DESIGN AND DEVELOPMENT OF POLYMERS FOR GENE DELIVERY

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Abstract | The lack of safe and efficient gene-delivery methods is a limiting obstacle to human gene therapy. Synthetic gene-delivery agents, although safer than recombinant viruses, generally do not possess the required efficacy. In recent years, a variety of effective polymers have been designed specifically for gene delivery, and much has been learned about their structure–function relationships. With the growing understanding of polymer gene-delivery mechanisms and continued efforts of creative polymer chemists, it is likely that polymer-based gene-delivery systems will become an important tool for human gene therapy.

Gene therapy can be defined as the treatment of human disease by the transfer of genetic material into specific cells of the patient[1]. Advances in molecular biology and biotechnology, and the completion of the Human Genome Project, have led to the identification of numerous disease-causing genes. It is not difficult to envision treatment of genetic diseases such as haemophilia[2], muscular dystrophy[3] or cystic fibrosis[4] through replacement of errant genes within the affected cells. Gene therapies are also being developed for cardiovascular[5], neurological[6–8] and infectious diseases[9], wound healing[10] and cancer[11–13] by delivering genes to augment naturally occurring proteins, to alter the expression of existing genes, or to produce cytotoxic proteins or prodrug-activating enzymes — for example, to kill tumour cells[11] or inhibit proliferation of endothelial cells to prevent restenosis[14]. Finally, it has been shown that expression of viral genes can result in immune responses, which has led to the concept of DNA vaccines[15].

Because of its broad potential, gene therapy has been heavily investigated during the past 15 years. The first clinical trial of gene therapy, for the treatment of severe combined immunodeficiency (SCID), was initiated in 1990[16]. However, it was not until April 2000 that Cavazzana-Calvo *et al.* reported the first clinical success with gene therapy, specifically the treatment of two infants with γc-SCID[17]. Also that year, Kay *et al.* reported positive data, including increased circulating levels of factor IX, in a haemophilia clinical trial[18] and Khuri *et al.* reported the successful completion of a Phase II clinical trial using a combination of gene therapy and traditional chemotherapy to treat recurrent squamous-cell carcinoma of the head and neck[19]. Considering that 863 gene-therapy clinical trials have been approved worldwide since 1989[20], the small number of successes is disappointing.

Gene therapy requires the identification of a therapeutic gene and transfer of that gene, often specifically to target cells, with high efficiency. Although short-term gene expression is sufficient for some applications, such as cancer therapies, long-term expression is needed for the treatment of chronic conditions, including most genetic diseases. For many applications it will be crucial to tightly regulate gene-expression levels. Finally, one must obviously accomplish each of these tasks in a way that is safe for the patient. Both toxicity/pathogenicity of the delivery vehicle and immune responses to the treatment must be considered. A key limitation to development of human gene therapy remains the lack of safe, efficient and controllable methods for gene delivery[21].

Methods of gene delivery

Gene-delivery vehicles can be divided into two categories: recombinant viruses and synthetic vectors. The majority of synthetic vectors, furthermore, can be divided into polymers (including polypeptides), which

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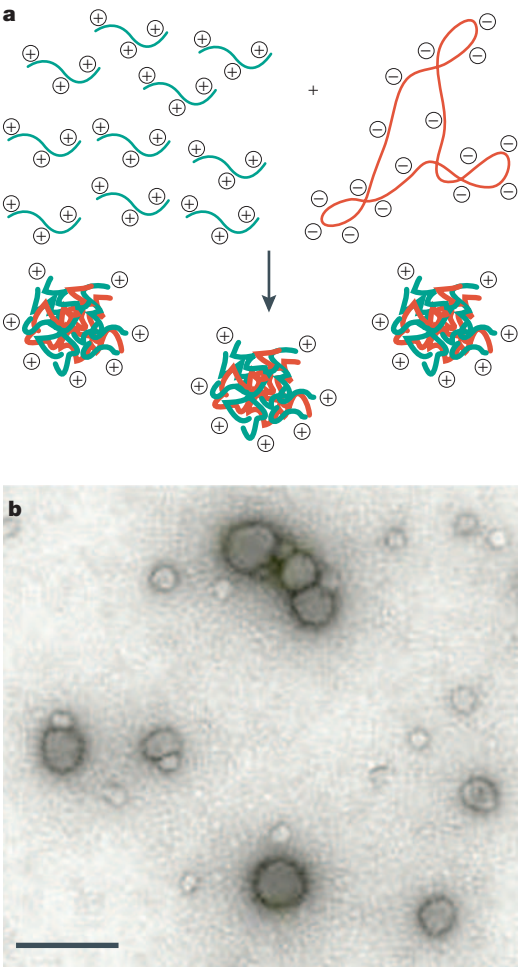

Figure 1 | **Polyplex formation.** Polyplexes are formed by electrostatic interactions between polycations and DNA. **a** | When aqueous solutions of a polycation and DNA are mixed, polyplexes form spontaneously. The interaction is entropically driven. For gene delivery, an excess of polycation is typically used, which generates particles with a positive surface charge. Each particle consists of several plasmid DNA molecules and hundreds of polymer chains and is 100–200 nm in diameter. **b** | Transmission electron micrograph of polyplexes comprising plasmid DNA and a polycation, in this case cyclodextrin-modified, branched polyethylenimine (PEI)[165]. Scale bar = 200 nm.

are the subjects of this review, and lipids. Each delivery method has specific advantages and disadvantages. To understand the problems facing polymer-based gene delivery vehicles and their current state of development, it is useful to briefly examine the alternative methodologies.

Viral vectors. The primary activity of a virus is to efficiently carry its genome from one host cell to another, enter the new target cell, navigate to the cell nucleus and initiate expression of its genome — albeit for the purpose of self-replication. Viruses such as retrovirus, lentivirus (for example, HIV), adenovirus, adeno-associated virus, herpes simplex virus and pox virus can be transformed into gene-delivery vehicles by replacing part of the genome of a virus with a therapeutic gene. Because viruses evolved essentially as sophisticated gene-delivery vehicles, such recombinant viral vectors are typically very efficient. Viral vectors have been used in the majority of gene-delivery studies reported in the literature and 69% of ongoing clinical trials[20]. For more information on recombinant viral gene delivery vectors, several excellent reviews have been published[22,23].

Safety concerns have been the primary bottleneck to the clinical application of viral gene delivery. Although recombinant viral vectors are rendered non-replicative, and therefore non-pathogenic, there still exists the possibility that the virus will revert to a wild-type virion or co-purify with replication-competent virions. Furthermore, viruses are inherently immunogenic, leading to difficulty with repeat administrations and the possibility of dangerous immune reactions. Other challenges with viral gene delivery include limitations in target-cell specificity and the costs of manufacturing viral-based gene therapies.

Synthetic vectors. Synthetic vectors provide opportunities for improved safety, greater flexibility and more facile manufacturing. In general, synthetic vectors are materials that electrostatically bind DNA or RNA, condense the genetic material into particles a few tens to several hundred nanometres in diameter (FIG. 1), protect the genes and mediate cellular entry. Such complexes of plasmid DNA with cationic lipids and polymers are known as LIPOPLEXES and POLYPLEXES, respectively. Various synthetic vectors, including (diethylamino)ether (DEAE)-dextran and calcium phosphate, have been used extensively for *in vitro* gene-transfer studies since the 1960s. However, the development of non-viral vectors for *in vivo* gene delivery has suffered from low gene-transfer efficiency, and in some cases toxicity and *in vivo* instability.

The use of cationic lipids for gene delivery was first reported by Felgner in 1987[24]. The lipid structures affect the interactions of the lipids with DNA and influence the membrane properties of liposomes formed from them, both of which have a strong influence on gene-delivery efficiency[25]. As a result of their relatively high efficiency, cationic lipids have been the most widely studied of the synthetic vectors[26]. 'Lipofection' has been routinely used in both *in vitro* and *in vivo* gene delivery studies, and in many human gene-therapy clinical trials[20]. However, lipid-based gene delivery has crucial limitations, including difficulty in reproducibly fabricating liposomes and DNA–liposome complexes, toxicity (for example, cell death) to some cell types *in vitro* and *in vivo*[27], and colloidal stability, especially upon systemic administration.

Cationic polymers, including off-the-shelf materials and polymers specifically designed for gene delivery, comprise a variety of chemistries. DNA-binding moieties — including primary, secondary, tertiary and quaternary amines, as well as other positively charged groups such as amidines — can reside in the polymer backbone, in pendant groups or in grafted oligomers.

LIPOPLEXES
Nanoparticles similar to polyplexes but formed by complexation of cationic lipids (or liposomes containing cationic lipids) and DNA.

POLYPLEXES
Nanoparticles, typically ~100 nm in diameter, formed by electrostatic complexation of cationic polymers (or polypeptides) and DNA.

The polymers themselves comprise linear, branched and dendritic structures. Because of the flexibility of polymer chemistries, it might be possible to provide the multiple functions required for efficient gene delivery while maintaining biocompatibility, facile manufacturing, and robust and stable formulation. As a result, cationic polymers have great potential for human gene therapy. However, poor gene-transfer efficiency has limited their clinical application. The specific advantages and disadvantages of several important classes of cationic polymers, with emphasis on recently designed gene-delivery materials, will be described in more detail below.

The gene-delivery problem

To escort genes from a solution (for example, in a vial) to the cell nucleus, gene-delivery vectors must navigate a series of obstacles, both extracellular and intracellular (BOX 1). Viruses have evolved functions to address each challenge. By contrast, synthetic vectors are generally unsatisfactory because they lack one or several of the necessary functions. Consideration of the important barriers to gene delivery, therefore, is necessary to understand the limitations of cationic polymers and is important for the rational design of new polymers.

Extracellular barriers. Gene-delivery vectors face an initial set of barriers in transporting genes from the test tube to the membrane of a target cell. These include physico-chemical challenges, such as binding and condensing plasmid DNA and maintaining the complex in solution, as well as *in vivo* barriers, including stability and survival in the blood stream, penetrating the blood vessel wall and surrounding tissue, and specific binding to the target cells of interest.

Gene packaging. Polyplexes protect DNA by sterically blocking the access of nucleolytic enzymes. Unprotected plasmid DNA is degraded by DNase within minutes, whereas plasmid DNA in polyplexes is stable for hours[28]. Gene-delivery vectors bind to and condense DNA into small, compact structures through electrostatic interactions between the negative phosphates along the DNA backbone and positive charges displayed on the vector material (FIG. 1a). The process of condensation is entropically driven[29], and polyplexes form spontaneously upon mixing of cationic polymers with plasmid DNA. The resulting particles are typically toroidal or spherical structures[30,31] with diameters ranging from about 30 to several hundred nanometres (FIG. 1b). Each polyplex particle most often comprises several DNA molecules along with many polymer chains. The structure and morphology of polyplexes seems to be kinetically controlled[32] and often depends on the order of mixing (for example, adding polymer to DNA solution or DNA to polymer solution). Efforts have been made to better understand polyplex formation[29,32,33], but improvements in the theoretical understanding of the process and physico-chemical characterization of the resulting complexes are needed.

Box 1 | Design criteria for non-viral vectors

- **Protection of DNA**
- **Packaging of large DNA plasmids**
- **Easy administration**
- **Serum stability**
- **Targetability to specific cell types**
- **Ease of fabrication**
- **Inexpensive synthesis**
- **Facile purification**
- **Robustness/stability**
- **Internalization**
- **Endolysosomal escape**
- **Nuclear transport**
- **Efficient unpackaging**
- **Infection of non-dividing cells**
- **Safety**
- **Non-toxic**
- **Non-immunogenic**
- **Non-pathogenic**

The structure of the polycation can affect DNA binding and condensation. For example, the number of cationic moieties has a strong effect on the polymer–DNA interaction. Several groups have reported that a minimum of six to eight charges in a polypeptide are required for efficient DNA condensation[34–36]. The same groups disagree, however, on the effect of the peptide sequence. Wadhwa *et al.* reported that addition of a single tryptophan (Trp) residue into polylysine oligomers increased DNA binding[34]. By contrast, Plank *et al.* found that Trp residues had no major effect on DNA binding and, in fact, decreased the 'DNA-compacting potency' of the polypeptides[35]. The difference might be to due the structures (linear versus branched) of the peptides studied. Additionally, placing the cationic moiety nearer to the synthetic polymer backbone and keeping the charges to a minimum separation along the polymer backbone correlated with increased DNA binding[37].

It is important to note that strong binding and efficient DNA condensation do not correlate directly with gene-delivery efficiency, probably because tight binding prevents transcription. A polymer must therefore balance sufficient binding strength to initially protect the plasmid with the ability to release the plasmid, perhaps by competitive binding of genomic DNA, cytosolic proteins or anionic membrane lipids[36,38].

Serum stability. The stability of polyplexes depends on the polymer structure and on the DNA/polymer charge ratio. Neutral polyplexes in physiological salt concentrations quickly form large aggregates, which are generally ineffective gene-delivery agents and can even be toxic due to embolization of the particulates in the lung. In contrast, positively charged polyplexes typically remain in solution. However, recent studies have

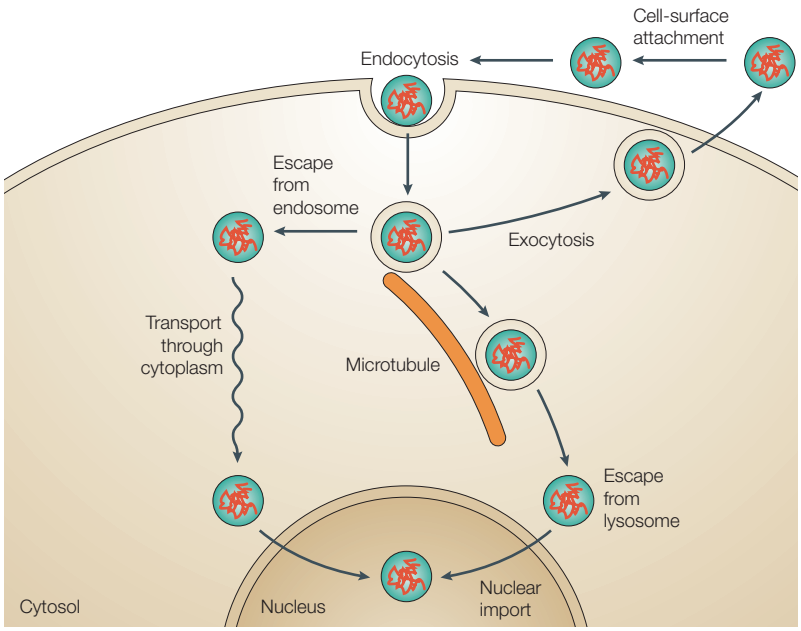

Figure 2 | **Barriers to intracellular trafficking of polyplexes.** Polyplexes must attach to the cell surface, be internalized (by endocytosis), escape from endolysosomes, move through the cytoplasm toward the nucleus and cross the nuclear membrane. Alternative pathways exist for several of these steps. In addition, the polyplexes must unpackage — DNA must be released by the polymer — but where unpackaging occurs is not known.

shown that the solubility and aggregate size of even positive complexes is time dependent[32]. Adsorption of serum albumin and other negatively charged proteins causes further aggregation and can lead to rapid clearance of the polyplexes by phagocytic cells and the reticuloendothelial system[39].

When grafted onto the polymer as a 'brush', modification of polyplexes with hydrophilic polymers such as polyethylene glycol (PEG)[40,41] and *N*-(2-hydroxypropyl)methacrylamide (HPMA)[41–44], oligosaccharides[41,45], sugars[46,47] and proteins[48], can stabilize polyplexes against salt-, protein- and complement-induced inactivation. Increased stability presumably results from steric effects that lead to decreased particle–particle and particle–protein interactions. The effect depends on the molecular mass of the hydrophilic polymer, the grafting density, and the method of attachment of the hydrophilic polymer to the polycation. PEGylation, however, has been shown to reduce internalization of untargeted polyplexes and to alter intracellular trafficking[49].

Cell-specific targeting. The degree of target-cell specificity required for a given gene therapy varies widely. For some applications, such as haemophilia, the identity of the transduced cells is of little concern so long as sufficient levels of the secreted therapeutic protein are produced. In cancer therapies, in which the goal is to kill the target cells, gene delivery to a very specific set of cells might be required. Polymers generally do not have the capacity for cell-specific targeting but provide flexible chemistry for the attachment of targeting moieties that allow both increased cell uptake and, often, cell specificity. Many membrane-bound receptor proteins can be used for targeting via RECEPTOR-MEDIATED ENDOCYTOSIS. Derivatization of polymers with glycosidic moieties[50–53], as well as other small-molecules such as folate[54], provides selective targeting to cell types that display the appropriate receptor protein. In two classic examples of protein-mediated targeting, asialoorosomucoid was attached to polylysine[55–58] to target the asialoglycoprotein receptor on hepatocytes, whereas Wagner and co-workers reported a method for targeting to a variety of cell types through the attachment of the iron-transport protein transferrin[30,59–62]. Other proteins might provide targeting to a more limited range of cells. Examples include epidermal growth factor (EGF)[63,64], antibodies or antibody fragments[65] and integrin-binding sequences[66].

The success of a targeting strategy depends on the conjugation chemistry, the length of spacer between ligand and polyplex, the ligand–receptor binding strength, and the number of targeting ligands per polyplex. A variety of common crosslinking chemistries have been used to attach targeting ligands to the polymers, including covalent bonds and biotin–streptavidin[63]. Care must be taken, however, to ensure that the ligand–receptor interaction is not disrupted by conjugation. Schaffer *et al.* found that for EGF–biotin/avidin–polylysine conjugates, short crosslinkers between the EGF and biotin interfered with EGF/EGF receptor binding, but that a 30-Å spacer allowed nearly unhindered binding and resulted in significantly higher gene-transfer efficiency[67]. There also exists a balance between the specific targeting interactions and nonspecific electrostatic binding to the cell surface. Cell uptake and gene expression were found to be specific only within a narrow window of polymer/DNA ratios near electroneutrality[67]. Finally, there is typically an optimal ligand valency, due to saturation of both receptor binding and the cell's internalization machinery[67]. Efficient cell-specific targeting therefore requires careful optimization of the various parameters that affect cell-surface binding.

Intracellular barriers. Following internalization, gene-delivery vehicles are challenged with a new set of intracellular obstacles (FIG. 2), and the vector needs to provide functionality to overcome each one. Although >95% of cells in culture typically internalize vectors (>100,000 copies per cell[68]), only a fraction, typically <50%, express the transgene. Polyplexes are generally internalized by endocytosis, and, once in the endocytic pathway, polyplexes can be trafficked to lysosomes, acidic vesicles filled with degradative enzymes[69]. DNA and vector must escape these compartments into the cytoplasm, be transported towards the nucleus and cross the nuclear membrane. At some point during this process, the vector must release the genes so that they can be transcribed in the nucleus.

Endolysosomal escape. Untargeted polyplexes bind electrostatically to the surface of cells and are internalized via adsorptive pinocytosis[70]. Alternatively,

RECEPTOR-MEDIATED ENDOCYTOSIS
The process by which cells internalize various nutrients and signalling molecules. Binding of the ligand to a specific plasma membrane-bound receptor protein generally initiates the formation of clathrin-coated pits and subsequently formation of a vesicle (the endosome) inside the cell. Viruses, drugs and delivery vectors can also be internalized by displaying the natural ligand or an analogue.

Box 2 | The proton-sponge hypothesis

'Proton-sponge' polymers[104,114,119], including polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimers (FIG. 3)**, which contain a large number of secondary and tertiary amines, exhibit pK_a values between physiological and lysosomal pH. Endolysosomes are acidified by the action of an ATPase enzyme that actively transports protons from the cytosol into the vesicle. These polymers, therefore, undergo large changes in protonation during endocytic trafficking. It has been proposed that proton-sponge polymers prevent acidification of endocytic vesicles, causing the ATPase to transport more protons to reach the desired pH. The accumulation of protons in the vesicle must be balanced by an influx of counter ions. The increased ion concentration ultimately causes osmotic swelling and rupture of the endosome membrane, which releases the polyplexes into the cytosol** (FIG. 4)**[182].**

polyplexes derivatized with targeting ligands bind to specific cell-surface receptors, in which case they are often internalized by receptor-mediated endocytosis. In either case, the polyplexes become localized within endocytic vesicles, which represent a hostile environment. The first vesicle, termed the early endosome, fuses with sorting endosomes from which the internalized material can be transported back to the membrane and out of the cell by exocytosis. More generally, however, polyplexes are believed to be trafficked into late endosomes, vesicles that rapidly acidify to pH 5–6 due to the action of an ATPase proton-pump enzyme in the vesicle membrane. Polyplexes can subsequently be trafficked into lysosomes, which further acidify to pH ~4.5 and contain various degradative enzymes. It is believed that much of the DNA becomes trapped in these vesicles and is degraded. Only DNA that escapes into the cytoplasm can go on to reach the nucleus.

Several strategies have been used to overcome this barrier. Concurrently treating cells at the time of TRANSFECTION with chloroquine, which is known to accumulate in the acidic vesicles and buffer their pH, results in improved gene delivery with some polymers[71]. Although this approach has been commonly used in *in vitro* studies, it is impractical for *in vivo* gene delivery. Other researchers have conjugated whole, inactivated adenovirus particles to polylysine, which enhanced gene transfer by up to 2,000-fold[72–75]. It was proposed that the enhancement was due to virus-mediated endosomal escape, but the virion might also provide functionality for addressing subsequent intracellular barriers. This approach is also impractical owing to the increased difficulty of preparing the vector and safety concerns, especially immunogenicity, raised by the virus. Alternatively, fusogenic viral[76–79] or synthetic[80–82] peptides can be attached to the polymer to provide endosomal escape. These peptides are typically pH-sensitive amphiphiles that undergo a structural change at acidic pH and disrupt the vesicle membrane. Gene transfer is typically enhanced one to three orders of magnitude in this fashion. Finally, certain materials, known as 'proton-sponge' polymers, are believed to mediate their own endosomal escape through a unique mechanism (BOX 2; FIG. 4).

Transport through the cytoplasm. Once released from endosomal compartments, polyplexes must move through the cytoplasm to the nucleus. However, the cytoplasm is concentrated with protein, microtubules and other organelles, all of which can hinder polyplex movement. Studies of mobility in the cytosol showed that diffusion is size-dependent[83] — DNA larger than 3,000 base pairs in length is essentially immobile[84]. One might expect relatively large polyplexes to be immobile as well. This fact, together with the known degradation of DNA in the cytosol due to the presence of cytosolic nucleases[85], presents an often-overlooked barrier to efficient gene delivery.

Positively charged polyplexes could move along microtubules by nonspecifically interacting with anionic microtubules or motor proteins, or they could rely on the natural transport of ENDOLYSOSOMES along the microtubules, for example[86]. Alternatively, polyplexes could be redistributed throughout the cell by the mixing that occurs during mitosis, such that some accumulates near (or in) the nucleus. The mechanisms of cytoplasmic polyplex transport need to be better characterized in order to facilitate design of improved polymeric vectors.

Nuclear localization. Because the genome and nuclear machinery are vital to the various functions of the cell, nature has isolated the nucleus behind a double-bilayer membrane with tightly regulated pores that allow import and export of a specific set of biomolecules. The nuclear pore complex (NPC), a 10^7-Da assembly of at least 30 distinct proteins, allows the passage of small molecules, but proteins larger than 10–20 kDa require active transport via specific nuclear import proteins (for example, IMPORTINS). Viruses have evolved mechanisms to utilize the nuclear import machinery of the cell. Polymers clearly do not have this capacity and rely largely on nuclear membrane breakdown during cell division for nuclear entry[87]. Transfection immediately before cell division is 30- to 500-fold more effective than transfection of cells at the beginning of their cell cycle[88]. As such, transport across the nuclear membrane, especially for non-dividing cells, seems to be a formidable obstacle to non-viral gene-delivery vectors.

It is well known that many proteins are naturally targeted to the nucleus by the presence of nuclear localization signals (NLS), which are short cationic peptide sequences that are recognized by importins. Due to their positive surface charge, it is possible that polyplexes could mimic NLS to a limited extent, but if so they must be very inefficient because very few vectors typically reach the nucleus[89–94]. Further, nucleotide sequences on the genes themselves could provide some nuclear targeting[89,90,95]. Nuclear import of polyplex vectors is one of the most poorly characterized steps in the gene-delivery process.

TRANSFECTION
Delivery of biomolecules, usually DNA or RNA, into mammalian cells in culture mediated by synthetic reagents such as polymers, polypeptides or lipids.

ENDOLYSOSOMES
Cytoplasmic vacuoles formed by inward budding of the cell membrane during endocytosis. The organelles, first known as endosomes, acidify and fuse with enzyme-carrying vesicles from the Golgi to become lysosomes.

IMPORTINS
Cytoplasmic proteins that bind nuclear localization signal peptides and, therefore, target proteins for transport across the nuclear membrane. Importins are recognized by the nuclear pore complex, a macromolecular assembly of more than 100 different proteins, and translocated (along with their cargo molecule) through the pore by an energy-dependent mechanism.

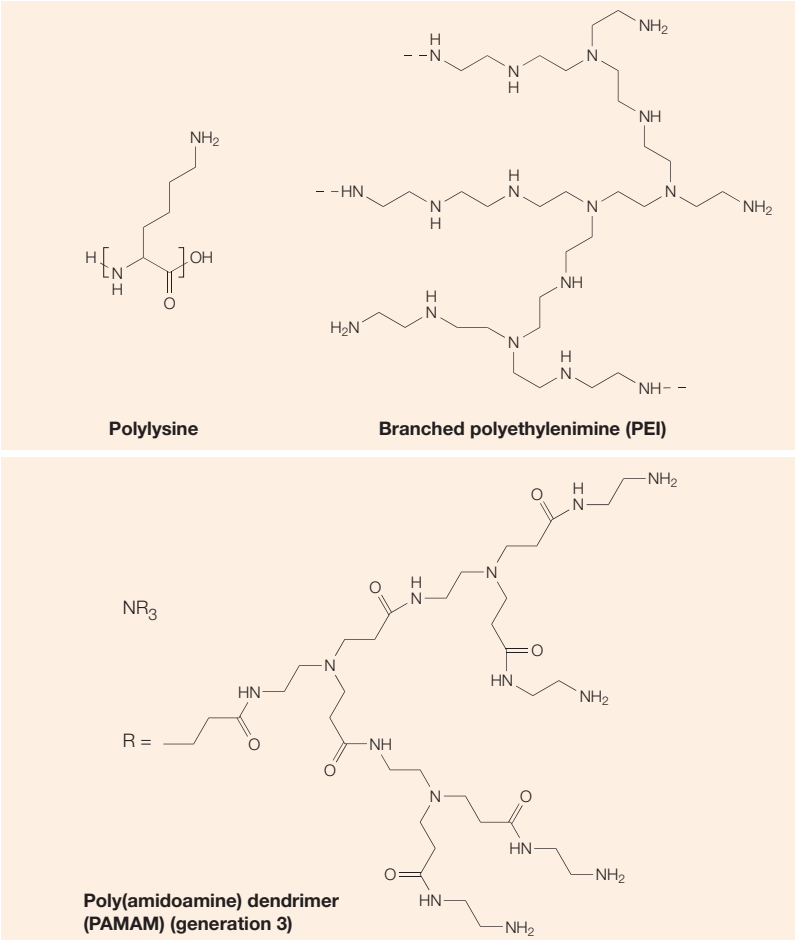

Figure 3 | **Structures of off-the-shelf gene-delivery polymers.** Polylysine, polyethylenimine (PEI) and polyamidoamine (PAMAM) dendrimer represent the most widely studied polymeric gene delivery vehicles of the 1990s.

Unpackaging. Just as incorporation into a polymer complex protects DNA from enzymatic degradation, complexation prevents binding of the proteins required for gene expression. A vector must therefore release its DNA at some point in the delivery process. Several studies have found that reducing the polymer/DNA binding strength, by reducing the number of positive charges[96], conjugation of PEG chains[97], or decreasing the polymer molecular mass[36], leads to increased gene expression. Polymers must clearly be designed to incorporate a mechanism for nonspecific or environmentally responsive release of the genes, ideally in the nucleus.

'Off-the-shelf' polymers for gene delivery

Many early gene-delivery studies used commercially available polymers. Because these materials were not designed for gene delivery, their efficacy as gene-delivery agents is somewhat serendipitous. Off-the-shelf polymers have nonetheless been widely studied and form the basis for much of the non-viral gene-delivery literature. However, significant problems face these polymers. Three of the major off-the-shelf polymers are described here.

Polylysine. Polylysine (FIG. 3) was one of the first cationic polymers to be used in the modern era of gene-delivery research[98]. Polyplexes of DNA and polylysine itself are poor gene-delivery vectors and require the addition of chloroquine for even moderate transfection activity. Addition of targeting ligands greatly enhances *in vitro* and *in vivo* delivery efficiency. Some of the first reports of polylysine-mediated gene delivery used conjugation of the asialoorosomucoid glycoprotein to target the asialoglycoprotein receptor on mouse hepatocytes *in vitro* and *in vivo*[55–57]. Shortly thereafter, a series of papers by Wagner and colleagues reported conjugation of the iron transport protein transferrin to polylysine[62,99]. These studies provided a basis for much of the subsequent literature, including some of the first studies of polyplex formation[30] and mechanisms of endolysosomal escape[75,76]. Polylysine–DNA complexes have also been targeted to specific cells through conjugation of sugars[51,100–102], folate[54], RGD-displaying peptides[66] and antibodies[103].

Early studies on polylysine were promising, but it now seems unlikely that polylysine-based polyplexes will find clinical applications because of their relatively low efficiency. This is generally accepted to be the result of poor escape from the endocytic pathway. In recent years, polylysine has been relegated to a role in mechanistic studies or as a point of comparison to more promising polymers.

Polyethylenimine (PEI). PEI (FIG. 3), one of the most effective gene-delivery polymers studied to date, has been used as a gene-delivery vector since 1995[104]. Importantly, PEI mediates gene delivery efficiently in the absence of any exogenous endosomolytic agent. PEI-containing polyplexes have been targeted to specific cell types by the conjugation of ligands including galactose[52], mannose[105], transferrin[106] and antibodies[107]. Additionally, PEI has been successful for *in vivo* gene delivery to a variety of tissues, including the central nervous system[108,109], kidney[110], lung[111,112] and tumours[113]. PEI gene delivery has been hindered by the polymer's relatively high cytotoxicity.

The relatively high gene-transfer activity of PEI is believed to be due in large part to efficient escape from the endocytic pathway through the proton-sponge mechanism (BOX 2)[104,114]. Because every third atom of the polymer is a nitrogen, PEI has a very high density of amines, only 15–20% of which are protonated at physiological pH[115]. This unique property makes PEI an extraordinarily strong proton sponge.

Because PEI is an off-the-shelf material, it is not surprising that its buffering capacity or other properties are sub-optimal for gene delivery. Forrest *et al.* reacted PEI with acetic anhydride to convert the primary and secondary amines to secondary and tertiary amides, respectively[116]. Such a change was expected to produce a poorer proton sponge by decreasing the number of protonable nitrogens in the polymer. Surprisingly, gene-delivery activity increased upon acetylation, and the polymer with acetylation on ~43% of the primary amines was as

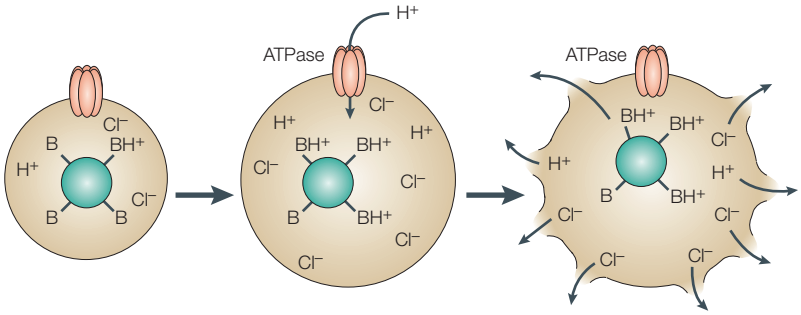

Figure 4 | **Schematic of the proton-sponge mechanism.** Protonation of the proton-sponge polymer (green) causes increased influx of protons (and counter-ions) into endocytic vesicles. Increasing osmotic pressure causes the vesicle to swell and rupture. See BOX 2.

much as 26-fold more efficient than unmodified PEI. In a similar study, Thomas and Klibanov modified all of the amino groups of PEI with a variety of chemical groups, including permethyl and perethyl, choline, amino acids and long-chain alkyl groups[117]. Most of the modifications reduced the gene-delivery activity of PEI, although addition of alanine marginally increased gene-delivery efficiency. Further, dodecylation and hexadecylation of low-molecular-mass PEI (2,000-Da) enhanced gene delivery in the presence of serum by five- to six-fold in comparison with unmodified 25-kDa PEI. These results do not necessarily contradict the proton-sponge hypothesis but suggest that buffering capacity might need to be balanced with other properties of the polymers. For example, the enhanced efficiency of these modified polymers could be due to weaker binding of DNA and, therefore, more efficient unpackaging of the DNA–polymer complexes.

Polyamidoamine (PAMAM). PAMAM ('Starburst') dendrimers are spheroidal, cascade polymers (FIG. 3), the size and surface charge of which are controlled by varying the number of 'generations' in the synthesis[118]. Because of the large number of secondary and tertiary amines on the polymer, PAMAM dendrimers are also thought to be proton sponges. Haensler and Szoka originally reported the use of PAMAM dendrimers for gene delivery[119]. They found that the sixth-generation dendrimer was better than higher and lower generations by ~10-fold. Due to its relatively high gene-delivery efficiency and good biocompatibility, PAMAM dendrimers have recently been used in several *in vivo* gene-delivery studies[120–123].

Partially degraded PAMAM dendrimers seem to be more effective vectors than the intact polymer. Heat treatment of the polymer in various solvolytic solvents (for example, water or butanol) degrades the polymer at the amide linkages, resulting in a heterodisperse population of 'fractured' dendrimers that show >50-fold enhanced transfection activity[124]. The mechanism of the enhancement seems to be twofold. First, the fractured dendrimer has greater flexibility, allowing a more beneficial interaction with the plasmid DNA[124]. Second, polyplexes containing fractured PAMAM seem to have enhanced solution stability in comparison to polyplexes containing intact polymer, which tend to aggregate[125].

Polymers designed for gene delivery

Many types of polymers have been specifically designed for gene delivery. In most cases, the polymers were designed to address a specific intracellular barrier, such as stability, biocompatibility and endosomal escape. The results have been mixed, with some polymers performing as well as, or even slightly better than, the best off-the-shelf polymers. None approach the efficiency of viruses, however. We cannot describe all of the polymers designed for gene delivery here. Rather, we review several new classes of polymers that seem to show promise.

Imidazole-containing polymers

Proton-sponge polymers are some of the best available off-the-shelf materials for gene delivery. However, they are limited by considerable cytotoxicity. It would be desirable to mimic the proton-sponge mechanism, which requires buffering capacity between physiological and lysosomal pH, in a polymer that is more biocompatible. Imidazole exhibits the required protonation properties (pK_a ~6) and is a component of several biomolecules (for example, the amino acid histidine), suggesting that polymers incorporating imidazole might be expected to show increased biocompatibility[126].

In one study, a homopolymer of histidine (pHis) was derivatized with gluconic acid to increase its solubility in aqueous solutions (G-pHis) (FIG. 5a)[127]. Ternary complexes of DNA, G-pHis and transferrin–polylysine conjugates (Tf–PLL) transfected COS-7 cells, in the absence of any exogenous endosomolytic agents, with efficiency similar to that of DNA/Tf–PLL complexes administered with chloroquine. Further, the polymer showed negligible toxicity at concentrations used in gene-delivery studies. Similarly, Ihm *et al.* reported a vinyl polymer with pendant imidazole moieties that showed higher transfection efficiency than PEI in at least one cell line[128].

A second approach is to derivatize polylysine with an imidazole-containing pendant group (FIG. 5a). Two chemistries have been reported to date. In the first, the ε-amino groups of polylysine or oligolysine were derivatized with histidine, which resulted in an amide linkage with a free primary amine remaining[129,130]. The imidazole group was therefore introduced without a reduction of the number of positive charges available for binding DNA. In the second, polylysine was derivatized with imidazoleacetic acid and yielded a similar amide linkage, but in the absence of the primary amine[131]. Both polymers were found to efficiently transfect a variety of cell types in the absence of any exogenous endosomolytic agent, and gene-expression levels increased with increasing imidazole content. Transgene-activity levels achieved by histidylated polylysine transfection were three to four orders of magnitude higher than with underivatized

polylysine[129]. In fact, Putnam *et al.* reported gene-expression levels comparable to those achieved with PEI polyplexes, yet without cytotoxicity.

Membrane-disruptive peptides and polymers

As described above, endosomal escape is a crucial barrier to efficient gene delivery. Many viruses have evolved specific acidic peptide sequences in their protein coat that are protonated at acidic pH and become fusogenic with the endosomal membrane, enabling delivery of their DNA or RNA cargo directly into the cytoplasm[132–134]. Synthetic peptides with similar pH-responsive, membrane-destabilizing mechanisms have been developed as endosomal-releasing agents in cationic polyplexes and lipoplexes[76,78,135–137]. However, there is the potential for eliciting an undesirable immune response to such foreign peptides. This has led to increased interest in the use of synthetic excipients that could enhance endosomal escape in a manner similar to that of viral fusogenic peptides. Towards this goal, a family of synthetic, acid-responsive polymers has been 'molecularly engineered' to enhance the efficiency of polyplex and lipoplex carrier systems[138]. The key feature of such polymers is that they are converted from being hydrophilic to hydrophobic when they are protonated within the acidic environment of the endosomes, which probably causes them to partition into, and therefore disrupt, the vesicle membrane.

Early work of Tirrell *et al.* showed that polyethylacrylic acid (PEAA) caused membrane disruption of liposomes at acidic conditions[139,140]. Inspired by this early work, Stayton, Hoffman and co-workers have designed and investigated a family of 'smart' pH-responsive polymers that incorporate the following components: pH-responsive functionalities, especially –COOH and anhydride groups; hydrophobic groups for interacting with the endosomal membrane; pendant groups that permit conjugation and/or ionic complexation of the macromolecular drug; and pendant groups that permit conjugation of a cell-targeting ligand[141–147]. Compositions have included polymers and copolymers of α-alkyl acrylic acids (for example, methacrylic acid (MAA), ethylacrylic acid (EAA), propylacrylic acid (PAA) and butylacrylic acid (BAA)) and their copolymers with alkyl acrylates or methacrylates. In addition to the acrylate-based polymers, another type of membrane-disruptive polycarboxylic acid polymer is based on copolymers of maleic anhydride with styrene, alkyl vinyl ethers and other hydrophobic monomers[147]. In this type of copolymer the anhydride group can be opened with alkyl alcohols or amides to control the ratio of the –COOH groups to hydrophobic groups in the resulting polymer.

Although the early work of Tirrell and co-workers described the acid-stimulated disruption of liposomes by PEAA[139,140], they did not investigate higher alkylacrylic acid polymers. Murthy, Cheung and co-workers found that PPAA was approximately 15 times more active than PEAA[141–143] and showed maximum haemolytic activity at pH ≤6.0, which is in the range of endosomal pHs. Haemolysis is a generally accepted method for characterizing the capacity of various agents to disrupt membranes. The much greater haemolytic activity of PPAA compared with PEAA at endosomal pHs was a result of the increase in the pK_a of PPAA to pH ~6.0, which resulted from the addition of just one methylene group. PBAA caused haemolysis even at physiological pH. PBAA homopolymer is therefore not useful as a gene-delivery vector, because it would probably induce whole-cell lysis at pH 7.4[142]. Further studies showed that compositions of methacrylic acid-acrylate ester copolymers and maleic anhydride copolymers (esterified with alkyl alcohols or amidified with alkyl amines) could be engineered to show efficient haemolysis in the endosomal pH range of 5.5–6.5[142,147]. A key variable for controlling the acid-induced haemolytic action of such synthetic polycarboxylic polymers and copolymers is ratio of –COOH groups to hydrophobic groups in the polymer.

The exceptional pH-dependent haemolytic activity of these polymers and copolymers, especially PPAA[141–148], motivated an investigation of its ability to enhance gene expression in cell culture when physically incorporated in cationic lipoplexes[143,145]. PPAA was found to enhance transfection significantly in 3T3 cells[143], and incorporation of PPAA also greatly improved the serum stability of these formulations[146]. The favourable transfection results obtained with PPAA motivated further *in vivo* evaluation. PPAA-containing lipoplexes significantly enhanced wound healing in thrombospondin-2-null knockout mice through the combined effects of altered extracellular matrix organization and greater vascularization[145].

The early success of these polymers and copolymers motivated recent work to design a new functionalized monomer, pyridyl disulphide acrylate (PDSA), that provides a means for conjugating or complexing thiol-terminated antisense oligonucleotides (AS-ODN) or small interfering RNA (siRNA) drugs directly to the endosomolytic polymer backbone through reducible disulphide bonds[149,150]. The disulphide linkages can also be reduced by glutathione in the cytoplasm, leading to release of the free drug in the cytosol after the backbone polymer has facilitated escape from the endosome.

A different approach to the design of pH-responsive, membrane-disruptive polymers involves the incorporation of acid-degradable bonds into a polymer backbone or its pendant groups. Several researchers have designed polymers with acid-degradable bonds[151,152], but most of the acid-sensitive groups (for example, hydrazone, acontityl and ester) degrade too slowly to avoid eventual lysosomal localization of the formulation. (It usually takes several hours to move from the early endosome to the lysosome.) Furthermore, only the two studies of Murthy *et al.* incorporated membrane-disruptive properties in the polymer backbone[151,152]. Acetal-containing polymers synthesized by Murthy *et al.* were very effective in delivering two AS-ODNs — anti-inducible nitric oxide synthase (iNOS), designed to reduce the inflammatory response of macrophages to express iNOS — and an anti-interleukin-1 receptor-associated kinase (IRAK), which acts to block the intracellular

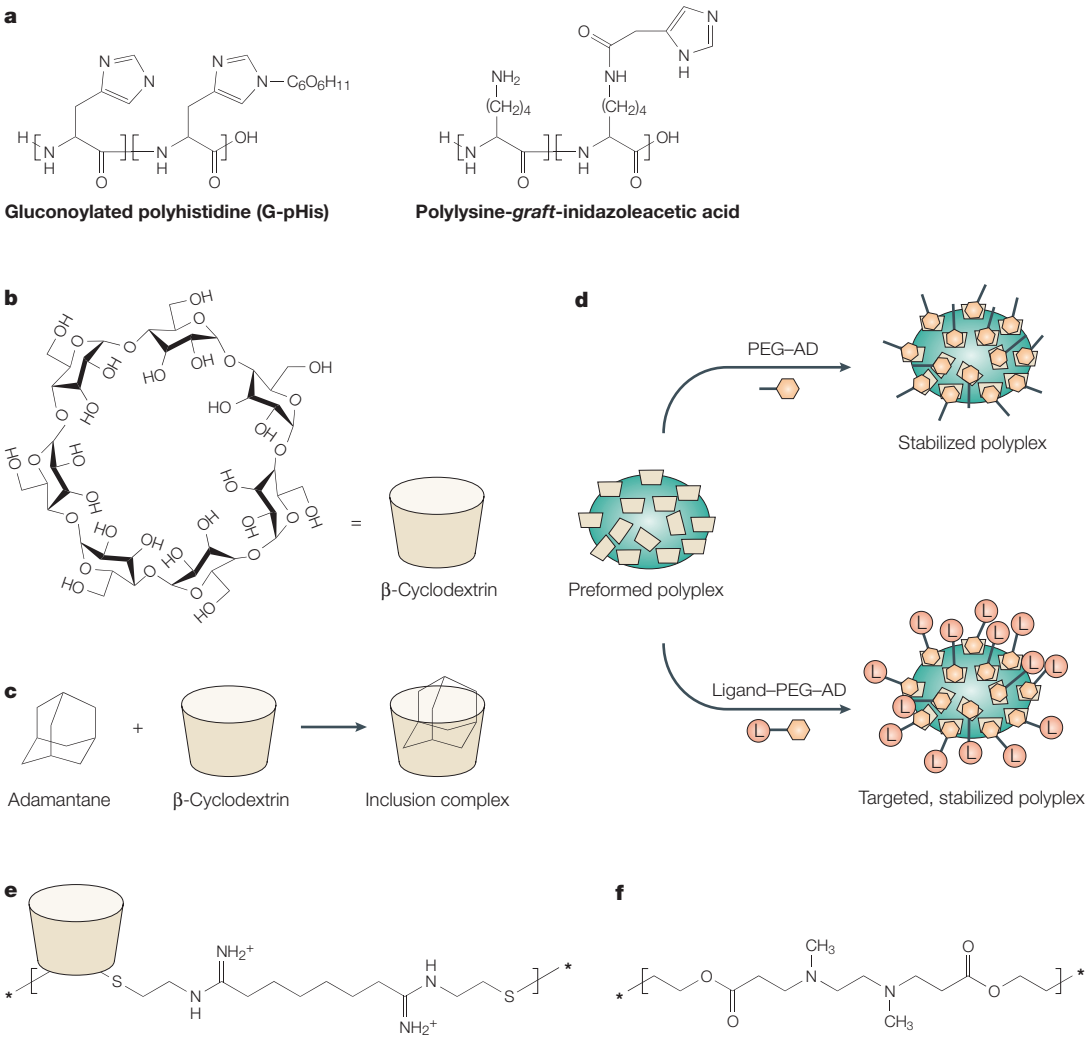

Figure 5 | **Structures of several polymers designed for gene delivery. a** | Imidazole-containing polymers generated by conjugation of a sugar group to polyhistidine[127] or histidylation of polylysine[131]. **b** | The 'cup' of β-cyclodextrin (CD) has a hydrophobic interior and hydrophilic exterior surface. **c** | β-CD is capable of binding non-polar molecules such as adamantane (AD) inside the cup to form inclusion complexes. **d** | Polyplexes containing β-CD can be decorated with adamantane-terminated shielding groups such as polyehtylene glycol (PEG) to improve serum stability or with targeting ligands via modification of the targeting group with adamantane[166]. **e** | Chemical structure of a β-CD-containing polymer. **f** | Structure of a typical poly(β-amino ester)[174].

signalling pathway leading to tumour-necrosis factor-α expression[151,152]. The polymer backbone was hydrophobic and membrane-disruptive by itself, and was modified with pendant PEG to enhance its solubility and mask its membrane-disruptive ability until it was endocytosed. The drug was complexed or conjugated at the end of the PEG, whereas the PEG was conjugated at the other end to the backbone via the acetal groups. The latter groups degraded within about an hour at endosomal pH, unmasking the backbone and enhancing the escape of the AS-ODN to the cytosol[151,152].

The precise pH-induced membrane-disruption activity profile of each of these 'smart' polymers and copolymers will depend on the hydrophilic nature of the nucleic acid drug (for example, DNA, RNA, AS-ODN or siRNA), the drug 'loading' (that is, the quantity of drug complexed or conjugated to the polymer backbone) and the carrier polymer composition. The modular character of these biomimetic copolymers permits precise control of the desired intracellular drug delivery.

INCLUSION COMPLEXES
Bi-molecular complexes in which the 'host' forms a cavity into which the 'guest' molecule binds through non-covalent (van der Waals) interactions.

Cyclodextrin-containing polymers

Cyclodextrins (CDs) are cup-shaped molecules comprising 6, 7 or 8 glucose units (called α-, β- and γ-CD, respectively; FIG. 5b). The exterior of the cup is hydrophilic and the interior is hydrophobic. CDs are therefore water-soluble and can form INCLUSION COMPLEXES with small, hydrophobic 'guest' compounds (FIG. 5c). CDs are biocompatible materials and are used in FDA-approved pharmaceutical formulations as solubilizing agents.

A new class of linear, CD-based polymers was introduced by Davis and co-workers in 1999 for gene-delivery applications[153]. These polycations contain

CDs in the polymer backbone and self-assemble with anionic nucleic acids to form condensed polyplex structures with diameters ~100 nm that can mediate cellular delivery. Because the polymers are synthesized by condensing a difunctionalized CD monomer with a difunctionalized co-monomer, polymer structure can be methodically controlled. The effect of CD size (α-, β- or γ-CD), charge centre (amidine or quaternary ammoniums), and charge density on gene-delivery efficiency and polymer toxicity were reported in a series of publications[154–158]. More details on this class of linear polymer is available in recent reviews[159,160].

Polycations containing pendant CDs have been synthesized to impart the pharmaceutically attractive properties of CDs (low toxicity and inclusion-complex formation) with 'off-the-shelf' polymers used for gene delivery. Uekama and colleagues synthesized a series of PAMAM dendrimer–CD conjugates by grafting CD (α-, β- and γ-CD) to generations of PAMAM dendrimers (G2, G3 and G4) and concluded that α-CD conjugation to G3 PAMAM dendrimers boosted *in vitro* and *in vivo* gene transfer without significant changes in polymer toxicity[161–163]. CD-grafted PEI (CD–PEI) also efficiently delivers nucleic acids to cultured cells with lower toxicity than the unmodified parent polycation[164,165]. Both CD–PAMAM and CD–PEI have been applied successfully as *in vivo* gene-delivery vectors[162,165].

A key feature of CD-containing polyplexes is that the particles can be readily surface-modified by inclusion-complex formation (FIG. 5d). Pun *et al.* demonstrated PEGylation of CD-containing polyplexes by modification with adamantane–PEG conjugates (the adamantane and CD form inclusion complexes with high association constants)[166]. This self-assembly approach to polyplex modification has also been used to functionalize CD-containing polyplexes with targeting ligands for cell-specific uptake, including galactose[166], transferrin[167] and insulin[164]. Transferrin-modified, PEGylated CD-based polyplexes containing DNAzyme administered by tail vein injection to tumour-bearing mice are well tolerated, even up to doses of 40 mg DNA per kg mouse, and are specifically internalized by tumour cells[168].

Degradable polycations

Two important barriers — polyplex unpackaging and cytotoxicity — have recently been addressed via synthesis of biodegradable polycations. Several cationic polyesters have been reported[169–172], including poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), a biodegradable mimic of polylysine[171]. PAGA showed no cytotoxicity under conditions in which polylysine reduced cell viability by 80%. In the presence of chloroquine, PAGA transfected cells threefold better than polylysine[171]. Furthermore, PAGA has shown efficacy in delivering plasmid DNA *in vivo*[173]. A similar poly(amino ester) with a hyperbranched structure has also been reported[172]. Transfection efficiency was one to two orders of magnitude less efficient than with PEI or PAMAM and was minimally toxic, but was tenfold better than with PAGA (without chloroquine).

Another class of degradable polymers for gene delivery are the poly(β-amino esters) (FIG. 5f)[174–176]. Initially, a set of three poly(β-amino esters) was shown to be capable of binding and condensing plasmid DNA with negligible cytotoxicity to cells in culture[174]. Subsequently, these authors used a combinatorial approach to synthesize 140 structurally unique poly(β-amino esters)[176]. This library was screened for solubility in aqueous buffer (pH 5.0) and DNA binding. Of the 70 water-soluble polymers 56 interacted sufficiently with DNA to allow transfection studies. Of these, six polymers were identified as 'hits,' and two of the polymers mediated gene delivery with efficiency similar to or exceeding that observed with PEI. Most recently, poly(β-amino esters) have been used as components of genetic vaccines[177] and for the delivery of the SUICIDE GENE diphtheria toxin to experimental tumours in mice[178].

Although the commonly used 25-kDa PEI is toxic to many cell lines, low molecular mass PEI (<2,000 Da) is essentially non-toxic but ineffective for gene delivery. A degradable polymer, initially similar in size to 25-kDa PEI, can be expected to mediate efficient gene delivery but yield relatively non-toxic degradation products. Following this idea, Forrest *et al.* crosslinked 800-Da PEI with small diacrylates to generate poly(β-amino esters)[179]. The degradable polymers were similar in structure, size (14–30 kDa), and DNA-binding properties to commercially available 25-kDa PEI, but mediated gene expression 2–16-fold more efficiently and were essentially non-toxic. One important interpretation of these results is that the degraded polymers can more readily unpackage to release free DNA in the cell.

Another novel approach has been to use disulphide-containing moieties that will be cleaved in a reducing medium such as the cytosol and nucleus. In one such polymer, poly[Lys-(AEDTP)], the lysine ε-amino groups are substituted with 3-(2-aminoethyldithio)-propionyl residues such that the amino groups interacting with the DNA phosphates are linked to the polymer via a disulphide bond[180]. Decondensation of polyplexes was observed in the presence of the reducing agents dithiothreitol and glutathione. Transfection of HepG2 cells was 50-fold more efficient with poly[Lys-(AEDTP)] compared with polylysine. Similarly, Gosselin *et al.* generated a novel vector by crosslinking 800-Da PEI with disulphide linker groups at two different amine/crosslink ratios to generate a set of reducible conjugates[181]. Although none of the polymers was as effective as the commonly used 25-kDa PEI, they were all less toxic.

Conclusions

A variety of polymers have been used in gene-delivery studies, but their effectiveness as gene-therapy vectors remains orders of magnitude poorer than viral vectors. As a result, polymers are generally considered unacceptable for clinical applications. The important extra- and intracellular barriers to efficient gene delivery are known. The lack of efficiency of polymer gene-delivery

SUICIDE GENE
A gene that, once expressed in a target cell, causes death of the cell — for example, by initiating apoptosis or making the cell susceptible to the activity of a prodrug. One example of the latter is herpes simplex thymidine kinase, which activates drugs such as acyclovir and ganciclovir.

vectors, nevertheless, results from a lack of functionality for overcoming at least one of these barriers. On the basis of the large number of studies of off-the-shelf gene-delivery polymers, much has been learned about the structure–function relationships of polymer vectors. This knowledge has been applied to the design and synthesis of new polymers, tailor-made for gene delivery, and a number of promising candidates have been reported in recent years. With a growing understanding of polymer gene-delivery mechanisms, it is likely that polymer-based gene-delivery systems will become an important tool for human gene therapy.

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Competing interests statement
The authors declare no competing financial interests.

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