many future directions for research in hydrogel-mediated gene delivery exist, and one particularly interesting approach focuses on photoresponsive hydrogels, wherein both mechanical and chemical properties can be easily modified with exquisite spatiotemporal selectivity.[1384] Moreover, design rules elicited from hydrogel platforms can be readily implemented for electrospun polymer mats, another promising class of tissue-engineering scaffolds.[1385] While many studies have preferred to encapsulate genetic cargo within coaxially generated polymeric microfibers and nanofibers to protect DNA from harsh processing conditions,[1386] it would be highly desirable to functionalize electrospun fibers with ECM coatings and effect gene delivery from these bioactive matrices.

7.2. Polyelectrolyte Multilayers

Layer-by-layer assembly is a rapidly evolving materials platform for nucleic acid delivery that combines exquisitely tunable release kinetics, co-delivery of diverse cargoes encompassing drugs,[1403] nucleic acids,[1404,1405] and imaging modalities[1406,1407] in a sequential or "scheduled" manner[1408] and a vastly diversifying substrate scope. The inherent simplicity of LbL synthetic methodologies and its unique capabilities have allowed LbL-based materials to address complex therapeutic challenges in creative ways.[1409] LbL coatings are assembled by alternately depositing two or more macromolecules that share complementary interactions with each other through electrostatic attractions, hydrogen bonding,[1410] DNA base-pairing, covalent bonds,[181,1411,1412] or metal-ligand chelation.[1413] Originally reported by Decher and Hong in 1991,[1414] the first reported synthesis of LbL coatings exploited electrostatic interactions to build alternating layers of poly(styrenesulfonate) and poly(allylamine hydrochloride) starting from a charged substrate.[1414] Deposition steps are interwoven with washing steps in order to remove unbound polymers, ensuring the formation of monolayers and cyclical charge reversion over the course of each immobilization sequence. The iterative repetition of deposition/washing steps can create multilayered architectures of controllable film thickness, composition, and hydrolytic stability. LbL assembly is well-suited for the encapsulation, protection, and release of therapeutic nucleic acids such as pDNA, siRNA, and others since the negative charge on nucleic acid backbones facilitates complexation with cationic polymers such as PLL,[1250,1415−1417] chitosan,[1418−1420] and PEI.[1421−1423] In the context of gene delivery, LbL coatings are typically synthesized in the following formats: (1) the traditional approach to LbL assembly employs planar substrates, onto which polyelectrolytes are sequentially immobilized, creating nanometer-thick multilayer films (Figure 46A).[1421,1424−1426] Further, nucleic acid cargoes can be impregnated within these films in the form of naked DNA or RNA, polyplexes,[1427] lipoplexes,[1424,1428] or simply as adenoviral capsids. (2) LbL coatings can be applied to nanoparticle[1429,1430] or microparticle[1431,1432] "cores" of desired shapes and sizes, such that the particle surface can be successively modified with polyelectrolytes, thereby transforming its interactions with cellular targets (Figure 46B).[1433] (3) Micron-sized polymeric capsules[1434−1438] composed of "free-standing" LbL multilayer films can be formed through sacrificial particle templates[1439−1442] or through template-free methods (Figure 46B).[1443]

In this section, we will discuss (1) engineering targeted film properties by optimizing layer architecture and LbL assembly conditions, (2) tuning degradation kinetics, triggering release using chemical and physical stimuli, and co-delivering multiple cargoes along individualized release trajectories to meet complex therapeutic objectives, and (3) applying LbL coatings to biomedically relevant substrates such as catheters, bandages, and stents. We will conclude by outlining challenges involved in the clinical translation of LbL-based vectors and directions for future research.

LbL film properties can be controlled by modifying polymer composition,[1445] layer architecture, that is the number, composition, and ordering of layer components,[1446] and finally by varying features of the solvent environment[1447] such as pH

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and ionic strength.[1249,1448,1449] Lynn and coworkers developed PBAE libraries[1450] to probe structure-property relationships that shed light on the relationship between film thickness, charge density, hydrophobicity, and the erosion profiles and release rates of anionic payloads.[1451−1453] Further, by combining PBAEs with contrasting payloads into a single coating, the authors were able to control the timing and sequence in which disparate pDNA payloads were delivered.[1451] In addition to hydrolytic cleavage and enzymatic degradation,[1425] they also explored the use of charge reversal as a film disassembly mechanism. This was accomplished either by incorporating pH-responsive tertiary amines within the polymer pendant groups[974] or through an ester hydrolysis to unmask carboxylate groups.[1454]

The use of an additional barrier layer to prevent interlayer diffusion can reduce the mobility of the nucleic acid payload and transform the release profile from a "bulk process" to a more sustained surface-mediated process.[1455] Oupicky and coworkers elegantly demonstrated this concept by comparing the degradation behavior of bioreducible LbL layers with and without a PEI interlayer.[1251] They concluded that, in the absence of the interlayer, the film degradation proceeded through the release of large micron-sized fragments of DNA and cationic polymer, culminating in a burst release in a reducing environment, while the interlayer-incorporating films degraded at a more controlled rate over the span of 5 d by breaking down into nanoparticles. Compared to tuning the release kinetics of a single payload, managing the pharmacokinetics of dual or multiple payload[1438,1456] systems presents a greater challenge, since the design space for multifunctional LbL assemblies is significantly more complex. Hammond and coworkers have reported several powerful case studies demonstrating the utility of multifunctional "onion-like" LbL nanoparticle platforms[1457] capable of co-delivering diverse cargoes such as siRNA and chemotherapeutics[1403] and biosensor peptides that can serve as urinary reporters for the recurrence of metastatic cancer.[1458] By focusing on polyelectrolyte composition, layer architecture, and the surface chemistry of the outermost shell, they identified critical design parameters for the electrostatic assembly of dual payloads towards cancer treatment.

Several responsive LbL assemblies have been engineered with the goal of disrupting the interactions holding layers together to release the payload on demand upon application of appropriate physical stimuli.[1444] LbL films have been used to enhance transfection efficiencies during ultrasound-mediated gene delivery by coating a microbubble or gas core with alternating layers of cationic polymers and DNA, thereby delivering a higher quantity of DNA than via ultrasound treatment alone.[1416,1459] Wang and coworkers employed polyphosphoester to engineer degradable multilayers for osteoblast regeneration.[788] Similarly, electrochemical[1460,1461] and electrical[1423,1462] triggers have also been used to engineer an on-demand film disassembly and cargo release. Incorporating redox-responsive moieties such as disulfide bonds that can undergo cleavage by glutathione within the reducing environment of the cytosol has been a popular strategy to tune the degradation kinetics of LbL films.[772] Disulfide bonds can be placed in the polymer backbone[1252,1463,1464] in the form of degradable crosslinkers[1440] to tune layer rigidity and stability. Additionally, the degree of cross-linking[181,1252] can also be modulated to extend the duration of a payload release. In addition to the temporal control afforded by LbL assembly, patterned films can also be easily created, allowing for spatial control[1253,1422,1423,1465] transfection outcomes and the creation of microarrays for high-throughput experimentation.

Unlike other platforms for local or substrate-mediated gene delivery,[1466] LbL coatings can be conformally applied on a broad range of surfaces, including highly tortuous geometries such as stents, without the need for pretreatment. Oupicky and coworkers coated a stainless steel mesh, which bears a net negative charge, with alternating layers consisting of either a bioreducible form of hyperbranched PAMAM or a PEI positive control and plasmid DNA interspersed between cationic layers.[1230] In contrast, Park and coworkers pre-treated the steel surface with dopamine-functionalized hyaluronic acid to facilitate an immobilization of DNA/PEI polyplexes, instead of relying on electrostatic interactions with the bare steel surface.[1467] LbL coatings are attractive alternatives to the use of polymer-coated (such as PLGA) stents since they function as a degradable matrix from which drugs or genes can be slowly eluted. In contrast to the bulk degradation of a polymeric matrix, LbL coatings not only offer more precise control of DNA release kinetics but also enhance the cellular internalization and endosomal escape of nucleic acid payloads within diseased vascular cells.[1468] Several examples of gene-eluting stents based on LbL platforms have been reported[1467,1469] including the use of PBAE-based films from Lynn and coworkers.[1470] While gene-eluting intravascular stents are long-term interventions for the treatment of atherosclerosis, other therapeutic contexts demand the application of DNA-loaded LbL films on catheter balloons.[1471−1473] Hammond and coworkers have pursued meshes or bandages as substrates for LbL-mediated gene silencing and demonstrated sustained release during the entirety of the wound-healing process.[1474] Percutaneous or intradermal gene delivery has been extremely challenging, since the stratum corneum bars even the diffusion of small-molecule drugs, ensuring that macromolecular therapeutics such as DNA-based vaccines, genetic medicines for skin cancer, and proteinaceous drugs cannot penetrate the skin.[1475] To penetrate the skin barrier, microneedle arrays have been developed as safer and pain-free alternatives to traditional needle-based administration. In the context of DNA vaccine delivery, the groups of Irvine and Hammond developed microneedle-based polymer multilayer "tattoos" wherein polymer-coated microneedle patches carrying both DNA vaccines and immune-stimulatory RNA were transferred into the epidermis to achieve a sustained month-long release and immune response, unlike an intradermal injection of DNA alone.[1476] Other studies have reported the use of PLGA microparticles[1477] and pH-responsive polymers[1478] to promote microneedle-mediated DNA vaccination, while Lynn and coworkers took advantage of their tunable PBAE platform to functionalize stainless steel microneedles with multilayers containing either DNA or a model protein.[1479] Wang and coworkers modified PCL-based microneedle arrays with polyelectrolyte multilayers consisting of a pH-responsive polymer and a plasmid DNA intended to treat subdermal tumors.[1480] These LbL-functionalized microneedle patches outperformed both intravenous injection as well as unfunctionalized DNA-loaded microneedles in inhibiting tumor growth. While microneedle-based DNA vaccination will undoubtedly regain relevance in the face of the coronavirus disease of 2019 (COVID-19) pandemic, polymers for LbL-based surface modification of microneedle patches must allow for tunable

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Table 5. Summary of Gene Therapeutics on the Market[a]

therapeutic name	disease target	manufacturer	approval granted	notes	approx cost
Gendicine	Squamous cell carcinoma	Shenzhen SiBiono Genetech	2003 China	Turned down by USFDA, withdrawn by EMA	$360 per dose
Macugen	Age-related macular degeneration	OSI pharmaceuticals	2005 US	Intravitreal injection every 6 weeks	$9000 per eye per year
Glybera	Lipoprotein lipase deficiency	UniQure	2012 EU	Withdrawn from market in 2017	>$1 M in total
Kynamro	Familial hypercholesterolemia	Genzyme corporation	2013 US	Rejected by EMA in 2012 & 2013	$176 000
Imlygic	Melanoma	Amgen	2015 US	First oncolytic virus approved	$65 000 in total
Strimvelis	Adenosine deaminase deficiency	Orchard Therapeutics	2016 EU	"bubble boy disease"	$665 000
Spinraza	Spinal muscular atrophy	Biogen	2016 US	First gene therapeutic for SMA in US.	$750 000 for the 1st year and $375 000 per year thereafter
Exondys 51	Duchenne's muscular dystrophy	Sarepta Therapeutics	2016 US	Conditional USFDA approval	$892 000/year
Kymriah	B-cell lymphoma	Novartis	2017 US	First USFDA-approved cell-based gene therapy	$475 000 in total
			2018 EU		
Luxturna	Leber congenital amaurosis	Spark Therapeutics	2017 US	Developed with Children's Hospital of Pennsylvania	$425 000 in total
			2018 EU		
Yescarta	B-cell lymphoma	Gilead Pharma	2017 US	Cheaper than Kymriah	$373 000
			2018 EU		
Defitelio	Veno-occlusive disease	Jazz Pharmaceuticals	2017 US & EU	ssODN mixture	$160 000 in total
Onpattro	Transthyretin-mediated amyloidosis	Alnylam Pharmaceuticals	2018 US	siRNA with lipid vehicles	$450 000
Zynteglo	Beta thalassemia	Bluebird Bio	2019 EU	Yet to seek USFDA approval	$1.8 M
Zolgensma	Spinal muscular atrophy	Novartis	2019 US	Most expensive to date	$2 M
Givlaari	Acute hepatic porphyria	Alnylam Pharmaceuticals	2019 US	GalNac-conjugated RNAi therapeutic	$575 000
Oxilumo	Primary hyperoxaluria type 1		2020 EU and US		$493 000
Leqivo	Reducing LDL cholesterol	Novartis	2021 EU		$15 000 per year
Tozinameran or BNT162b2	Vaccine for SARS-COV2	Pfizer/BioNTech	2020 US and EU, emergence use	mRNA vaccines based on lipid NPs	~$20 per dose
mRNA-1273	Vaccine for SARS-COV2	Moderna	2020 US, emergence use authorization		~$40 per dose

[a]Prices gathered from press releases.

release kinetics, DNA dose control, and payload protection against heat and mechanical stress.

Although LbL research has been gradually moving away from manual film assembly in favor of automated production methods that employ liquid-handling robots, the scalability, robustness, and reproducibility of the LbL coating process needs further improvement. Incorporating process control modules to maintain pH and ionic strength within the narrow windows demanded by precise LbL assembly will be a step towards creating reproducible formulations. Additionally, LbL nanoparticles have so far been restricted to spherical geometries and systematic explorations of size and shape in conjunction with LbL film composition and architecture have the potential to be productive avenues for future research.

7.3. Polymer Brushes

Polymer brushes are typically employed as cell-instructive coatings and non-fouling surfaces in biomedical research, and hence their gene delivery capabilities have remained under-investigated. Gautrot and coworkers, who have been enthusiastic proponents of polymer brushes in gene delivery, argue that, unlike drug delivery, where polymer brushes may be limited by their low loading capacities, gene delivery presents no such obstacles.[1481] Compared to small-molecule drugs, which require high dosages, moderate loadings of nucleic acid therapeutics would suffice to bring about the desired clinical outcomes. Further the versatility of surface-initiated (SI) polymerization tools such as SI-RAFT and SI-ATRP allows for orthogonal control over particle core composition, size, and shape and polymer brush architecture, composition, density, and thickness. For instance, graphene,[1482] nanodiamonds,[1483] and magnetic nanoparticles[1484] have been employed, imparting a highly desirable mixture of properties originating from physically interesting inorganic cores and chemically tunable polymer coronas. This modularity is more challenging to achieve with other platforms, making polymer brushes an ideal approach to independently probe the effects of physical as well as chemical properties of polyplexes and arrive at meaningful structure-property relationships, unlike with free polymer chains, where an independent control of these attributes is near-impossible.[1485] Investigators must however be cautioned that interactions between substrate-bound polymer brushes and nucleic acids bear very little resemblance to what is

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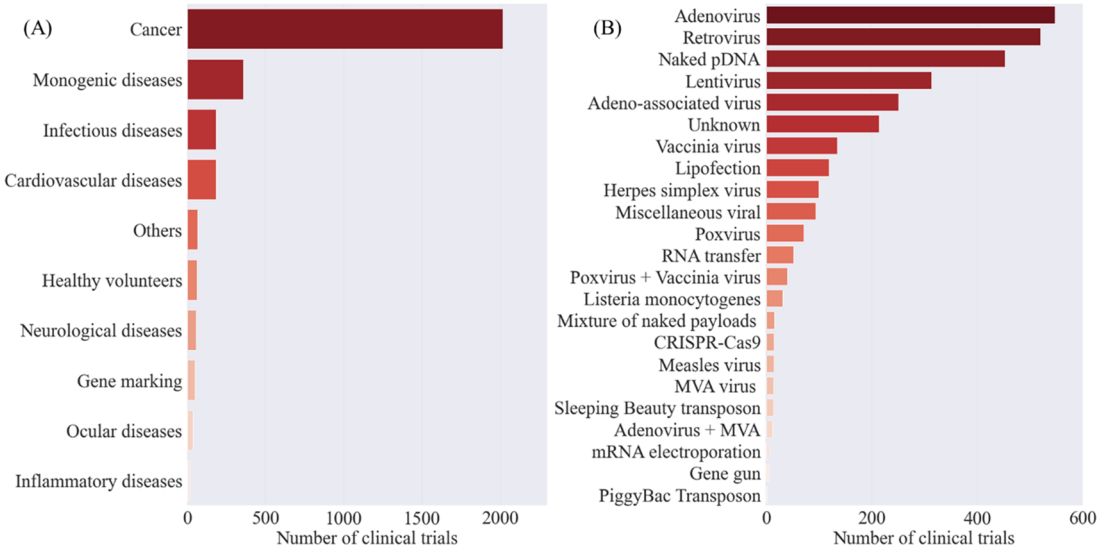

Figure 47. Summary of clinical trials by (A) therapeutic area, (B) delivery modality. Data obtained from *The Journal of Gene Medicine*. Copyright 2019 John Wiley and Sons. Database updated December 2019.

observed with their free polymer counterparts. The grafting density of polymer brushes, in particular, has been shown to be the most critical determinant of nucleic acid binding affinity, loading density, and release rate. While sparsely grafted polymer brushes (where the reduced grafting density $\Sigma < 1$) can be easily obtained through "grafting-to" approaches, densely bound polymer brushes (where $\Sigma > 1$) require the controlled immobilization of polymerization initiators at sufficiently high surface densities.[1486] Moreover, nucleic acids may bind to cationic brushes via one of two binding configurations: superficial adsorption, where they do not penetrate the brush layer, or brush infiltration, wherein they overcome steric barriers to bind to charged sites within the brush. While the choice of binding configuration is dependent on brush density, with denser brushes forbidding infiltration, the size, stranded-ness, and the backbone composition of the nucleic acid payload also play key roles. Gautrot and coworkers demonstrated that smaller oligonucleotides (10−22 bp) easily permeate even densely grafted polymer brushes but bind weakly and result in low levels of loading.[1487] The adsorption of larger payloads such as pDNA is hindered by high-density brushes. This results in low pDNA loading levels, but these complexes display extremely strong binding affinities. Gautrot's group also demonstrated that RNA payloads are much more easily captured by polymer brushes, irrespective of grafting density, and that the grafting density can be modulated in order to attain the desired loading of RNA payloads.[1488] Further, they have also drawn attention to the role played by buffer composition, ionic strength, and pH in influencing the brush conformation and swelling and ultimately deciding the fate of DNA complexation.[1481] Although polymer brushes are attractive tools for producing serum-stable and highly efficient polyplexes, their synthesis and biological testing must be accompanied by rigorous physicochemical characterization via SPR, ellipsometry, light-scattering techniques, and themogravimetric analysis. Further, research on polymer-brush-functionalized nanoparticles must move past a heavy reliance on PDMAEMA and incorporate polycationic brushes containing varied charge centers, monomer distributions, and architectures. The effect of particle size and curvature on the brush conformation and DNA complexation is also an apt subject for further studies. We would also like to point out the promise of using mixed-brush systems comprised of PAA and polycationic polymers such that the PAA termini can be decorated with RGD motifs[1362] as well as growth factors in order to modulate biointerfacial behavior.

In this section we highlighted some interesting examples of non-traditional material design approaches to polymeric gene delivery. These examples demonstrate that hybrid polymer engineering approaches employing tissue-engineering scaffolds, crosslinked hydrogels, engineered nanoparticle templates, and polyelectrolyte multilayer coatings can be as powerful as traditional polymer synthetic approaches.

8. CLINICAL OUTLOOK FOR POLYMER-MEDIATED GENE THERAPY

Synthetic advances and an improved understanding of structure-function relationships have accelerated progress in ex vivo and in vivo delivery applications of polymeric vehicles. Yet very few polymers have progressed to clinical trials and testing in human subjects. In this section, we will focus on the clinical translation of gene therapeutics, restricting our attention to synthetic vehicles such as lipid nanoparticles and polymers. We will begin our clinical perspective by drawing attention to recently approved gene therapy products. Then we will describe clinical trials involving lipid-based and polymeric vehicles and discuss promising developments from these nonviral clinical studies, particularly with lipids.

Although the first demonstration of gene therapy was published in 1972, it was not until 2017 that the United States' FDA (USFDA) granted approval for the clinical use of gene therapeutics.[1489] The intervening years have witnessed an explosion of clinical trials and USFDA approvals with several gene therapeutics reaching the market.[1489] Salient examples have been mentioned in Table 5 with notes on approval history and cost.

All but six of the above therapeutics are based on viral vectors, which may have contributed to the high treatment costs.[1490] Recognizing the importance of developing synthetic alternatives to engineered viruses, there have been increasing efforts to test formulations based on lipids and polymers in the

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Table 6. Summary of Clinical Trials Involving Polymeric Vehicles

No.	vehicle	sponsor	therapeutic name	payload type & target	condition	phase	status	identifier
1	PEI	Genetic Immunity	DermaVir	plasmid DNA expressing 15 HIV antigens	HIV/AIDS	2	Completed	NCT00711230
2	Cyclodextrin based polymer and adamantane PEG stabilizer	Calando Pharmaceuticals	CALAA-01	siRNA targeting M2 subunit of ribonucleotide reductase (R2)	Solid tumors	1	Terminated	NCT00689065
3	PEI	Senesco Technologies, Inc.	SNS01-T	RNAi-resistant DNA plasmid expressing non-hypusinable eIF5AK50R + eIF5A targeting siRNA	B-cell lymphoma	2	Unknown	NCT01435720
4	PEG−PEI-cholesterol Lipopolymer-	EGEN Inc	EGEN-001	IL-12 Plasmid	Colorectal peritoneal carcinomatosis	2	Terminated	NCT01300858
5		Gynecologic Oncology Group			Ovarian cancer	2	Completed	NCT01118052
6						1		NCT01489371
7	Poloxamer CRL1005− benzalkonium chloride	Astellas	VCL-CB01	CMV vaccine	End organ disease	2	Completed	NCT00285259
8			ASP0113			2	Completed	NCT01903928
9			ASP0113			3	Active, not recruiting	NCT01877655
10	Mixture of polymer/siRNA	Arrowhead Pharmaceuticals	ARC-520	siRNA targeting HBV proteins	Hepatitis B	1	Completed	NCT01872065
11						2	Terminated	NCT02065336
12	PEI	University Hospital, Toulouse	CYL-02	plasmid DNA encoding SST2 + DCK::UMK genes	Pancreatic adenocarcinoma	2	Recruiting	NCT02806687
13	Spherical nucleic acids	Northwestern University	NU-0129	siRNA targeting Bcl2L12	Recurrent glioblastoma	1	Active, not recruiting	NCT03020017
14	biodegradable polymeric matrix	Silenseed Ltd	siG12D LODER	anti KRASG12D siRNA	Pancreatic ductal adenocarcinomas	1	Completed	NCT01188785
15					Advanced pancreatic vancer	2	Recruiting	NCT01676259
16	PEI	Anchiano Therapeutics Israel Ltd.	DTA-H19	plasmid diphtheria toxin A (DT-A)	Diphtheria	2	Completed	NCT00595088
17			BC-819		Ovarian cancer	2	Completed	NCT00826150
18			DTA-H19		Pancreatic neoplasms	2	Completed	NCT00711997
19			BC-819		Non-muscle invasive bladder cancer	2	Terminated	NCT03719300

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Table 7. Summary of Clinical Trials Involving Lipids, Electroporation, and Transposons

No.	vehicle	sponsor	therapy name	payload type & target	condition	phase	status	identifier
1	GAP-DMORIE−DPyPE	US Army Medical Research and Material Command	Tetravalent dengue vaccine	DNA vaccine	Dengue vaccine	1	Completed	NCT01502358
2	Lipid NPs	Arbutus Biopharma	PRO-040201	siRNA APB	Hypercholesterolemia	1	Terminated	NCT00927459
3			TKM-080301	siRNA PLK1	Cancer	2	Completed	NCT01262235
4			TKM-100201	siRNA VP24, VP35 and Zaire Ebola l-polymerase gene	Ebola	1	Terminated	NCT01518881
5		Imperial College London	pGM169/GL67A	plasmid DNA expressing CFTR	Cystic Fibrosis	2	Completed	NCT00789867
6								NCT01621867
7		Silence Therapeutics	Atu027	siRNA PKN3	Advanced cancer	1	Completed	NCT01808638
8		Nitto Denko Corporation	ND-L02-s0201	siRNA SERPINH1	Fibrosis	1	Completed	NCT01858935
9		Alnylam Pharmaceuticals	ALN-VSP02	siRNA targeting KIF11 and VEGF	Solid tumors	1	Completed	NCT01158079
10			ALN-PCS02	siRNA targeting PCSK9	Hypercholesterolemia	1	Completed	NCT01437059
11			Patisiran (ALN-TTR02)	siRNA targeting abnormal transthyretin.	Transthyretin (TTR)-Mediated Amyloidosis	3	Approved	NCT01960348
12		Dicerna Pharmaceuticals, Inc.	DCR-MYC	siRNA targeting MYC	Solid tumors	1	Terminated	NCT02110563
13		SynerGene Therapeutics, Inc.	SGT-53	p53 plasmid DNA	Glioblastoma	2	Terminated	NCT02340156
14					Metastatic Pancreatic Cancer	2	Recruiting	NCT02340117
15					Pediatric cancers	1	Recruiting	NCT02354547
16		MD Anderson Cancer Center	EphA2−DOPC	siRNA EPHA2	Advanced cancer	1	Recruiting	NCT01591356
17	Electroporation	University of Pennsylvania	RNA CART19 cells	ex vivo cell therapy messenger RNA anti-CD19 CAR	Hodgkin Lymphoma	1	Terminated	NCT02624258
18	Lipid NPs	Translate Bio, Inc.	MRT5005	mRNA encoding CFTR	Cystic Fibrosis	1	Recruiting	NCT03375047
19		National Cancer Institute (NCI)	(NCI)-4650	mRNA vaccine	Cancer	2	Terminated	NCT03480152
20	Sleeping Beauty Transposon		Sleeping Beauty Transposed PBL	CD-19 specific CAR	Cancer	2	Recruiting	NCT04102436
21	Lipid NPs	Moderna TX, Inc.	mRNA-2416 + Durvalumab	mRNA encoding Human OX40L	Solid tumors	1	Recruiting	NCT03323398
22			mRNA-1273	mRNA encoding S-2P antigen	SARS-CoV-2 vaccine	3	Approved	NCT04470427
23		Pfizer and BioNTech SE	BNT162b2 or Tozinameran			3	Approved	NCT04368728
24		Genprex, Inc.	DOTAP:Chol-TUSC2	plasmid encoding TUSC2 gene	non-small cell lung cancer	1	Active, not recruiting	NCT01455389
25			GPX-001		Small cell lung cancer		Not yet recruiting	NCT04486833

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clinic. As of December 2019, 3025 clinical trials have been initiated using therapeutic nucleic acids. The recent approvals granted to Givosiran, Oxlumo, and Leqivo (siRNA therapeutics functionalized with GalNAc residues) represents an exciting development for siRNA-glycan conjugates.[1491] At the time of submitting this manuscript, two lipid-based mRNA vaccines for SARS-CoV-2, mRNA-1273 (Moderna), and BNT162b2 (Pfizer) released interim results from their respective Phase 3 trials, based on which emergency use authorization was granted by the FDA and the European Union (EU).

If we break down the trials by disease area (Figure 47A), cancer treatment emerges as the most widely targeted therapeutic area, a trend we attribute to advances made by molecular biologists in understanding the genetic basis of cancer progression.[1492] Surprisingly, inherited disorders underlying monogenic diseases constitute a rather small proportion of clinical trials, possibly because these disorders are extremely rare among the population and present financial challenges to development.[1493] We believe that ocular disease, vaccine development for infectious diseases such as SARS-CoV-2, and cardiovascular diseases will constitute a great proportion of clinical trials in the years to come. A breakdown by delivery modality reveals a stark picture: nonviral methods such as lipofection, gene guns, electroporation, and naked payload delivery constitute a small fraction of the more than 3000 trials initiated thus far (Figure 47B). Although the delivery landscape was historically dominated by adenoviruses, retroviruses, and lentiviruses, adeno-associated viruses are quickly emerging as safer viral alternatives, since they have been shown to elicit more predictable and less severe immune responses during clinical trials.[44] Now, we will briefly survey recent clinical developments with drugs composed of polymers/nucleic acids, compare the contrasting clinical fates of polymeric vehicles with those of lipid vehicles, and conclude with some recommendations for improving clinical outcomes of polymeric vehicles.

The Davis lab has extensively reported on the systemic administration of siRNA-based therapeutics using polymeric delivery platforms not only in primate models[120] but also in human subjects.[113,995,996,1494] Clinical trials conducted with CALAA-01 (No. 2 in Table 6), a cyclodextrin-based delivery system for siRNA silencing of ribonucleotide reductase subunit 2, have been described in detail in another report,[112] where a detailed analysis of clinical trials conducted up to 2015 can be found. Therefore, we will restrict our attention to more recent clinical candidates in this Account. PEI features in 7 of 19 trials involving polymers, underscoring the versatility of PEI-based vehicles across therapeutic applications ranging from acquired immunodeficiency syndrome (AIDS) vaccination (No. 1 in Table 6), diphtheria vaccination (No. 16 in Table 6), and cancer treatment (Nos. 3, 12, and 17−19 in Table 6). Among active PEI-based trials, the combination therapy CYL-02 that consists of plasmid DNA encoding SST2 (a tumorigenesis suppressor) and a chemotherapeutic, gemcitabine (No. 12 in Table 6), seems particularly promising for the treatment of pancreatic ductal adenocarcinoma, a leading cause of death. This nonviral therapeutic developed by the University Hospital, Toulouse, resulted in mild toxicities, and no serious adverse events were recorded. CYL-02 DNA was detected in blood and tumors, while therapeutic RNAs were detected in tumors. The authors noted that nine patients exhibited disease symptoms for six months following treatment, while two of these patients experienced long-term survival.[1495] Since this therapeutic is well-tolerated and led to disease stability, it will be interesting to examine results from phase 2 studies towards the end of next year. For the same disease, another combination therapy is under clinical investigation (No. 15 in Table 6). This siRNA-based therapeutic from Silenseed Ltd. has not provided the composition of the "miniature biodegradable biopolymeric matrix" employed to encapsulate the drugs and nucleic acids.

Spherical nucleic acids, where nucleic acids and polycations are conjugated to a gold nanoparticle core, have also entered the clinical pipeline, with NU-129 (No. 13 in Table 6) being tested in glioblastoma patients. In phase 0 or early phase 1 studies, no significant toxicities were seen in a cohort of eight patients. Since two patients reported adverse events (one grade 3, one grade 4) and were removed from the trial, tumor tissue could be collected from only six of the eight patients. Since gold nanoparticles can be quantified via inductively coupled plasma-mass spectrometry, gold accumulation was verified in the tumor tissue of all six of these patients.[1496] Finally, an investigational therapeutic (BC-819, No. 19 in Table 6) that relies on the tendency of the diphtheria toxin to be expressed specifically in malignant cells, reported its phase 2 results recently.[1497] This PEI-complexed plasmid DNA was found to be well-tolerated among 38 patients and did not contribute to toxicity during an intravesical therapy of non-muscle invasive bladder cancer. However, this trial did not progress to phase 3 due to a lack of efficacy.

We have tabulated a representative list of 22 clinical trials involving lipid nanoparticles, of which three have already gained FDA approval that are current as of the publication date of this review. We have also highlighted recent trials involving electroporation and the Sleeping Beauty transposon systems. We draw attention to some notable examples that entered Phase 3 clinical trials successfully. Patisiran (No. 11 in Table 7) is an RNA interference therapeutic agent marketed by Alnylam Therapeutics that relies on the encapsulation of a double-stranded siRNA within lipid nanoparticles to inhibit a hepatic synthesis of transthyretin.[1498] This is the first lipid-based gene therapeutic to be granted FDA approval (2018) and has renewed industry interest in lipofection as a viable nonviral platform.

The approval of two lipid-based mRNA vaccines for SARS-COV-2 has lent further impetus to the clinical translation of non-viral gene delivery platforms. For mRNA-1273, a vaccine efficacy rate of 94.5% was reported, with 90 of the COVID-19 cases occurring in the placebo cohort and only 5 in the vaccinated cohort.[13] All 11 instances of severe illness occurred in the placebo group. Results from the trials of BNT162b2 indicated a vaccine efficacy above 95%.[1499] While lingering concerns about the use of PEG in mRNA-1273 and BNT162b2 persist, we anticipate that unpleasant side effects resulting from both the inherent immunogenicity of mRNA as well as the presence of anti-PEG antibodies in some patients will spur the development of PEG alternatives such as carbohydrates, polyoxazolines, and zwitterionic moieties. The success stories of lipid nanoparticle platforms such as mRNA-1273, BNT162b2, and Patisiran motivate us to learn from the design philosophies of lipid nanoparticle development and apply these to polymeric gene delivery.

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9. CONCLUSIONS AND OUTLOOK

Because of breakthroughs in synthetic tools and physicochemical characterization methods, polymeric vehicles for gene delivery have grown in sophistication, multifunctionality, and precision. As more and more creative examples of polymer architectures and biofunctional monomers continue to be developed, we have witnessed unprecedented improvements in the properties and delivery capabilities of polymeric vehicles. Serum stability, immune evasion, payload protection, and intracellular trafficking are formidable biological barriers that demand numerous material properties be engineered and calibrated with care. Several classes of polymeric materials highlighted in this Account have juggled these competing design requirements to demonstrate exquisite spatiotemporal control in vivo and ex vivo. These improvements have allowed us to both visualize and manipulate the complex cascade of biological events leading up to intracellular gene delivery and to harness a delicate web of intermolecular interactions, ultimately facilitating the desired polyplex-cell interactions. For instance, researchers have innovated ingenious polymer design strategies to navigate the toxicity−efficiency trade-off through decationization and the use of hydrophobic motifs, to alleviate aggregation in serum-rich environments while ensuring payload integrity through a triggered shedding of hydrophilic stealth layers and to facilitate highly precise delivery of genetic cargoes to specific cellular targets through the use of variegated targeting moieties. Ultimately, successful gene delivery approaches benefit from an interdisciplinary effort and a balance between investigating fundamental mechanistic questions and solving development challenges that may hinder clinical translation.

Surprisingly, progress in polymer chemistry and engineering has not been accompanied by a commensurate progress in the clinical translation of polymeric gene delivery vectors. We believe that clinical progress has been hindered by the workflows that are currently being used for the biological evaluation and screening of polyplex formulations. Typically, formulations that do not achieve efficient delivery during in vitro screening are excluded from subsequent in vivo studies. For instance, Langer and coworkers[1500] employed a statistical design of experiments to optimize formulation parameters using an in vitro evaluation. After having triaged inconsequential process parameters during in vitro studies, they again employed DoE to reduce the in vivo experimental burden to further optimize the lipid nanoparticle composition. This approach assumes that in vitro gene delivery experiments are good predictors of in vivo outcomes, an assumption that has been called into question repeatedly.[1500] Instead of screening polyplex libraries in vitro before identifying a small subset of promising candidates for further in vivo evaluation, some groups have eschewed in vitro studies altogether, reasoning that experimental conditions during cell culture do not faithfully reproduce physiological barriers faced by formulations within living organisms.[1501] Dahlman and coworkers have improvised a powerful approach for boosting in vivo experimental throughput by employing multiplexed signals in the form of DNA barcodes to tag chemically distinct lipid formulations. Recognizing the reliability, rapidity, and large multiplexing bandwidth afforded by storing and retrieving information from oligonucleotide strands, they demonstrated a simultaneous in vivo analysis of over 150 nanoparticles using their customized workflow, Joint Rapid DNA Analysis of Nanoparticles (JORDAN).[1502] We believe that adopting similar high-throughput in vivo experimental platforms will allow us to explore the polymer design space more efficiently and in a physiologically relevant environment. Currently, polymers have underperformed relative to lipids when tested in clinical gene therapy settings, with no polymer candidate having reached phase 3. This is rather surprising, given that polymers offer incontestable advantages over lipids when we consider reproducibility and scalability. We posit that this performance differential can be bridged if polymer formulations are optimized through multiplexed in vivo studies rather than a sequential strategy where in vitro screening is followed by in vivo validation.

Secondly, logistical planning of preclinical studies is critical to facilitate agile transitions from early phase development to preclinical studies, ensuring the timely submission of investigational new drug dossiers.[1503] Proper planning of in vitro and in vivo pharmacokinetic studies that measure absorption, distribution, metabolism, and excretion properties, immunogenicity evaluation via antibody screening, and toxicology studies that identify dosing ranges and quantify the toxicity induced by repeat dosing are essential. The clinical potential of polymeric vehicles can be fully realized only if we work in a coordinated fashion with clinicians, regulators, and entrepreneurs when the discovery and development processes are still in their nascency.

A number of challenges should be addressed for polymers to tackle critical therapeutic challenges: (1) The question of whether polymers that are highly efficient with a certain cell type can extend their performance across diverse cell types has not been sufficiently investigated. We do not yet know whether polymer structure and composition should be tailored independently for each cell type, given that endocytosis pathways are known to be cell-type-dependent. (2) On a similar note, the tissue specificity of engineering polyplexes also remains an open question, and the lack of clarity on this aspect has hindered in vivo translation. While synthetic vector platforms based on lipid nanoparticles have established design guidance for liver-targeted and lung-targeted delivery, similar investigations are still at their nascent stage with polymers. (3) The overlap in polymer design criteria across multiple nucleic acid modalities (mRNA, pDNA, RNP, etc.) must be probed in detail. While some investigators have reported that certain nucleic acid payloads have more stringent design spaces for polymeric vectors than others, other studies have laid claim to "universal" delivery platforms that are functional across a broad selection of nucleic acid cargoes. (4) Although biodegradable polymers are considered most favorably in the light of regulatory approval, the long-term safety profile of these vehicles must be evaluated, and the immune responses to degradation products must be examined in detail. (5) Synthetic chemists must develop monomers that possess theranostic capabilities, by coupling delivery functionalities with imaging capabilities (such as Raman imaging,[1504] magnetic resonance imaging (MRI), or aggregation-induced emission (AIE)). Theranostic polyplexes will combine delivery efficiency with a detailed mechanistic view of intracellular events that are often challenging to monitor via traditional microscopy. (6) Modular approaches to polymer synthesis must be developed since specialty monomers are often difficult to polymerize. Polymer chemists must continue to develop post-polymerization approaches that allow us to plug in arbitrary ratios of desired functionalities on polymer scaffolds

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of precisely controlled lengths and architectures. (7) Advances in experimental automation, high-throughput polymerization, and data science must be leveraged to develop a materiomics approach to polymeric vector discovery.[1505] Accompanied by an in-depth characterization, polymer synthesis and processing are well-poised to tackle fundamental biological questions and ultimately facilitate the widespread clinical deployment of polymeric biomaterials in therapeutic gene delivery.

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ABBREVIATIONS

ABC, Accelerated blood clearance; AEA, Acrylamidoethylamine; AEMA, *N*-(2-Aminoethyl) methacrylamide; AFM, Atomic force microscopy; AIDS, Acquired immunodeficiency syndrome; AIE, Aggregation-induced emission; APMA, *N*-(3-Aminopropyl)methacrylamide; APNBMA, 5-(3-(Amino)-propoxy)-2-nitrobenzyl methacrylate; ASGPR, Asialoglycoprotein receptors; ASO, Antisense oligonucleotides; ATPase, Adenosine triphosphatase; ATRP, Atom transfer radical polymerization; AzEMA, 2-Azidoethyl Methacrylate; Bcl2L12, B-cell lymphoma 2-like protein 12; BDNF, Brain-derived neurotrophic factor; BIP, 2,6-Bis(1-methylbenzimidazolyl)pyridinyl; BMA, Butyl methacrylate; BMP, Bone morphogenetic protein; BPEI, Branched polyethyleneimine; BSA, Bovine serum albumin; CARPA, complement activation-related pseudoallergy; CBD, Carbohydrate-binding domains; CBMA, Carboxybetaine methacrylate; CD, Cyclodextrin and circular dichroism; CFTR, Cystic fibrosis transmembrane conductance regulator; CHE, 2-Cyclohexylethyl; CLIC, Clathrin-independent carrier; CMV, Cytomegalovirus; COVID-19, Coronavirus disease of 2019; CPMG, Carr−Purcell−Meiboom−Gill pulse sequence; CPP, Cell-penetrating peptides; CPT, Camptothecin; CRISPR, Clustered regularly interspaced short palindromic repeat; CTA, Chain transfer agent; CuAAC, Copper-catalyzed azide−alkyne click chemistry; DAB, Diaminobutane-dendrimer; DCK::UMK, Deoxycytidine kinase::uridine monophosphate kinase; DEAE, Diethylaminoethyl; DEAET, 2-(Diethylamino)ethanethiol hydrochloride; DLS, Dynamic light scattering; DMAE, 2-(Dimethylamino)-ethyl; DMAEMA, 2-(Dimethylamino)ethyl methacrylate; DMAPMA, *N*-[3-(*N*,*N*-dimethylamino)propyl]-methacrylamide; DMBA, *N*,*N*′-dimethylbutylamine; DMEA, *N*,*N*′-dimethylethanolamine; DMPC, 1,2-Dimyristoyl-sn-glycero-3-phosphocholine; DNA, Deoxyribonucleic acid; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DOSY, Diffusion ordered spectroscopy; DOTA, 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid; DOTAP, 1,2-Dioleoyl-3-trimethylammonium propane; DOX, Doxorubicin; DPT, *N*-[*N*-(3-aminopropyl)-3-aminopropyl]; DPyPE, 1,2-Diphytanoyl-sn-glycero-3-phosphoethanolamine; DTS, DNA nuclear targetting sequences; EAA, Ethyl acrylic acid; ECM, Extracellular matrix; EDC, 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide; EDI, Azido-functionalize PAMAM dendrimer; EGF, Epidermal growth factor; eIF5A, Eukaryotic Translation Initiation Factor 5A; ELS, Electrophoretic light scattering; EMA, European Medicines Agency; EPHA2, Ephrin type-A receptor 2; EPR, Enhanced permeability and retention; ER, Endoplasmic reticulum; FCS, Fluorescence correlation spectroscopy; FDA/USFDA, Food and Drug Administration; FGF, Fibroblast growth factor; FITC, Fluorescein isothiocyanate; FTIR, Fourier-transform infrared spectroscopy; GAG, Glycosaminoglycan; GalNAc, *N*-acetyl-D-galactose; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GAP-DMORIE, (±)-*N*-(3-Aminopropyl)-*N*,*N*-dimethyl-2,3-bis(cis-9-tetradecenyloxy)-1-propanaminium bromide; GEEC, GPI-Anchored protein-enriched early endosomal compartment; GFP, Green fluorescent protein; GlcNAc, *N*-acetyl-D-glucose; GMA, Glycidyl methacrylate; GPI, Glycosylphosphatidylinositol; GSH, Glutathione; GTPase, Guanosine Triphosphatase; HA, Hyaluronic acid; HBV, Hepatitis B Virus; HDR, Homology-directed repair; HEMA, 2-Hydroxyethyl methacrylate; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; HGF, Hepatocyte growth factor; HIF, Hypoxia-inducible factor; HIV, Human immunodeficiency viruses; HPLC, High presssure liquid chromatography; HPMA, N-(2-Hydroxypropyl)-methacrylamide; HSQC, Heteronuclear single quantum coherence spectroscopy; IL, Interleukin; ITC, Isothermal titration calorimetry; JORDAN, Joint Rapid DNA Analysis of Nanoparticles; LODER, Local drug eluter; MAS, Methacryloxysuccinimide; MAT, Methacrylamidotrehalose; MPC, 2-Methacryloyloxyethyl phosphorylcholine; MRI, Magnetic resonance imaging; MSC, Mesenchymal Stem Cells″; N/P Ratio, Ratio of amine groups in the polymer vector to phosphate groups within nucleic acid payloads; nBMA, *n*-Butyl methacrylate; NGF, Nerve growth factor; NHEJ, Non-homologous end-joining; NHS, *N*-Hydroxysuccinimide; NHSA, *N*-(Acryloxy)succinimide; NHSMA, *N*-(Methacryloxy)succinimide methacrylate; NLS, Nuclear localization sequence; NMP, Nitroxide-mediated polymerization; NMR, Nuclear magnetic resonance spectroscopy; NOESY, Nuclear Overhauser effect spectroscopy; NPCs, Nuclear pore complexes; NPs, Nanoparticles; NTA, Nitrilotriacetic acid or Nanoparticle tracking analysis; ODN, Oligodeoxynucleotides; OEGMA, Oligoethylene glycol methacrylate; OEI, Oligoethylenimine; P4VPQ, Poly(*N*-methyl 4-vinylpyridine iodide); PAA, Poly(acrylic acid); PAAs, Poly(amidoamines); PAEM, Poly(aminoethyl methacrylate); PAEMA, Poly(2-aminoethyl-methacrylamide); PAMA, Poly(amidoamine); PAMAM, Poly-(amidoamine); PAsp(DET), Poly(*N*-[*N*′-(2- aminoethyl)-2-aminoethyl]aspartamide); PAsp(TEP), Poly(*N*-(*N*′-{*N*″-[*N*‴-(2-aminoethyl)-2-aminoethyl]-2-aminoethyl}-2-aminoethyl)-aspartamide); PBAE, Poly(*β*-amino ester); PBL, Peripheral blood lymphocytes; PBMA, Poly(butyl methacrylate); PBS, Phosphate-buffered saline; PCL, Poly(*ε*-caprolactone); PCSK9, Proprotein convertase subtilisin/kexin type 9; PDA, Polydopamine; PDADMAC, Poly(diallyldimethylammonium chloride); PDMA, Poly(*N*,*N*-dimethylamino-2-ethylmethacrylate); PDMAEA, Poly(*N*,*N*-dimethylamino-2-ethylacrylate) or Poly(2-(dimethylamino)ethyl acrylate); PDMAEMA, Poly-(*N*,*N*-dimethylamino-2-ethylmethacrylate) or Poly(2-(dimethylamino)ethyl methacrylate); pDNA, Plasmid DNA; PDTEMA, Poly(*N*-[2-(2-pyridyldithio)]ethyl methacrylamide); PEG, Poly(ethylene glycol); PEGA, Poly(ethylene glycol) acrylate; PEGEEMA, Poly(ethylene glycol) ethyl ether methacrylate; PEGMA, Poly(ethylene glycol) methacrylate; PEHA, Pentaethylenehexamine; PEI, Poly(ethylenimine); PFG, Pulsed-field gradient; PFP, Pentafluorophenyl; PFPA, Pentafluorophenyl acrylate; PFPMA, Pentafluorophenyl methacrylate; PGA, Poly(glutamic acid); PGAA, Poly-(glycoamidoamine); PGBA, Poly(glycidylbutylamine); PGEA, Ethanolamine-functionalized poly(glycidyl methacrylate); PGMA, Poly(glycidyl methacrylate); PHPMA, Poly(*N*-(2-Hydroxypropyl)methacrylamide); PIC, Polyion complex; PKN3, protein kinase N3; PLA, Poly(lactic acid); PLG, Poly(L-glutamate); PLGA, Poly(lactic-co-glycolic acid); PLK, Poly(L-lysine); PLK1, Serine/threonine-protein kinase; PLL, Poly(L-lysine); PLLA, Poly(L-lactic acid); PLMA, Poly(lauryl methacrylate); PMAA, Poly(methacrylic acid); PMAG, Poly-(2-deoxy-2-methacrylamido glucopyranose); PMMA, Poly-(methyl methacrylate); PMPC, Poly(2-methacryloyloxyethyl phosphorylcholine); PMPD, Poly[*N*-(3-(methacryloylamino) propyl)-*N*,*N*-dimethyl-*N*-(3-sulfopropyl) ammonium hydroxide]; PnBA, Poly(*n*-butyl acrylate); PnBMA, Poly(*n*-butyl methacrylate); PNIPAM, Poly(*N*-isopropyl acrylamide); PO-

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EGMA, Poly(oligoethylene glycol methacrylate); POSS, Polyoctahedral silsesquioxanes; PPA, Poly(phosphoramidate); PPG, Poly(propylene glycol); PPI, Poly(propylenimine); PS, Poly(styrene); PSS, Poly(sodium 4-styrenesulfonate); PTBP, Poly(tributyl-(4-vinylbenzyl)phosphonium chloride); PTEP, Poly(triethyl-(4-vinylbenzyl)phosphonium chloride); PTMAEMA, Poly((2-trimethylamino)ethyl metacrylate chloride); PTX, Paclitaxel; PVBTMA, Poly((vinylbenzyl) trimethylammonium); PVDMA, Poly(2-vinyl-4,4-dimethylazlactone); PVP, Poly(*N*-ethyl-4-vinylpyridinium bromide); QPDMAEMA, Quaternized PDMAEMA; RAFT, Reversible addition−fragmentation chain transfer; RES, Reticuloendothelial system; RISC, RNA-induced silencing complex; RLU, Relative luminescence units; RNA, Ribonucleic acid; RNPs, Ribonucleoproteins; ROMP, Ring-opening metathesis polymerization; ROP, Ring-opening polymerization; ROS, Reactive oxygen species; SAM(S), Self-assembled monolayer(s); SANS, Small-angle neutron scattering; SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; SAXS, Small-angle X-ray scattering; SBMA, Sulfobetaine methacrylate; SEM, Scanning electron microscopy; SERS, Surface-Enhanced Raman Spectroscopy; siRNA, Small interfering RNA; SLS, Static light scattering; SMA, Spinal Muscular Atrophy; SPAAC, Strain-promoted azide−alkyne cycloaddition; SPR, Surface plasmon resonance; SSOs, Splice-switching oligonucleotides; TALENS, Transcriptor activator-like nucleases; TAPP, 5,10,15,20-Tetrakis(4-aminophenyl) porphyrin; TAR, Transactivation response element″; TCPS, Tissue culture polystyrene; TEM, Transmission electron microscopy; TEPA, Tetraethylenepentamine; TLR, Toll-like receptor; TMCC, 2-methyl-2-carboxytrimethylene carbonate; TNF-α, Tumor necrosis factor alpha; TRAIL, TNF-related apoptosis-inducing ligand; TREN, Tris-(2-aminoethyl) amine; UCF, Ultracentrifugation; UV, Ultraviolet; VBC, Vinyl benzyl chloride; VEGF, Vascular endothelial growth factor; VIPER, Virus-inspired polymer for endosomal release; XPS, X-ray photoelectron spectroscopy

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