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Targeted polymers for gene delivery

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Over the past decade, significant research has been done in the area of polymer-mediated gene delivery. Synthesis of new polymers and modifications to existing polymers has resulted in polyplexes with improved *in vitro* and *in vivo* transfection efficiencies. Targeting has been an important aspect of this research, and various strategies for obtaining selective and enhanced gene delivery to the target site have been evaluated. This review covers the different aspects involved in polyplex targeting. Development of targeted polyplexes involves a careful consideration of the target site, the targeting ligand and the physicochemical properties of the polyplex itself. The need to redirect the polyplexes by using the 'shield and target' approach by reducing nonspecific interactions with negatively charged components, while conferring specificity by incorporating targeting ligands, is discussed. Basic chemistry involved in modifying polymers is covered and examples of targeting strategies used for tissue-specific gene delivery are discussed. Targeting is also discussed in the broader context of developing safe and effective polymeric vectors for *in vivo* gene delivery. Maximum benefit of targeting can be obtained when it is used as part of a multi-functional complex containing elements designed to improve gene delivery and reduce overall toxicity of the polyplex.

Keywords: gene delivery, polymers, targeted delivery

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1. Introduction

Gene therapy involves the introduction of a therapeutic gene into the body to overcome the effects of an absent or a defective gene. This approach provides an advantage over the use of conventional small molecules because it allows for the development of therapies that are curative instead of merely palliative. Although it was originally targeted towards treatment of hereditary diseases (e.g., cystic fibrosis, adenosine deaminase deficiency), gene therapy is now also applied for the treatment of acquired diseases (e.g., cancer, AIDS) [1]. Several clinical trials have been approved in this area over the past few years for both inherited disorders and infectious diseases (for details of these studies, see [201]).

Therapeutic genes can be delivered to the site of action in the body via two different approaches. In the *ex vivo* approach, the therapeutic gene is introduced into cells obtained from the patient's body and cultured *in vitro*. These modified cells are then re-introduced into the patient's body [2,3]. The *ex vivo* approach generally results in extremely high gene transfer efficiencies because it is easier to optimise *in vitro* conditions for transfection. However, the need to obtain and culture cells from the patient's body makes this approach tedious and time consuming. Moreover, the invasive nature of this approach makes it inconvenient and, thus, unsuitable for routine clinical use. The second method for gene therapy is the *in vivo* approach. In this case the therapeutic gene (either integrated into viral vectors or complexed with non-viral vectors) is introduced directly into the body. On the one hand, this direct approach provides greater flexibility and is better suited for clinical use [4]; however, it also provides lesser control over the transfection process and adds greater variability in the results obtained.

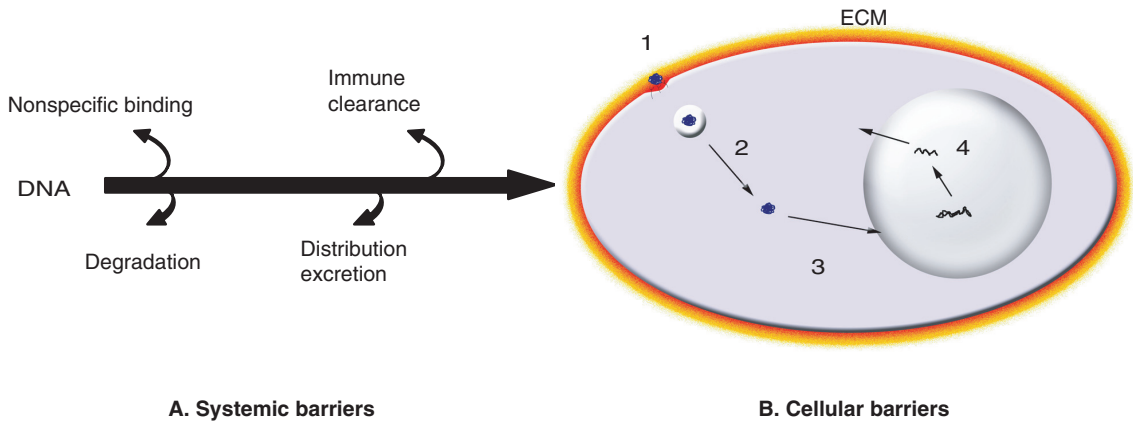

Figure 1. Stages of *in vivo* transfection. A) Systemic – affects the concentration of delivered gene that reaches the target organ or cell. **B**) Cellular – affects the concentration of delivered gene that enters the target cell and reaches the nucleus. In terms of delivery, most nonviral vectors have to undergo three main steps for successful gene expression to occur: (**1**) get internalised into the cell; (**2**) escape from endosome into the cytoplasm; and (**3**) migrate to and across the nuclear membrane. The final stage of transcription and translation (**4**) affects the duration and extent of therapeutic protein production.
ECM: Extracellular matrix.

2. Barriers to gene delivery

From a delivery point of view, the *in vivo* transfection process can be divided into two main stages: i) systemic – involving the *in vivo* biodistribution of the vector, which affects the concentration of therapeutic gene reaching the target cell; and ii) cellular – involving the intracellular disposition of the vector, which affects the concentration of the therapeutic gene reaching the cell nucleus (**Figure 1**). There are several barriers to gene delivery at each of these stages. Systemic barriers include degradation of DNA by plasma nucleases, opsonisation of DNA complexes by negatively charged serum components, uptake by the reticulo-endothelial system, and distribution of DNA to non-target tissue [5]. Cellular barriers include internalisation at the cell surface, endosomal release, cytoplasmic degradation and translocation into the nucleus [6].

3. Vectors for gene delivery

A significant amount of research has been conducted on developing efficient vehicles for delivering nucleic acids into the target cell nucleus. Viral vectors are based on the use of replication-deficient viruses for delivering genes into cells and are constructed from the pro-viral form of the virus [7,8]. Viruses used as gene delivery vectors include retroviruses, adenoviruses, adeno-associated viruses, herpes simplex virus, influenza virus and hepatitis B virus [9,10]. Viral vectors possess the innate ability to gain entry into cells efficiently and thus have high transfection efficiencies. However, concerns about viral infection and immunogenecity still pose a significant hurdle in the clinical use of viral vectors. These concerns have been highlighted by cases of toxicity and mutagenecity observed in patients treated with viral vectors [11,12]. Nonviral or synthetic vectors have long been pursued as a safer alternative to viral vectors. Most nonviral methods are based on the use of plasmid DNA, either used alone or in combination with a synthetic delivery agent. Currently, there are two main classes of nonviral gene delivery agents: one based on the use of cationic lipid or liposomes (lipofection), and the other based on the use of cationic polymers (polyfection). The first reports of using cationic polymers for DNA delivery can be traced back to 1973, much before that of cationic lipid [13]. However, until recently, research in the area of polymer-mediated gene delivery lagged behind that of lipid-based delivery vectors [13]. Recent advances in the development of polymers have regenerated the interest in polymer-mediated gene delivery.

4. Polymer-mediated gene delivery

Various cationic polymers, both naturally occurring and synthetic, have been studied as potential vectors for gene delivery (**Figure 2**). These include proteins such as histones and cationised human serum albumin, polypeptides such as poly-L-lysine (PLL) and poly-L-ornithine, and polyamines such as polyethylenimine (PEI) and starburst polyamidoamine (pAMAM) dendrimers [14-18]. Both linear and branched forms of polymers have been used. Copolymers containing hydrophilic segments (such as polyethylene glycol [PEG] or dextran) have also been synthesised [19-21].

All cationic polymers interact with the anionic phosphate backbone of DNA and condense the plasmid into nanoscale particles [22,23]. The concentrations of polycation and DNA in the polyplex are generally expressed in terms of the N/P

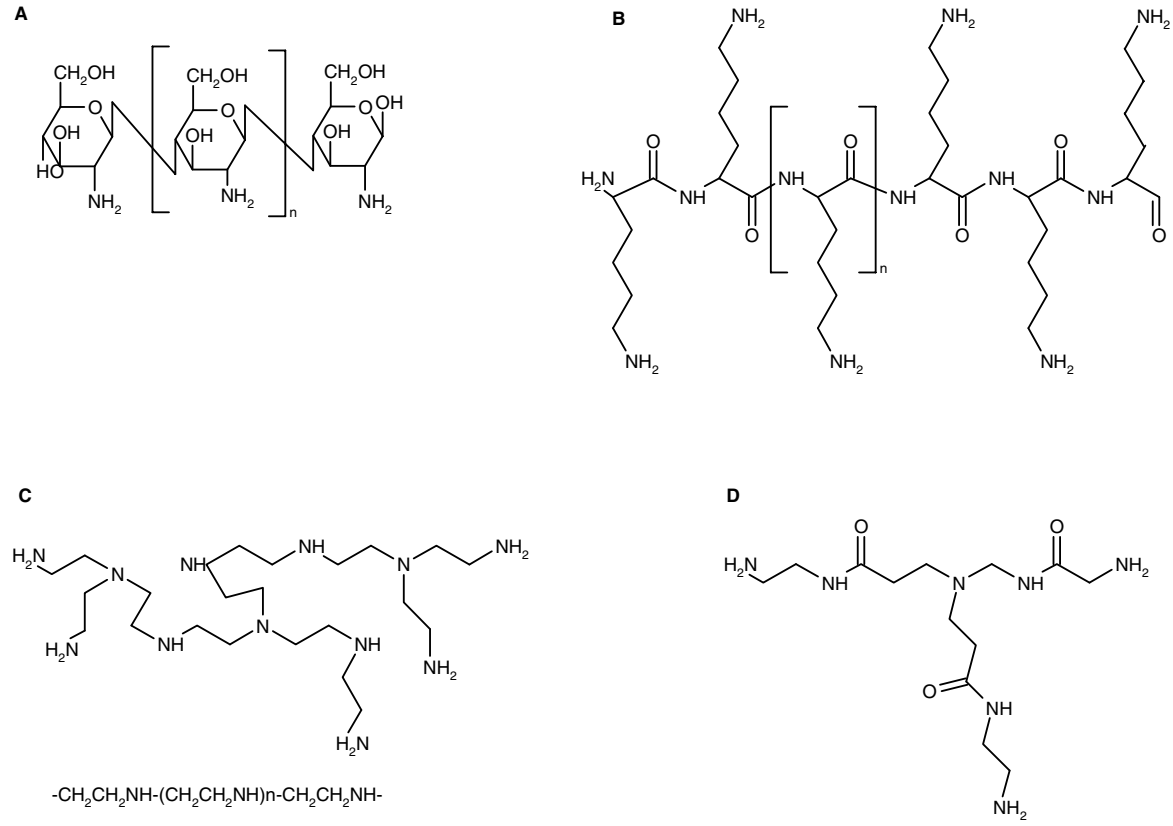

Figure 2. Chemical structures of representative cationic polymers that have been used for gene delivery. A) Chitosan; **B**) poly-L-lysine; **C**) polyethylenimine (linear and branched); **D**) polyamidoamine dendrimers.

ratio: the ratio of polymer nitrogens to DNA phosphates. The N/P ratio required for complete complexation of DNA varies with the polymer used and also with the molecular weight of the polymer. Higher molecular weight polycations are more efficient in compacting DNA and form smaller particles than low-molecular-weight polycations. In most cases, DNA is completely condensed at N/P ratios of 2 – 3 forming polyplexes possessing slightly negative or close to neutral zeta potentials. However, higher N/P ratios (> 5) are required for successful transfections. Polyplexes formed at higher N/P ratios tend to be in the range of 100 – 200 nm and possess an overall positive surface charge (+ 20 – 40 mV). The excess positive charge on polyplexes is hypothesised to promote cell entry via electrostatic interactions with negatively charged heparin sulfate proteoglycan (HSPG) residues on the cell surface [24-26]. Recently, a model based on cell entry using syndecans, a class of transmembrane HSPGs, as receptors has been proposed. Binding of polyplexes to syndecans was observed to result in the clustering of syndecans into cholesterol-rich rafts, which in turn triggered the internalisation of polyplexes via the endocytic pathway [27]. HSPGs are ubiquitously expressed on adherent cells; probably the reason for the wide variety of cells that can be transfected using polycations. However, this advantage turns into a liability *in vivo* where polyplexes interact non-specifically with anionic components. Once endocytosed, the polyplex has to escape from the endosome into the cytoplasm. In case of some polymers, such as polylysine, this step can pose a significant hurdle to efficient transfection because the polyplex is trapped in the endosomes and eventually the DNA gets degraded in the lysosome [28]. Gene expression can be improved by using endosomolytic agents such as chloroquine. Other polymers such as pAMAM dendrimers and PEI can overcome this barrier due to their large buffering capacity over a wide range of pH and the resulting 'proton-sponge effect' [15,29,30]. These polymers are only partially protonated at physiological pH; even after binding to DNA, only one to three nitrogens in PEI are protonated [31]. In the acidic environment of endosomes, the ability of the polymer to capture protons leads to osmotic swelling and endosomal disruption.

5. The 'shield and target' approach

As mentioned above, polyplexes usually possess a net positive surface charge, which facilitates cell uptake. Unfortunately, this excess positive charge can also result in nonspecific interactions of the polyplexes with a variety of negatively charged components *in vivo*. Nonspecific binding of polyplexes to negatively charged cell membranes can lead to uptake into

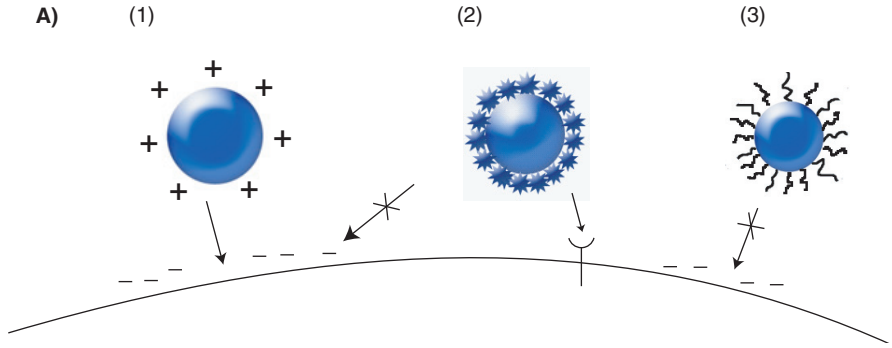

Figure 3A. Methods to shield and target the polyplex for *in vivo* gene delivery. Shielding the nonspecific interactions of the polyplexes (**1**) by ligand (**2**) or shielding polymer (**3**).

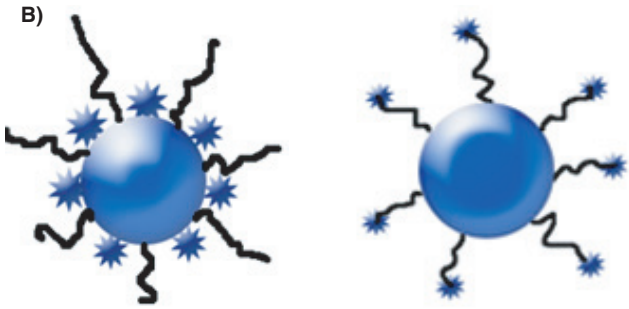

Figure 3B. Methods to shield and target the polyplex for *in vivo* gene delivery. Alternative methods to attach the ligand on the polymer.

non-target cells. Furthermore, binding to negatively charged plasma proteins following *in vivo* administration results in aggregation of the polyplexes. These aggregates are believed to be responsible for the sequestration of polyplexes in the pulmonary capillary bed [32]. Consequently, although *in vivo* delivery of non-targeted polyplexes leads to high levels of gene transfer to the lungs, it also leads to significant toxicity. Binding to serum components also can lead to opsonisation and clearance via the reticulo-endothelial system [33,34]. Furthermore, cationic polyplexes are known to activate the complement system. Thus, there are two aspects of the problem that have to be addressed. First, the polyplex has to be shielded from the nonspecific interactions; and second, a targeting capacity has to be conferred on the polyplexes [35,36]. The most widely used approach to address this problem is to replace the nonspecific electrostatic interactions between cells and the polyplexes with a cell-specific interaction that triggers receptor-mediated endocytosis of the DNA complexes. It is possible to achieve both shielding and targeting by incorporating a high density of targeting ligand into the polyplex (**Figure 3A**). However, this may not be feasible in some cases; for example, steric hindrance caused by the size of the ligand may limit the extent to which the polymer can be modified with the ligand. A more popular option is the incorporation of hydrophilic nonionic polymers such as PEG into the polyplex [37,38]. In general, inclusion of such shielding agents in the polyplex lowers the zeta potential and reduces the nonspecific interactions of the polyplex. However, it must be noted that modification of cationic polymers with shielding agents can have a complex effect on the overall transfection process depending on the type, size and amount of the shielding agent incorporated into the polyplex. Another factor is whether the cationic polymer is modified before or after polyplex formation [31,39,40]. For example, block or graft copolymers of PLL and PEG exhibit lower DNA-binding strengths compared with unmodified PLL [41]. Thus, whereas 'pegylation' may increase stability of polyplexes toward aggregation in serum, the loosely bound DNA could be less resistant to degradation. These contrasting effects make it difficult to predict the effect of shielding agents on the *in vivo* biodistribution of polyplexes. The effect of shielding agents on DNA binding can be partly circumvented by modifying the polymer after polyplex formation [42]. Even in this case, the effect of modification on *in vivo* circulation times is variable and is seen to be dependant on the type and density of shielding agent used [43]. Because the presence of shielding agents can interfere with the interaction of polyplexes with cell membranes, it can lead to reduced cell uptake. This loss of functionality can be regained by introducing cell-specific ligands into the polyplex (**Figure 3B**). When both shielding and targeting ligands are incorporated on the polymer, the targeting ligand can be incorporated directly on the cationic polymer or on the shielding agent. The latter approach may provide an advantage by improving the accessibility of the ligand to the receptor.

6. Ligands used for targeted polyplex delivery

In general, ligands can be grouped into two categories based on the targeting approach used. One approach is to incorporate ligands that allow us to target the polyplexes physically under the influence of an externally applied force. For example, polyplexes can be combined with paramagnetic nanoparticles such as iron oxide and subsequently be

Table 1. Small molecules and synthetic peptides used as targeting ligands.

Ligand/ receptor	Polymeric system	Target organ/cell	Reference
Galactose/ ASGPR	Gal–PLL	Hepatoma	[65]
	Gal–PEI	Hepatoma	[114]
Mannose/ mannose receptor	Mann–PLL	Macrophages	[115]
	Mann–PEI	Antigen-presenting dendritic cells	[116]
Folate/folate receptor	Folate–PEG–folate–PEI	Tumour	[117]
	Folate–PEG–PLL	Tumour	[66]
Synthetic peptides ASGPR	YEE(GalNAcAH)3–PLL	Hepatocyte	[59]
	THALWHT–K_{16}	Airway epithelial cells	[104]

ASGPR: Asialoglycoprotein receptor; GAL: Galactose; PEG: Polyethylene glycol; PEI: Polyethylenimine; PLL: Poly-L-lysine.

controlled by using an external magnetic field [44]. Using this method of 'magnetofection', transfection efficiencies of polyplexes can be enhanced in a number of different cell lines and also in primary cell cultures. Transfection efficiencies of PEI–DNA complexes in human umbilical vein endothelial cells (HUVECs) were observed to be almost 300-fold higher when combined with magnetic particles than those obtained with PEI–DNA alone. Magnetofection has also been used to obtain improved localised gene expression in the ilea of rats and vasculature of rabbit ears [45]. Other studies have used light or heat to obtain target-specific gene delivery and expression [46-48]. Photochemical-induced transfection is based on the use of photosensitisers that localise in endocytic vesicles [49]. Activation with light induces photochemical reactions that lead to the rupture of endosomes and releases the contents into the cytoplasm. Incubation of tumour cells with a photosensitiser, aluminium phthalocyanine disulfonate (AlPcS2a), before transfection with PEI–DNA polyplexes leads to up to 30-fold higher expression of GFP as compared with using PEI–DNA alone [50]. Cell survival was also studied using the herpes simplex virus-thymidine kinase (*HSVtk*) gene along with ganciclovir treatment. Notably, the use of relatively low DNA doses (1 μg/ml) and PEI (N/P ratio = 4) did not have an effect on cell survival unless the photochemical treatment was performed, which increased the cytotoxicity to 90%. This ability to transfect cells at low DNA doses and N/P ratios may be used to obtain targeted gene expression in cells that are exposed to light after administration of low doses of polyplexes. Physical targeting of polyplexes represents a novel approach that holds significant potential. Even so, these approaches are still in their infancy and need to be further developed for *in vivo* applications.

Most targeting approaches are based on the affinity of the ligand to a specific cellular receptor. Receptor-mediated targeting has been attempted using both natural ligands and synthetic derivatives, and includes proteins, peptides, carbohydrates or small molecules [51] (**Tables 1** and **2**). Protein ligands include epidermal growth factor (EGF), fibroblast growth factor and transferrin. Several studies have also used monoclonal antibodies generated toward a particular cell-specific antigen as targeting ligands [55-57]. An alternative approach has been to use synthetic peptides that are designed to mimic the specificity of natural ligands [58-60]. Among small molecules used as ligands, several studies have used galactose-modified polymers to target the asialoglycoprotein receptor, which is selectively expressed on hepatocytes [61-63]. Folate-mediated targeting has been used to selectively transfect rapidly dividing tumour cells, which overexpress the folate receptor [64-68].

7. Attachment of ligands to polymers

The presence of readily accessible amine groups on the cationic polymers provides a convenient method for introducing shielding and targeting ligands. A common approach is to modify the amine using the *N*-hydroxy succinimide (NHS) moiety, which forms amide and imide bonds with primary and secondary amines, respectively. The carboxyl groups of PEG can be coupled to the amine in the presence of NHS and carbodiimide reagents. In many cases, a hetero-bifunctional linker is used to conjugate the ligand to the polymer. One such linker that is widely used is *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), containing an NHS moiety on one end and a 2-pyridyldithiol group on the other end, which is specific toward sulfhydrl groups [69-71]. Use of sulfhydryl groups is especially convenient when conjugating peptides, because this functional group can be easily included during peptide synthesis in the form of cysteine. Alternatively, a sulfhydryl group can be introduced on the polymer by using reagents such as dithiobis(succinimidyl propionate) [72]. Hetero-bifunctional PEG containing an NHS moiety on one end and a sulfhydryl-reactive maleimide or vinyl sulfone group has also been used [73]. As an alternative approach to chemically attaching ligands on the polymer, cationic polypeptides containing the ligand of interest can also be generated biologically by using recombinant DNA technology. Thus, peptides containing targeting domains have been generated in the form of recombinant fusion proteins [75,76].

8. Examples of targeted polymer-mediated gene delivery

Several studies have been conducted using various polymers and targeting ligands for selective gene delivery. These studies have provided valuable insights into the various factors affecting targeted polymer-mediated gene delivery. Some examples are presented here, categorised based on the target

Table 2. Proteins used as targeting ligands.

Ligand/ receptor	Polymeric system	Target organ/cell	Reference
Asialooroso mucoid/ ASGPR	ASOR–PLL	Hepatocyte	[86]
Transferrin/ Tf-R	Tf-PEI–Tf-PEG–PEI	Tumour	[36]
EGF/EGFR	EGF–PLL	Lung cancer cells	[118]
	EGF–PEG–PEI	Tumour	[83]
	EGF–histone fusion protein	Tumour	[84]
Antibody	Anti-HER2–PEI	Tumour	[72]
	Anti-PECAM–PLL	Lung endothelial cells	[97]
	Fab-PEI–anti-OV3 surface antigen	Ovarian cancer	[80]
	Anti-JL1–PLL	Leukaemia specific	[56]

ASGPR: Asialoglycoprotein receptor; ASOR: Asialoorosomucoid; EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; GAL: Galactose; PECAM: Platelet endothelial cell adhesion molecule; PEG: Polyethylene glycol; PEI: Polyethylenimine; PLL: Poly-l-lysine; Tf: Transferrin.

tissue/organ. **Tables 1** and **2** list some of the targeting strategies reported in literature.

8.1 Targeting tumours

There has always been a critical need for targeted drug delivery in cancer therapy [77,78]. Because therapeutic genes are often aimed at inducing cell death and/or inhibiting cell proliferation it is necessary for gene delivery to be highly cell specific. In addition, in many cases long-term expression of anticancer genes is not required; in fact, it is often undesirable. In this regard, the transient gene expression provided by nonviral vectors is especially suited for cancer therapy. Certain accessible solid tumours can be treated with localised gene delivery directly into the tumours. However, systemic administration becomes necessary when treating inaccessible tumours or metastasised cancer cells. The main problem in using polyplexes for tumour therapy is the nonspecific interaction *in vivo*. Shielding with polymers and/or targeting ligands has been used to improve tumour uptake. In addition, the longer circulation times that are obtained by shielding leads to passive targeting of the tumours through the 'enhanced permeation and retention' effect [79]. Antibodies can be designed to be selective toward antigens present on the cancer cell. PEI was modified with PEG and the Fab′ fragment of the OV-TL16 antibody for targeted delivery of luciferase plasmid (pCMV-luc) into ovarian cancer cells [80]. The antibody binds specifically to the OV3 surface antigen, which is expressed on ovarian cancer cells. The Fab′ fragment was chosen instead of the entire antibody because it has a lower molecular weight and, therefore, lower steric hindrance during DNA–polymer complex formation. The Fab–PEI–PEG polyplexes showed increased cell binding than unmodified or pegylated polyplexes in OVCAR cells, which overexpress the OV3 antigen. At lower N/P ratios (2.5 – 3.5), gene expression using the Fab–PEI–PEG polyplexes was selective toward OVCAR cells compared with NIH3T3 cells, which do not express the OV3 antigen; expression levels were also ~ 80-fold higher than with unmodified or PEGylated PEI. Chiu *et al.* used linear PEI (25 kDa) modified with trastuzumab (Herceptin®, Genentech): a HER-2-specific monoclonal antibody [72]. Modification resulted in selectively increased gene expression in HER-2-overexpressing cells (SK-Br-3) compared with that in MDA-MB-231 cells which have low HER-2 expression levels. Expression levels using the modified PEI were around 10-fold higher than those obtained with unmodified PEI and were highest at N/P ratio 10.

Dash *et al.* modified polylysine-based polyplexes with a hydrophilic multivalent polymer, poly-[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA) [81]. The shielding effect of the polymer imparted greater stability to the modified polyplexes toward disruption with bovine serum albumin. Although particle sizes of pHPMA–PLL polyplexes were slightly larger (50 versus 90 nm), they did not increase after incubation with 10% fetal calf serum. In contrast, sizes of unmodified polylysine–DNA complexes increased almost 20-fold after a similar incubation. Even so, modification of PLL resulted in a twofold decrease in cell uptake (in K562 human leukaemia cells) compared with that using unmodified PLL. Notably though, further modifying the pHPMA–PLL polyplexes with transferrin more than regained the loss of function. Cellular uptake with transferrin (Tf)–pHPMA–PLL polyplexes was sixfold higher than that with untargeted pHPMA–PLL polyplexes. Furthermore, transfection efficiency (β-galactosidase) increased 15-fold over both unmodified PLL and untargeted pHPMA–PLL polyplexes. *In vivo* targeted tumour delivery was studied by Ogris *et al.* using PEI-based polyplexes that were modified with transferrin alone, or with polyethylene glycol and transferrin [82,83]. Zeta potentials of the polyplexes decreased in the following order: PEI > Tf–PEI > PEG–PEI > PEG–Tf–PEI. High levels of luciferase expression were obtained in tumours with both Tf–PEI and PEG–Tf–PEI polyplexes after systemic administration. However, pegylated polyplexes resulted in gene expression almost exclusively restricted to the tumour, whereas use of Tf–PEI polyplexes resulted in gene expression in many other organs. Administration of unmodified polyplexes (PEI–DNA) led to expression mainly in the lung and toxicities including death due to lung embolism were observed, especially when higher molecular weight PEI (800 kDa) was used [42]. In another study by the same group, surface-shielded polyplexes were used to treat Neuro2A tumour-bearing mice with a gene expressing tumour necrosis factor-α (TNF-α) [82].

Following systemic administration via the tail vein, significant TNF-α gene expression was observed within the tumour, compared with much lower levels in other tissues. Repeated application of surface-shielded TNF-α complexes resulted in pronounced haemorrhagic tumour necrosis: a hallmark of the antitumour activity of TNF-α. Necrosis was focused specifically to the tumour, resulting in significant inhibition of tumour growth in treated animals as compared with the control groups. No systemic toxicity was observed. Some researchers have attempted to develop multifunctional recombinant fusion proteins as delivery vectors. Dai *et al.* designed a gene delivery carrier based on a recombinant histone ($H1^0$) polypeptide consisting of three functional domains: the DNA-binding histone, an EGF receptor (EGFR) target domain (GE7) and an endosomolytic domain (HA20) derived from the influenza virus [84]. Gene delivery using this polypeptide was compared with other fusion proteins containing only histone, or histone-GE7 and histone-HA20. Gene expression was observed only in cells expressing the EGFR (SK-OV-3 and BEL-742). No expression was observed in EGFR-negative U2OS cells. *In vitro* and *in vivo* transfection efficiencies were highest when GE7–histone–HA20 was used in combination with histone–GE7. Histone alone, or with either GE7 or HA20, did not result in good transfection efficiency. Ziady *et al.* targeted the serine protease inhibitor (serpin) enzyme complex receptor in human hepatoma cells using peptides containing the five amino acid recognition sequence for the receptor [85]. Polylysine was modified with the peptides through an SPDP linker, resulting in polyplexes with varying degrees of linker densities and ligand densities. DNA condensation was performed in the presence of a high salt concentration, resulting in polyplexes of 17 – 20 nm in size. Polymers with the highest degree of modification (26% of lysines in the polymer were modified) failed to either condense DNA or transfect cells. Lower degrees of modification (3.5 and 7.8%) had better transfection efficiencies. Surprisingly, lower ligand densities led to higher levels of peak gene expression. The effect on duration of expression was more complex and depended on whether a high- or low-affinity ligand was used in addition to ligand density. Depending on the peptide ligand used, optimum ligand densities varied from 8 to 40 per complex.

8.2 Targeting the liver

The asialoglycoprotein receptor (ASGPR) is one of the most widely used targets in the development of liver-specific polymeric carriers. This receptor, which is uniquely expressed on hepatocytes, mediates cellular uptake through the specific recognition and endocytocis of ligands with terminal galactose or *N*-acetylgalactosamine residues. The first reported use of this approach was by Wu and Wu in 1988, in which polylysine was modified with an asialoglycoprotein: asialoorosomucoid (ASOR) [86]. Administration of the targeted polyplex via tail vein injection into mice resulted in increased uptake of the polyplexes into the liver and higher liver-specific gene expression (chloramphenicol acetyl transferase gene). Subsequently, several studies have been conducted to better understand the factors affecting the hepatocyte-specific gene expression via the ASPGR [87]. Refinements in this approach have led to the use of galactose- or lactose-modified polymers instead of the initially used ASOR. Nishikawa *et al.* studied the correlation between the physicochemical properties, *in vivo* biodistribution and gene expression mediated via DNA–polylysine–galactose polyplexes [88]. The factors affecting the targeting and expression of plasmid DNA were identified by studying Gal–PLLs with various molecular weights (average molecular weights: 1800, 13,000, 29,000) and varying ligand density (number of galactose residues/molecule of polymer). This study showed that both the molecular weight of the polymer and the ligand density affect the uptake and gene expression. As expected, DNA condensation was more efficient using higher molecular weight polymers. Following *in vivo* administration, uptake into hepatocytes increased proportionally to an increase in the ligand density. However, extensive modification of the polymer hindered its ability to condense DNA, which in turn reduced gene expression. Interestingly, all the polyplexes administered *in vivo* had a slightly negative zeta potential, instead of the excess positive charge that is usually associated with polyplexes. The authors attributed the selective uptake of the polyplexes into hepatic parenchymal cells to this slight electronegativity. Particle sizes of polyplexes were < 200 nm, which is necessary for passage through the fenestrate of the hepatic sinusoids. Larger particles that are excluded from the fenestrae are observed to be cleared by Kupffer cells, whereas smaller particle sizes have been reported to lead to prolonged hepatic gene expression. Perales *et al.* reported the formation of Gal–PLL polyplexes of exceptionally small size (around 20 nm) obtained by the slow addition of the modified polymer to DNA in the presence of high NaCl concentration [89]. *In vivo* administration of these polyplexes (via the caudal vena cava) resulted in prolonged gene expression of Factor IX up to 136 days as observed by western blotting.

More recently, Hashida has reported optimisation studies using branched PEI with different molecular weights (1800, 10,000, 70,000). Similar to the polylysine study reported above, it was found that lower molecular weight polymers were less efficient than their higher molecular weight counterparts [90]. Higher molecular weight polymers with greater ligand densities were required for successful gene transfer *in vivo* (Gal_{153}–$PEI_{70,000}$) compared with *in vitro* (Gal_{19}–$PEI_{10,000}$). *In vivo* gene expression was observed after portal vein injection, but not after tail vein injection. This was attributed to nonspecific interactions with anionic components *in vivo*; an indication that incorporation of targeting ligands may not be sufficient to effectively shield PEI–DNA polyplexes.

In another study, Collard and colleagues investigated the use of low-molecular-weight carriers based on derivatives of a synthetic peptide containing a polylysine chain, CWK_{18} [91]. Two derivatives, one containing a targeting ligand

(triantennary *N*-glycan with terminal galactose residues; Tri–CWK_{18}) and the other containing PEG (PEG–CWK_{18}), were studied. Complex formation of DNA with Tri–CWK_{18} alone was not sufficient to mediate liver-specific uptake even with a high density of targeting ligand present. Optimal targeting to hepatocytes was obtained only when the glycopeptide was combined with PEG–CWK_{18}. Moreover, it was necessary to crosslink the two peptides with glutaraldehyde in order to obtain gene expression. Using this formulation 60% of the administered dose could be targeted to the liver with most liver-targeted DNA localised to hepatocytes. Gene expression (human α_1-antitrypsin) was detected in plasma using enzyme-linked immunosorbent assay; levels increased above baseline values 5 – 9 days after intravenous administration of the crosslinked Tri–CWK_{18}/PEG–CWK_{18} DNA condensates. Removal of the terminal galactose residues from Tri–CWK_{18} resulted in no gene expression in the liver. Some studies have shown increased gene expression when targeted polylysine carriers were conjugated with fusogenic peptides. Nishikawa and colleagues report the use of a polyornithine-based carrier modified with galactose and a fusogenic peptide, mHA2 [92]. The function of the peptide is to destabilise cellular membranes and enhance endosomal escape of the polyplexes. They report an increased uptake of polyplexes into parenchymal cells of the liver compared with nonparenchymal cells. Luciferase gene expression also increased 300-fold compared with polyplexes that lacked the fusogenic peptide.

8.3 Lung delivery

Polyplex aggregates formed following *in vivo* administration can become entrapped in the pulmonary endothelium. This process of passive targeting can lead to significant levels of transfection in the lung. In addition, lung can be directly targeted using aerosol-mediated delivery. Indeed, several papers have reported successful lung transfection by using plain polycation–DNA complexes both via systemic injections and aerosols [93-95]. However, the high, localised concentration of polyplexes can also lead to severe toxicities. The 'shield and target' approach can help in obtaining safer vectors for lung delivery [20,83,96]. Successful transfection of pulmonary epithelium was obtained by using linear PEI modified with the antiplatelet endothelial cell adhesion molecule (PECAM) antibody [97]. PECAM is expressed in high levels on endothelial cells and has been used successfully for pulmonary targeting. Modification of the polymer with the antibody lowered the zeta potential of polyplexes from around +25 mV to around +6 mV at an N/P ratio of 6, and increased *in vitro* transfection efficiencies in lung endothelial cells. Following tail vein injection, increased lung uptake and higher gene expression (luciferase) was obtained with Ab-PEI–DNA complexes compared with unmodified polyplexes. In another study, pegylation of PEI-based polyplexes increased lung-specific gene expression following tail vein injection [20]. However, on intratracheal delivery, transfection efficiencies using pegylated polyplexes were significantly lower than unmodified polyplexes. Inclusion of transferrin in the pegylated polyplexes resulted in slightly higher expression, but was still lower than PEI–DNA alone.

8.4 Brain delivery

Delivery into the brain can be achieved either via systemic delivery or by localised injection into the brain parenchyma. The blood–brain barrier continues to be the main hurdle for systemic brain delivery. Systemic targeting has been attempted by using ligands that promote transcytocis across the brain capillary endothelium. Success has been limited and variable. Delivery strategies have also been designed to target each cell type in the brain. The ability of the cholera toxin b-chain (CTb) to improve neuronal transfection efficiencies was studied using polylysine-based polyplexes (K_{100}). CTb binds to ganglioside GM1, which is expressed at high levels on the neuronal surface [98]. Gene expression levels (pCMV-LacZ) in undifferentiated PC12 cells increased 30-fold when CTb-K_{100} was used. Transfection efficiency decreased when performed using differentiated PC12 cells, but still was higher than that with unmodified polylysine. Similar studies have been performed using the C fragment of the tetanus toxin [99]. In another study, neurotensin was used as a targeting ligand for neuron-specific gene transfer [100]. Neurotensin-modified polylysine was able to selectively transfect N1E-115 and HT-29 cell lines that expressed the neurotensin receptor.

9. Expert opinion and conclusion

The concept of gene therapy is extremely powerful because it provides us an opportunity to develop a cure rather than merely treat symptoms. Unfortunately, it has been difficult to fully harness the enormous potential of this approach. Part of the problem is the lack of safe and effective methods to deliver exogenous genes to the site of action. At the core of any therapeutic approach lies the issue of efficacy and safety. On the one hand, viral vectors provide fairly high and reproducible transfection efficiencies. Paradoxically, the same vectors also result in severe short- and long-term toxicities that undermine their positive attributes. Nonviral vectors, on the other hand, have lower toxicities, but are less efficient in delivering genes *in vivo*. In the case of *in vivo* gene therapy the extent and spread of gene expression is primarily governed by the pharmacokinetic and tissue distribution profiles of the gene delivery vector. Distribution of the administered DNA to nontarget tissue may not only reduce the efficacy of therapeutic effect, but can also pose a risk because nonspecific, unregulated gene expression may lead to undesirable side effects. Thus, targeting becomes a major component in developing safer and efficacious gene delivery approaches.

It is apparent from the studies presented in this review that developing a successful targeting approach depends on a myriad of factors. Obviously, it will require a coordinated effort from various disciplines of research. In this regard, three main areas of focus can be identified: gaining a comprehensive

understanding of the target site; improving the design of targeting ligands; and developing safer, more effective polymers for gene delivery. The targeting strategy will have to be adapted to suit the organ or disease target. A better understanding of the target site, in anatomical, physiological and molecular terms, is crucial. Not only will this help understand how the microenvironment of the target site affects transfection efficiency, but it may also help in identifying newer, more specific cell-surface targets [101]. Receptor density and availability, recycling and internalisation processes need to be well understood. There are some reports indicating that receptor turnover rate can be affected by the affinity of the targeting ligand [102,103]. Development of better targeting ligands is obviously critical. Recently, several high-affinity peptide ligands have been identified using phage libraries [58,104,105]. These ligands have several advantages over the corresponding naturally occurring protein ligands. Their smaller size improves accessibility to the target site; they are easier to manipulate and to use in modification reactions; and in some cases they do not possess the non-essential biological properties of their corresponding naturally occurring ligands.

Finally, targeting cannot be viewed in isolation from the other aspects of polymer-mediated gene delivery. The goal of nonviral gene delivery has always been to find the middle path and obtain virus-like efficiencies without the associated disadvantages. The flexibility offered by polymeric systems has made them attractive templates for the proposed development of the so-called 'artificial virus' [106,107]. In general, an ideal vector would be designed to maximise the efficiency at each stage of the transfection process. At the systemic level, shielding agents and targeting ligands can improve gene delivery to the target site. At the cellular level, cell-specific targeting ligands can improve specificity. Cell-penetrating peptides, which have been observed to facilitate efficient transfer of macromolecules into the cell cytoplasm, can be incorporated into the polyplex [108,109]. Inclusion of fusogenic peptides can improve endosomal transfer. Furthermore, nuclear uptake could be enhanced by incorporating a nuclear localisation signal [110]. Finally, an additional layer of targeting can be achieved by using tissue/cell-specific gene promoters [111]. Combining targeting at both the cellular level and the transcription level will allow us to obtain precise selectivity of gene expression. In addition, inclusion of biodegradable bonds in the polyplex, such as the disulfide or ester bond, could improve transfection and reduce the toxicity of the polymer; for example, biodegradable polymers have been synthesised by crosslinking low-molecular-weight polycations (such as 800 Da PEI) via reversible disulfide bonds [112,113]. These polymers exhibit lower toxicities than their high-molecular-weight counterparts. Modification of polymers with shielding agents may interfere with intracellular steps such as endosomal release and thereby reduce transfection efficiencies. In order to overcome this problem, the vectors can be designed such that the polymer is modified through bonds that are cleaved once they the polyplex reaches the target cell. Similar 'modular' approaches can be used to develop a vector that dynamically adapts to the environment: similar in part to viruses.

Theoretically, the ideal vector described above is easy to envision and certainly is an attractive goal. Many of these approaches (either alone or in combination) have been studied and in some cases successfully utilised to improve transfection efficiencies. However, there could be significant practical hurdles to develop a vector that contains all the aforementioned characteristics. One problem is chemistry. Although it is relatively easy to modify polymers with a variety of ligands, there are still only a limited variety of functional groups to choose from. As the number of modifications increases, the chemistry involved may get increasingly complicated and difficult to control. Inclusion of ligands through labile bonds could further complicate the issue. Furthermore, it is not always easy to characterise the final derivatised polymer. This is particularly the case when poorly characterised, off-the-shelf polymers are used. In this regard, the use of better-defined low-molecular-weight polymers could be helpful. In addition, the effects of modifying the polymer can be varied and complex. Thus, it is necessary to determine the effect of incorporating ligands on the physicochemical characteristics of the polyplex. The type of ligand used and the degree of modification need to be optimised to ensure that modification of the polymer does not negatively affect its basic function. In light of the above discussion, it is important to remember the final goal is the development of an effective and safe gene delivery system that is targeted toward specific disease states. The gene delivery approach and the complexity of the vector needed may vary depending on the disease target.

In the final analysis, research in the area of targeted polymers is constantly progressing. A proper understanding of the different aspects affecting the success of the delivery will allow us to obtain safer and more effective gene delivery systems.

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