Expert Opinion

Polymeric nanoparticles for gene delivery

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Since the evolution of the concept of gene therapy, delivering therapeutic genes to the diseased cells has been a major challenge. Although viral vectors have been shown to be efficient in delivering genes, the issue of their safety is still to be solved. Meanwhile, the field of developing nonviral expression vectors has seen considerable progress. As compared with viruses, these are relatively safe but are confronted with the problem of poor transfection efficiency. With the growing understanding of the biology of gene transfection, and the continued efforts at enhancing the efficiency of nonviral expression vectors, it could soon become a preferred option for human gene therapy. In this review, the potential of polymeric nanoparticles as a gene expression vector is discussed. Furthermore, the importance of understanding the pathophysiology of disease conditions in developing gene expression vectors is discussed in Section 6.

Keywords: conjugates, gene delivery, gene expression, gene therapy, polymers, targeting

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1. Introduction

The unfurling of the human genome has opened new opportunities for the field of medicine in the form of gene therapy. Gene therapy is an approach of treating disease by either replacing a defective gene or modifying the expression of a gene by introducing genetic material/DNA into the cells. Therapeutic prospects of this approach not only include the genetic disorders (such as cystic fibrosis and haemophilia) but also many somatic diseases, such as slowing the progression of tumours, stopping neurodegenerative diseases and fighting severe viral infections such as AIDS. The therapeutic components of gene therapy include plasmid DNA, antisense oligonucleotides (AS-ODNs) and small interfering RNA. The first successful attempt of gene therapy on humans was performed in 1990 and since then ~ 900 clinical trials for gene therapy have been approved [201]. So far, Vitravene™ (Isis Pharmaceuticals, Inc.), an AS-ODN that is used for treating a condition called cytomegalovirus retinitis in AIDS patients, is the only genetic product that has been approved in the US. In this case, a bicarbonate buffered solution of the oligonucleolotide (ODN) is given as a localised intravitreal injection. Recently, Gendicine (Shenzhen SiBiono GeneTech Co., Ltd), an adenoviral-based gene therapy product, has been approved by the Chinese State FDA for the treatment of head and neck squamous cell carcinoma. It is a replication incompetent, recombinant human adenovirus that is engineered to contain the human p53 tumour suppressor gene.

Despite the considerable interest that is generated in gene therapy and the phenomenal pace at which research has advanced, delivery of genes to the target cells remains the most formidable challenge. Polynucleotide molecules (e.g., DNA or RNA) are large, hydrophilic macromolecules with a net negative charge. These are very labile in the biological environment and do not cross biological membranes effectively. The need for an effective and safe gene delivery system is thus quite obvious. Among other reasons, the lack of efficient gene delivery systems is the major

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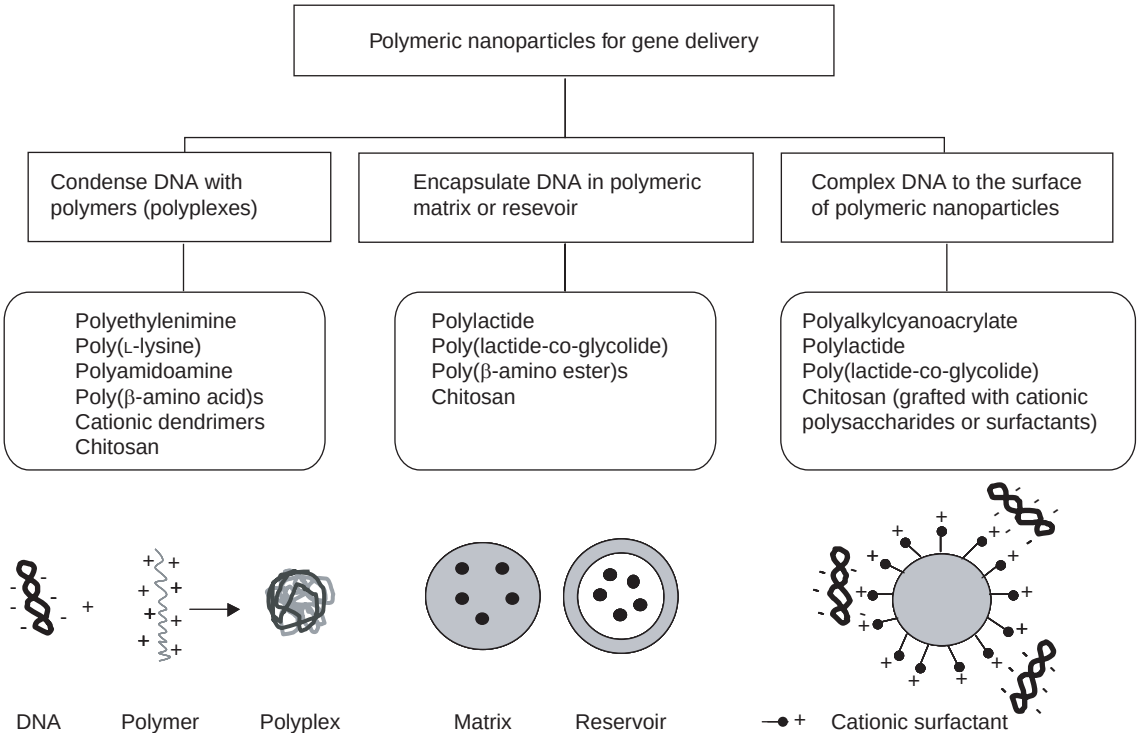

Figure 1. A schematic showing the different types of polymeric nanoparticles for gene delivery.

impediment in the development of gene therapy products. Crucial to the success of gene therapy modalities is the efficiency of gene expression vectors (viruses, liposomes or nanoparticles (NPs) used to deliver plasmid DNA). In general, gene expression vectors can be broadly categorised into the viral and non-viral vectors. Viral vectors include the use of genetically engineered retroviruses, adenoviruses, adeno-associated viruses and other viruses that have been used for gene transfer procedures. Safety concerns with viral vectors were raised by the death of Jesse Gelsinger, an 18-year-old patient who volunteered for adenovirus-based gene therapy in 1999. After his treatment with 3.8×10^{13} virus particles, which is believed to be the highest dose tried in humans, Jesse died from acute respiratory failure and multiple organ collapse 4 days after the treatment [1]. In 2002, two young boys who were enrolled into a gene therapy trial for the treatment of severe combined immunodeficiency developed a form of leukaemia [2]. The retroviral vector that was used in this particular trial is known to cause insertional mutagenesis and was responsible for the development of leukaemia in these patients [3]. Although these two cases sparked a big debate over the safety of viral vectors for gene therapy, they brought forward the importance of stringency of safety assessments, patient monitoring and reporting adverse events in gene therapy trials.

Nevertheless, for any gene delivery approach, its safety is the main reason for selecting protocols for *in vivo* studies. A major part (63%) of clinical trials in gene therapy is in Phase I where the safety of gene transfer protocol, including the choice of gene delivery vector, is tested. Only 2% of the protocols have been successful to continue onto Phase III studies [201]. Thus, the current consensus is to develop suitable vector systems that are minimally invasive (safe) and highly efficient for gene therapy in humans. This has steered research towards the development of non-viral vectors for gene delivery. These include the NPs that are prepared using cationic lipids and polymers as well as mechanical methods such as electroporation. Although these vectors are relatively safer as compared with viral vectors, so far the main hurdle has been the inability to achieve efficient gene delivery to the target cells and to obtain sustained gene expression for therapeutic effect. The purpose of this article is to review the potential of non-viral polymeric NPs for gene delivery applications (Figure 1). For the purpose of this review, NPs are those polymeric systems that, either due to condensation with DNA or its encapsulation, form particles that are in the nanometer range.

2. Gene delivery systems/vectors

Viruses have the inherent ability to infect host cells and thus offer an efficient mechanism of introducing foreign genes into cells. It has been shown that the binding of a single virus to the cell membrane is sufficient to infect the cell [4]. However, this high efficiency of gene transfection is counter balanced by the immunogenic and pathogenic response of the body to viral

vectors. Furthermore, these vectors are limited by the size of the genetic material they can carry. The capability of the gene delivery vector to carry large transgene constructs is essential for the delivery of a gene with its associated regulatory elements. An adenovirus can hold up to 7.5 kb [5], whereas an adeno-associated virus can carry only 2.5 – 4.5 kb of genetic material [6]. The high costs that are involved in the production and packaging of viral particles and the strict regulatory guidelines have diverted a lot of attention towards developing non-viral vectors for gene delivery. The non-viral vectors are relatively less expensive, easier to make and are non-immunogenic and relatively safe for administration to humans. In view of the fact that, unlike viral vectors, these vectors do not intrinsically possess properties for delivering genes to the nucleus, their gene transfection efficiency is limited (Figure 2). This has heralded highly exciting developments in the field of polymer science and has also given rise to a new genre of gene delivery vectors, which are hybrids of viral and non-viral vectors. The incorporation of viral capsid proteins or fusogenic peptides to liposomes is one such attempt [7].

At this juncture, it is important to mention that the efficiency of gene transfection depends on the adeptness of gene delivery and that of gene expression in the target cells. Higher gene expression can be achieved by the use of strong promoters and enhancers, whereas efforts at increasing the efficiency of gene delivery are still in their infancy. The capability of gene delivery is usually expressed in terms of the percentage of cells that are transfected on exposure to the gene delivery vector and the fraction of the administered dose of DNA that reaches the nucleus of the cells. Furthermore, the efficiency of expression must consider the levels and the duration of gene expression. Recently, efforts at improving the competence of gene transfection of the non-viral polymeric vectors are gaining high impetus. This makes it all the more important to understand the biological aspects of gene transfer and expression for successful development of such strategies.

3. Barriers to gene delivery

To be successful, gene delivery vectors need to overcome several physical and biological barriers before the gene can be delivered to its target site; the nucleus of a cell. Physical barriers include the formulation of polymeric gene delivery systems, which ideally should be able to condense DNA to a size that can easily gain access into cells and can maintain the stability and biological activity of DNA. The physicochemical characteristics of vectors have a major influence on their biodistribution and pharmacokinetics and thus affect the therapeutic efficacy [8]. Local gene delivery either by direct injection into the tissue, intra-arterial injections for the liver, direct instillation of gene-carrying vectors in the lungs, or intratumoural injections has the advantage of preventing unwanted exposure of gene delivery vectors to the systemic circulation. This also circumvents the interaction of vectors with the blood components and prevents opsonisation of the vectors by the reticulo-endothelial system (RES). However, such localised gene delivery requires that the polymeric NPs should be capable to diffuse within the tissue. Systemic administration of NPs is most advantageous for reaching the disseminated target tissues throughout the organism (e.g., in case of tumour metastasis) or tissues that are inaccessible for direct injections of NPs. For systemically administered NPs, the first and foremost barrier is to cross the vascular endothelium and the basement membrane to reach the target tissues. In addition, biodistribution of NPs can be altered via opsonisation by plasma proteins and their subsequent clearance by the RES. For efficient delivery of genes to the target tissues, it becomes imperative to avoid opsonisation of NPs and also to impart long-circulating properties to the NPs [9]. Protecting the surface of NPs with hydrophilic polymers such as PEG can prevent rapid opsonisation of NPs, subsequent to their intravenous administration. The surface of polymeric NPs can also be decorated with some tissue-specific ligands to facilitate active targeting of NPs to the tissue of interest. Thus, it is important to understand a correlation between NP characteristics and the pharmacokinetics of their biodistribution to develop an efficient targeted gene expression system.

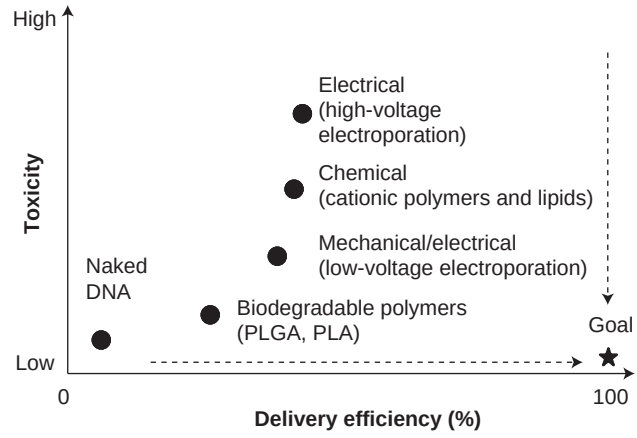

Figure 2. Comparison of delivery efficiency versus toxicity for various DNA transfection methods. Goal is to achieve higher transfection efficiency with minimal toxicity. Reprinted with permission from Macmillan Publishers Ltd: LUO D, SALTZMAN WM: Synthetic DNA delivery systems. *Nat. Biotechnol.* (2000) **18**(1):33-37 [107]. Copyright (2000).
PLA: Polylactide; PLGA: Poly(lactide-co-glycolide).

Furthermore, following introduction *in vivo*, gene delivery vectors are required to cross a series of hurdles in order to reach the nucleus. They lose a significant portion of the DNA molecules at each successive step (Figure 3). These barriers include the physical and chemical stability of the gene delivery vector in the systemic circulation or the extracellular space; association and internalisation of the vectors into cells by endocytosis; intracellular trafficking and release of DNA or DNA vector into the cytoplasm; cytoplasmic translocation of DNA or DNA vector to the nucleus; and the nuclear uptake of DNA [10].

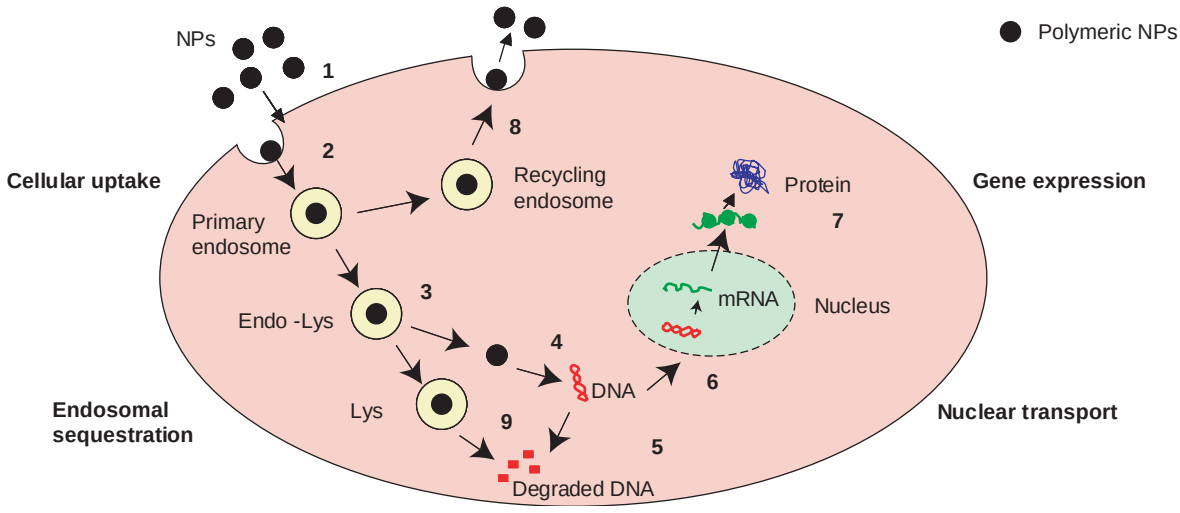

Figure 3. Schematic drawing of steps involved in gene delivery using polymeric NPs. 1) Cellular association of NPs; 2) internalisation of NPs into the cells by endocytosis; 3) endosomal escape of NPs; 4) release of DNA in the cytoplasm; 5) cytosolic transport of DNA; 6) nuclear uptake of DNA; 7) expression of the gene; 8) exocytosis of NPs; 9) degradation of DNA either in Lys or in the cytoplasm. Major barriers include: cellular uptake of NPs, endosomal sequestration of NPs, nuclear transport of DNA/NPs and low levels of gene expression.
Lys: Lysosomes; NP: Nanoparticle.

The efficiency of vectors for cytoplasmic delivery of DNA depends on their association with cell membranes and the endosomal release of vectors [11]. Gene delivery vectors associate with the negatively charged cell surface and are internalised into cells by means of endocytic mechanisms. Cell recognition and association of NPs to the cell membrane can be enhanced by the use of targeting ligands, which can bind to specific receptors on cell membranes. This can promote not only the association and binding of NPs to the cell surface, but can also increase the cellular internalisation by means of receptor-mediated endocytosis. The major bottleneck for polymeric gene delivery is that, following endocytosis, the polymeric vectors may get sequestered within the endosomal compartment. Viruses have evolved highly efficient inherent mechanisms for escaping the endosomal compartments and reaching the cytoplasm. These include the presence of fusogenic or membrane-disruptive peptides in the viral coats, which allow the viral particles to readily escape into the cytoplasm. However, transfection efficiency of the non-viral vectors is highly dependent on the efficiency of endosomal escape of the vectors. This has galvanised active research to develop strategies to enhance the endosomal escape of non-viral polymeric vectors, in order to improve the efficiency of gene transfection.

4. Polymeric nanoparticles

The earliest chemical methods for DNA delivery dates back to the 1950s. The concept that was used to enhance DNA entry into the cells was to complex negatively charged DNA molecules with polycationic proteins in the presence of high salt concentrations. Since then, the increased understanding of the molecular mechanisms of gene transfer in cells has increased the enthusiasm of polymer scientists to synthesise potentially versatile, custom-made polymers that can deliver genes. This has led to rational designing of more sophisticated polymers with added functionalities to help condense DNA and overcome the intracellular barriers for efficient gene delivery to the cells.

The ideal desired characteristics for a non-viral polymeric delivery system for gene therapy include the following:

- the polymer must condense/package DNA into a size that is small enough to gain access into the cell;
- the polymer must be capable of stabilising DNA during formulation and should be stable after *in vivo* administration;
- the polymer must possess some functional elements to facilitate its escape from the endosomes into the cytoplasm
- the polymer should unpackage DNA inside the cell in its active form; and
- the presence of targeting elements to target particular cell types for gene transfection and nuclear localisation of DNA or its vector.

Polymeric gene delivery vectors must maintain their physical stability in the presence of serum proteins and high ionic strength, and also protect DNA from nucleases that are present in the extracellular spaces. Advances in polymer chemistry have led to the development of polymeric NPs wherein the polymer can condense and protect DNA or package DNA without condensing it. Polymeric NPs for gene delivery can be formed by simple condensation of DNA

with polymers such as poly(L-lysine) (PLL), polyethylenimine (PEI), polyamidoamines, polyimidazoles; or by encapsulating DNA into polymers such as poly(ethylene oxide), polylactide, poly(lactide-co-glycolide), polyalkylcyanoacrylates; by complexing DNA to the surface of preformed polymeric NPs grafted with cationic surfactants or polysaccharides. These are the three basic types of polymeric NPs that are under investigation for gene delivery (Figure 1). The NPs that are formed either by condensation, encapsulation or complexation of DNA have very distinct characteristics and varying transfection efficiencies, making them suitable for different gene delivery applications. These differences primarily arise due to the disparity in the basic chemical structure of the polymers and the methods used to formulate NPs. Herein, some of the main polymeric nanoparticulate systems, which are under extensive investigation as gene expression vectors, are reviewed.

4.1 Condensation of DNA with polymers

4.1.1 Polyethylenimine

Since 1995, when the first report identifying PEI as a vector for gene delivery appeared, this polymer has been extensively used in gene transfection protocols and has been hailed as the most versatile gene delivery vector [12]. PEI is a cationic polymer, principally available in two forms: linear and branched, with different molecular weights. Every third atom in PEI is an amino nitrogen, which can be protonated over almost the entire pH range, thus offering a considerable buffering capacity to PEI [13]. Electrostatic interaction between these positively charged amino groups (N) in PEI and the negatively charged phosphates (P) in DNA molecules allow condensation of DNA, resulting in the formation of complexes called polyplexes. The N/P ratio, PEI molecular weights and the salt concentration during formulation can strongly influence the DNA condensation process and the diameter of the polyplexes that are formed. Condensation of DNA with PEI can be initiated at a N/P ratio of 0.5 in the presence of low concentrations of salt, where individual DNA strands are packed together in a core structure and the uncondensed DNA loops surround the condensed core [14]. Polyplexes (~ 50 nm) are formed at high charge ratios (N/P > 6.0), whereas aggregation of complexes is observed at lower N/P ratios and high salt concentration [15,16]. Such cationic polyplexes that form between PEI and DNA allow efficient gene transfection. PEI with a molecular weight of > 70,000 Da has a higher transfection efficiency than the low molecular weight PEI (Figure 4), as the larger PEI molecules may afford better entry of the polyplexes into the cells and offer more protection to the plasmids that they carry [17]. PEI is one of the most investigated polymers among the non-viral vectors that have been tested for gene transfection efficiency *in vitro* [12].

PEI polyplexes that are used for gene transfection are usually positively charged and thus interact favourably with negatively charged cell membranes; therefore are subsequently internalised into cells by means of adsorptive endocytosis [18].

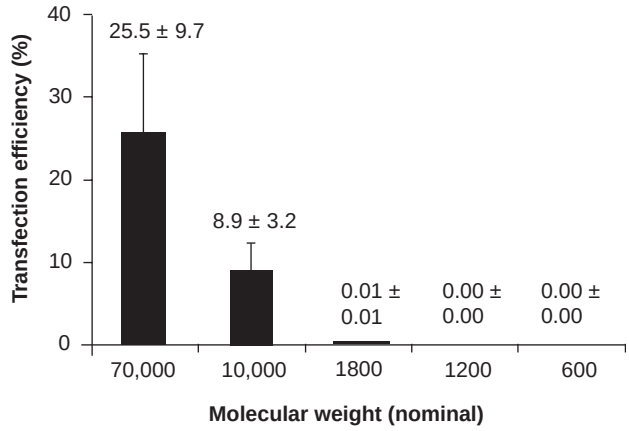

Figure 4. Importance of the molecular weight of cationic polymers for effective condensation of DNA that is required for efficient gene transfection. Transfection efficiencies of five molecular weights of polyethylenimine, pH 7.0, 3 days post-transfection. The cell line EA.hy926 was transfected with polyethylenimine supplied by Polysciences to produce these results. Error bars represent plus one standard deviation ($n \geq 4$). Reprinted from GODBEY WT, WU KK, MIKOS AG: Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* (1999) **45**(3):268-275 [17]. Copyright (1999), with permission from Elsevier.

Different ligands can also be conjugated to PEI to increase the internalisation of polyplexes into cells. Transferrin that is coupled to PEI polyplexes has been shown to improve cellular internalisation in transferrin receptor-expressing tumour cells [19]. The higher efficiency of gene transfection that is afforded by PEI polyplexes is partly because of its high buffering capacity at acidic pH. This led to the hypothesis of the 'proton sponge effect'; the buffering of the acidic pH in the endosome by PEI causes proton accumulation and the subsequent influx of chloride ions into the vesicle. Furthermore, osmotic swelling by the influx of water leads to the rupture of the endosomal or lysosomal membrane, releasing the polyplexes to the cytoplasm of the cell. The finding that bafilomycin A1, a potent inhibitor of endosomal acidification, can selectively inhibit transgene expression with PEI polyplexes and not with cationic lipids, confirmed the proton sponge hypothesis for the mechanism of gene transfection with PEI [20]. However, additional mechanisms proposing the rupture of lysosomes due to direct interaction of PEI with the membrane have also been suggested. Furthermore, this protonation of PEI in endosomes strengthens the electrostatic interactions with DNA and prevents dissociation of DNA from PEI. Nuclear import of PEI–DNA polyplexes usually occurs during cell division and, thus, gene expression depends on the stage of the cell cycle for transfection of the cells [21]. It has been shown that gene transfection using polyplexes made with linear PEI is less dependent on the cell cycle compared with branched PEI. In fact, it has been shown that intact

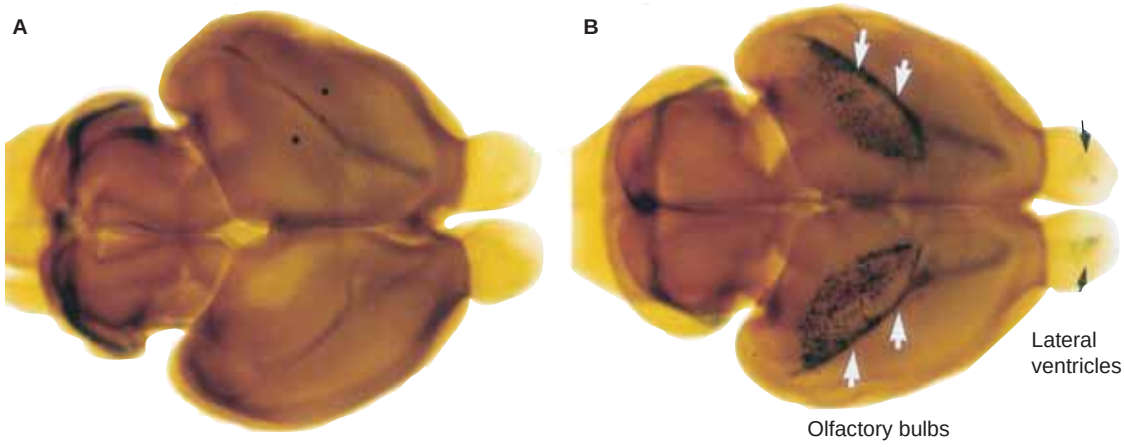

Figure 5. Importance of formulation conditions to achieve efficient gene transfer *in vivo*. Expression of the *lacZ* gene following a single intraventricular injection in the newborn mouse brain. A reporter plasmid DNA encoding for β-galactosidase construct used at 0.5 mg/ml in 5% glucose was complexed with 22-kDa polyethylenimine (amino group/phosphate ratio = 6). A total of 2 ml of the solution was injected into the lateral ventricle of newborn mice that were anaesthetised by hypothermia on ice. The animals were killed 24 h later. Dissected brains were fixed, treated as whole mounts for X-gal staining and clarification. Compared with the controls (**A**), transgene expression is found throughout the brain from the lateral ventricles to the olfactory bulbs (**B**). Reprinted from GOULA D, REMY JS, ERBACHER P *et al.*: Size, diffusibility and transfection performance of linear polyethylenimine/DNA complexes in the mouse central nervous system. *Gene Ther.* (1998) **5**(5):712-717 [27]. Copyright (1998), with permission from Macmillan Publishers Ltd.

PEI–DNA polyplexes that are made with linear PEI can actually enter the nucleus of the cell [16]. However, one of the major shortcomings of PEI is its cytotoxicity. Despite this, modifications of PEI, including PEG–PEI [22], and biodegradable PEI [23] continue to be often used as polymers for gene transfer, as the cells that remain viable after transfection with PEI express relatively high levels of protein.

PEI polyplexes have been used for *in vivo* delivery of therapeutic DNA, ODNs or therapeutic RNA molecules as part of experimental gene therapy. For the delivery of plasmid DNA, PEI of any molecular weight can be used, whereas small nucleic acids such as RNA molecules are preferentially complexed with low molecular weight PEI [24]. Systemic administration of PEI polyplexes with tumour suppressor gene *p53* every 3 days for 3 weeks in an orthotopic bladder cancer model resulted in a 70% reduction in tumour size. A 14-fold higher reporter gene expression was observed in the tumour as compared with the lungs on intravenous injection of the respective polyplexes [25]. Moreover, the toxicity that was observed was minimal as compared with other reports with PEI polyplexes. The authors attributed the reason for preferential accumulation in tumours (as compared with lungs) and the low cytotoxicity to the use of low N/P ratio for polyplexes. This results in marginally positive zeta potential of polyplexes as compared with the strong positive charge at higher N/P ratios, and can potentially reduce the interaction of polyplexes with the serum proteins, thus making it ideal for *in vivo* systemic delivery to the tumour.

Linear PEI of molecular weight 22 kDa was used for successful *in vivo* transfer of the cloned somatostatin receptor subtype 2 (*sst 2*) gene in transplantable models of primary and metastatic pancreatic carcinomas in hamsters [26]. The peptide somatostatin negatively regulates cellular proliferation by means of its cell-surface receptor subtypes (sst1, sst2 and sst5). It has also been observed that there is a loss of *sst2* gene expression in human pancreatic adenocarcinoma and in pancreatic cancer-derived cell lines. Thus, correcting the deficit of the *sst2* gene in pancreatic cancer cells can slow the tumour growth and can make the tumours responsive to somatostatin treatment. A plasmid DNA containing the murine *sst2* gene was complexed with linear PEI at a N/P ratio of 10:1. PEI–DNA complexes were injected intratumourally into the pancreatic tumour model in hamsters. This *in vivo sst2* gene transfer resulted in significant inhibition of growth progression for both pancreatic primary tumour and hepatic metastases 6 days after the intratumoural injection of PEI–DNA polyplexes. Localised delivery of transgene was attempted by stereotactic intracerebral injection of polyplexes made with linear PEI, into the brain (Figure 5). Polyplexes formulated with 22-kDa PEI in 5% glucose produced complexes with a mean size of 30 – 100 nm [27]. These polyplexes showed increased diffusibility in cerebrospinal fluid of newborn and adult mice, diffusing from a single site of injection throughout the entire brain ventricular spaces. Transgene expression was detected in both glial and neuronal cells, using the β-galactosidase reporter gene. The small size and increased stability of the NPs formed in glucose was responsible for the increased diffusivity of the polyplexes [27]. This shows that particle size of the polyplexes can influence the transfection ability of the complexes and it is critical to optimise the

formulation conditions, so as to achieve efficient gene transfer *in vivo*.

4.1.2 Poly(L-lysine)

The lysine moiety of the polypeptide PLL has ε-amine groups, which are positively charged at physiological pH and, thus, can ionically interact with negatively charged DNA molecules. Laemmli *et al.* first investigated the PLL–DNA complexes in 1975 [28]. The size of complexes that are formed depends on the PLL:DNA ratios, molecular weight of PLL and the salt concentration. PLL has been shown to condense DNA into toroid- or rod-shaped complexes with their size ranging from 25 – 80 nm. The transfection efficiency of PLL is lower than that of PEI and has been found to result from the differences in the intracellular trafficking of the complexes. Unlike PEI, PLL has a low buffering capacity and is, therefore, not as efficient as PEI in escaping the acidic endosomes. As a result, the PLL–DNA complexes are transferred to lysosomes where the plasmid DNA is degraded, resulting in low transfection efficiencies [29]. Another major concern with PLL is its relatively high cytotoxicity and its slow degradation in the body, which suggests that it would be excreted at a very slow rate [30,31].

The biodistribution of DNA polyplexes that is achieved with PLL depends on the molecular weight of the PLL used [32]. Polyplexes that are prepared with low molecular weight PLL (20 kDa) are mostly localised in the liver (80%), lungs and kidneys. On the other hand, polyplexes that are made with high molecular weight PLL (211 kDa) had a long circulation time with only 47% of these being taken up by the liver. The positive charge on the PLL–DNA polyplexes makes them vulnerable to interaction with the negatively charged serum proteins and the blood cells. Low molecular weight PLL polyplexes show marked aggregation in the blood, whereas no aggregation is observed with high molecular weight PLL polyplexes in the blood.

PLL has been widely used for gene transfection studies *in vitro*. However, the major limiting factors are its cytotoxicity and moderate transfection efficiency. Thus, many different modifications of PLL and its conjugates with targeting ligands have been prepared to overcome some of these limitations for gene delivery applications [33]. PEG has been conjugated to PLL to prepare a block copolymer of PLL–PEG. This copolymer has reduced cytotoxicity, improved protection from nucleases and improved solubility of polyplexes, which is favourable for *in vivo* applications [34-36]. Moreover, the presence of PEG prevents interactions with serum proteins and favours a long circulation time for the polyplexes. Biodegradable NPs have been prepared using PLL-graft-polysaccharide copolymers and poly(D,L-lactide), by using a solvent evaporation method or the diafiltration method [37]. NPs that are prepared using these co-polymers had bifunctional surfaces with positively charged amino groups of PLL and the polysaccharide moieties. This increased the adsorption capacity of NPs for polynucleotides and allowed the introduction of ligand (carbohydrate) moieties on the NP surface for ligand-mediated recognition of specific receptors. The formulation resulted in NPs, as small as 60 nm in diameter, and showed excellent dispersive stability in phosphate-buffered saline. A preferential distribution of dextran moieties over PLL was observed in the outer surface of NPs, using a PLL-graft-dextran copolymer. The presence of dextran chains on the NP surface can be potentially useful to prevent nonspecific interactions with serum as well as for receptor-mediated targeting to specific cells.

Furthermore, to allow internalisation into cells by receptor-mediated endocytosis, cell-specific ligands such as asialoglycoprotein, galactose, transferrin, folate or specific antibodies have been covalently attached to PLL [33]. This not only increases the transfection efficiency by means of receptor-mediated endocytosis, but also allows the NPs to be targeted to specific tissues or organs. Another approach to enhance the transfection efficiency of PLL is to facilitate the escape of PLL–DNA polyplexes from the acidic endosomal vesicles into the cytoplasm. This has been attempted by the addition of endosomolytic compounds to the polyplexes, such as chloroquine, fusogenic peptides and so on [38]. Histidylated PLL has been used to increase the endosomal escape of NPs and thus improve the efficiency of gene delivery [39].

4.1.3 Polyamidoamines

Polyamidoamines (PAAs) are synthetic water-soluble polymers, with tertiary amino groups that are arranged regularly along the polymer backbone. Unlike other polyamines, the protonation of the amine groups in PAAs are independent events, thus providing very sharp acid-base dissociation constants to the polymer. Some of the PAAs have been shown to display a marked conformational change, due to modification of their average charge during movement from neutral to acidic pH [40,41]. This property of PAAs is useful in two ways for efficient intracellular delivery of DNA. First, the polymer–DNA complexes stay compact on intravenous administration (where the pH is 7.4) and thus protect DNA from nuclease degradation. Second, following internalisation into the cells by an endocytic process, the PAA–DNA complexes would allow a pH-triggered release of DNA owing to the pH-dependent conformational changes in the polymer chain. In addition, it is believed that on protonation in endosomes, PAAs display intra-endosomal swelling and can induce membrane damage by inherently destabilising the endosomal membranes, thus allowing intracellular delivery of DNA. PAAs have been shown to afford significant protection of DNA from the lysosomal DNase II enzymes at acidic pH 5.5, possibly due to steric hindrance by polymer chains. PAAs can form complexes with DNA resulting in toroid-shaped complexes with sizes of 80 – 150 nm in diameter, which protect DNA from nuclease degradation and have been shown to mediate transfection *in vitro* with comparable transfection ability as that of PEI [42].

4.1.4 Cationic dendrimers

Cationic dendrimers, unlike other polymers, are unique in possessing nanoscale core-shell architectures, characterised by

multiple (dendritic) branches amplifying from the core to the surface giving rise to a radial symmetry. Their precise structures, well-defined functional surfaces, sizes and shapes, and the ability to introduce targeting functionalities distinguish dendrimers as an attractive non-viral gene delivery vector. Tomalia and colleagues synthesised polyamidoamine (PAMAM) dendrimers ranging in size from 1 – 13 nm, which mimic the dimensions of histones; the natural proteins responsible for DNA scaffolding in cells [43,44]. The synthesis of PAMAM dendrimers can be described as the addition of different generations of dendrimer branches around a molecular core to produce divergent branched structures. Typically, ethylenediamine is used as the core, and multiple reaction sequences of alkylation of primary amines and amidation of ester groups are allowed to generate repeat alanine units and primary amine terminal groups [43]. Amine-terminated PAMAM dendrimers can be used to complex DNA by means of electrostatic interactions between DNA and positively charged amino groups of the PAMAM dendrimers. Some of the critical parameters that affect electrostatic binding of DNA and dendrimers include the pH and ionic strength of the solutions, dendrimer:DNA ratios and the time allowed for complex formation [45]. Furthermore, intentionally heat-degraded PAMAM dendrimers were found to result in a 50-fold increase in the transfection activity compared with the unheated control dendrimers [46]. This prompted researchers to investigate heat-activated dendrimer degradation, using a variety of solvents. The results from such studies suggest that the flexible, random, hyperbranched polymer that was formed as a result of the heat-activated solvolysis of dendrimers was responsible for enhanced transfection ability [46]. Thermally degraded PAMAM dendrimers are now available commercially and are called fractured or activated dendrimers. Successful *in vivo* gene transfection with PAMAM dendrimers has been shown in a prostate cancer model in severe combined immunodeficiency mice [47]. Intratumoural injection of plasmid DNA encoding the Fas ligand complexed with PAMAM dendrimers induced significant growth suppression and apoptotic cell death in xenograft tumours in mice.

4.2 Encapsulating DNA with polymers

4.2.1 Chitosan

Chitosan is a linear cationic polysaccharide, obtained by partial alkaline deacetylation of chitin; a polymer that is found in the exoskeleton of crustaceans. Chitin is a copolymer that is made of two subunits, D-glucosamine and *N*-acetyl-D-glucosamine, that are linked together by 1 → 4 glycosidic bonds. Chitosans are biodegradable and biocompatible polymers and can thus be potentially safe and non-toxic carriers for gene delivery. Moreover, derivatives of chitosan can be synthesised, with relative ease, to hydrophobically modify chitosan or to conjugate different chemical entities for targeting chitosan NPs to specific tissues or organs. The mucoadhesive nature of chitosan makes it an ideal polymer for preparing NPs that are useful for mucosal immunisation after oral administration [48].

NPs for gene delivery can be prepared by complexing plasmid DNA with chitosan [49] or encapsulating DNA inside the chitosan NPs [50]. The high density of amino groups that are present in the glucosamine backbone of chitosan can be protonated and, therefore, offer the opportunity to complex chitosans with negatively charged DNA molecules, owing to the electrostatic interactions. Self-assembling chitosan–DNA complexes, in the size range of 150 – 500 nm were first prepared by mixing a solution of chitosan with plasmid DNA [51]. The particle sizes of the complexes depend on the molecular weight of chitosan used, but not on the buffer compositions. Chitosan, hydrophobically modified using deoxycholic acid, can form spherical self-aggregates with a mean diameter of 160 nm in aqueous media [49]. Charge complexes that are formed between these self-aggregates of modified chitosan and plasmid DNA have been shown to transfect COS-1 cells in culture. The transfection efficiency of chitosan self-aggregate–DNA complexes was reported to be more than that achieved with plasmid DNA alone but lower than liposomal formulations.

DNA can be encapsulated in chitosan NPs, prepared by a complex coacervation method, yielding particle sizes in the range of 200 – 500 nm. Mao *et al.* have studied the influence of various parameters of NP preparation on the size of chitosan–DNA NPs [50]. The size of particles was optimised to 100 – 250 nm using a N/P ratio of 3 – 8. The zeta potential of these particles was +12 to +18 mV at a pH < 6.0 and nearly zero at pH 7.2. Chitosan NPs could partially protect DNA from nucleases. The transfection efficiency was found to be cell dependent and lower than that achieved with Lipofectamine™ (Invitrogen) DNA complexes in human kidney epithelial (HEK) 293 cells. However, the presence of serum in the cell culture medium did not interfere with the transfection using chitosan NPs. Co-encapsulation of chloroquine (a lysosmolytic agent) with plasmid DNA in chitosan NPs was tried as an approach to facilitate the endosomal escape of NPs and to enhance gene transfection. This did not lead to a significant improvement in the transfection efficiency of chitosan NPs, suggesting the possibility of a cell-trafficking mechanism, in addition to endocytosis as with PEI [50]. Derivatives of chitosan have been prepared by conjugating the amino groups of chitosan with various chemical entities (e.g., lactosylated chitosan [52], trimethylated chitosan [53], galactosylated chitosan [54], pegylated chitosan) to afford tissue targeting. Lactosylated chitosans bear a galactose residue and can thus potentially be used to target cells expressing galactose membrane lectins, such as hepatocytes. *In vitro* transfection studies on HeLa cells with lactosylated chitosan demonstrated a gradual increase of gene expression over time (from 24 – 96 h), whereas the gene expression with PEI was transient [52]. This may be due to a slow release of plasmid or a slower endosomal escape of chitosan–DNA NPs. Galactosylated chitosan-graft-dextran was prepared by first coupling chitosan with lactobionic acid and then grafting dextran onto this chitosan [54]. Dextran was found to improve the stability of galactosylated chitosan–DNA

complexes in water. Efficient gene transfection was shown with this system in cells, which express asialoglycoprotein receptors (Chang liver cells), indicating the specific interaction of receptors and galactose residues on chitosan. In order to improve the dispersion stability of chitosan–DNA NPs, surface modification with PEG has been attempted. Pegylation of chitosan helped to improve the stability of NPs as well as preventing any aggregation of NPs, owing to the higher hydrophilicity of pegylated chitosan NPs [50]. Pegylation of chitosan was shown to have no adverse effect on transfection efficiency of NPs.

Chitosan NPs are also considered a very appealing carrier choice for orally delivered mucosal immunisation strategies, owing to the mucoadhesive properties of chitosan. Orally administered chitosan–DNA NPs can adhere to the gastrointestinal epithelium and transfect epithelial or immune cells present in the gut-associated lymphoid tissue [55]. *In vitro* studies have shown that chitosan can enhance transcellular and paracellular transport of drugs across intestinal epithelial monolayers [56]. Roy *et al.* have demonstrated successful mucosal immunisation via oral gene delivery using DNA that is complexed with chitosan [57]. Chitosan–DNA NPs were administered orally in a murine model of peanut allergen-induced hypersensitivity, and levels of serum and secreted antibodies were detected 4 weeks after the first immunisation. Substantial differences in the antibody levels were found for NP-immunised mice as well as for the control mice or the mice immunised with naked DNA. The efficiency of orally administered chitosan–DNA NPs for gene expression was studied using the *lacZ* gene in the same mice model. Mice that were fed with NPs showed high levels of gene expression in both stomach and small intestines as compared with mice fed with plasmid DNA.

4.2.2 Gelatin

Gelatin is a biocompatible and biodegradable biopolymer that is obtained by hydrolytic degradation of porcine or bovine collagen. It has been explored for gene-delivery applications either as gelatin–DNA coacervates or DNA encapsulated inside gelatin NPs. A salt-induced complex coacervation process has been used to prepare DNA–gelatin nanospheres [58]. The presence of salt as a desolvating agent facilitates nanosphere formation by promoting electrostatic interactions between positively charged gelatin and negatively charged DNA. High levels of DNA loading (25 – 30% w/w) have been reported for such systems. Transferrin has been covalently bound to the surface of gelatin NPs, encapsulating plasmid DNA with the endosomolytic agent chloroquine and calcium [59]. Optimum transfection of HEK 293 cells with DNA–gelatin nanospheres required the presence of calcium and transferrin ligand on the surface of the nanospheres. Transferrin is known to facilitate the cellular uptake of NPs by means of transferrin receptor-mediated endocytosis. Transfection enhancement by calcium that is present in the nanosphere was attributed to the possible role of calcium in facilitating the release of DNA from the gelatin matrix by competing with DNA for electrostatic interactions with gelatin [59]. Cultured human tracheal epithelial cells (9HTEo) were successfully transfected with gelatin nanospheres, encapsulating plasmid DNA encoding for cystic fibrosis transport regulator. Transfection with gelatin nanospheres resulted in cystic fibrosis transport regulator expression in > 50% of the cells [59]. Gelatin NPs that encapsulate plasmid DNA can also be prepared using a solvent precipitation method under controlled conditions of temperature and pH. Kaul and Amiji have prepared long-circulating NPs encapsulating plasmid DNA using PEG-modified gelatin [60]. Reporter plasmid DNA encoding for β-galactosidase (pCMV-β) was encapsulated in gelatin and pegylated gelatin NPs. Gene transfection *in vitro* in Lewis lung carcinoma cells showed efficient β-galactosidase expression starting from 12 h until 96 h post-transfection. Gene expression was also evaluated after *in vivo* administration of DNA–gelatin NPs in mice bearing Lewis lung carcinoma. Biodistribution studies confirmed the long-circulating properties of pegylated gelatin NPs as compared with gelatin NPs. Only 18% of the recovered dose of pegylated gelatin NPs was present in the liver 2 h after intravenous administration as opposed to 80% for gelatin NPs [61]. The presence of hydrophilic PEG chains on the surface of NPs prevents macrophage-induced opsonisation by the RES (liver and spleen), and thus imparts long-circulating properties to NPs. Moreover the residence half-life in the tumour of pegylated gelatin NPs (121 h) was significantly higher as compared with the gelatin NPs (19 h), providing a greater opportunity for uptake by tumour cells. Significant expression of β-galactosidase was observed in the tumour following intravenous and intratumoural injections of DNA–pegylated gelatin NPs in mice bearing Lewis lung carcinoma [60].

4.2.3 Poly(β-amino ester)s

The rising concerns about the cytotoxicity of cationic polymers has increased the interest in the rational design of cationic polymers, which are biodegradable, non-toxic and contain additional functionalities that can alter the intracellular trafficking of NPs for improved transfection. It requires fine tuning of the chemical structure of the polymer (in a reproducible manner) so that either condensation of DNA with the polymer or the endosomal escape of the polyplexes can be achieved more efficiently to increase the gene transfection abilities of the polymer. This necessitates defining clear structure–function relationships for the polymers and has opened doors to a whole new arena of polymer science. It involves the application of combinatorial chemistry approaches to polymer development. Langer's research group has identified one such polymer library approach to define the structure–function relationships of polymer-based DNA delivery [62]. Poly(β-amino ester)s constitute one such new class of synthetic biodegradable cationic polymers with tertiary amines in their backbone. Unlike other cationic polymers, these are hydrolytically degradable polymers and are generally

less cytotoxic than the polycations such as PEI (Figure 6). Using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, these polymers and their degradation products have been shown to be non-toxic relative to PEI in NIH 3T3 cell line [63].

The use of polymer libraries and high-throughput cell-based screening assays have led to the identification of ~ 46 new poly(β-amino ester)s, which mediate *in vitro* gene delivery more efficiently than either PEI- or lipid-based vectors [64-66]. Many of these polymers have been shown to possess pH-dependent solubility, and are thus suitable for the preparation of NP formulations that can trigger the polymer degradation and release of encapsulated DNA in the acidic pH in endosomal vesicles [67]. These polymers, owing to their polycationic nature, condense DNA into complexes of the order of 50 – 200 nm and can, therefore, be used for gene transfection. Improvement in the synthesis protocols and optimisation is required for obtaining reproducible molecular weights of the polymer, so that polymer properties can be compared directly. The use of poly(β-amino ester)s for *in vivo* application requires further tests on the toxicity of polymers to firmly establish their biocompatibility. The MTT assay is a preliminary test for polymer biocompatibility; it has been shown that the self-assembled polymer–DNA complexes may be more toxic to the cells than the free polymers themselves [64].

4.2.4 *Polylactide/poly(lactide-co-glycolide)*

Polylactide (PLA), and poly(lactide-co-glycolide) (PLGA) are the biodegradable and biocompatible polymers that were used for formulating NPs and are approved for human use by the FDA. Plasmid DNA is entrapped into the polymeric matrix, which not only protects DNA from nucleases but also allows a control over the DNA release kinetics from NPs. The main advantage of such NPs is the slow release of DNA from the NPs, which facilitates sustained levels of gene expression. PLGA NPs are generally formulated using w/o/w double-emulsion solvent-evaporation technique, using poly(vinyl alcohol) (PVA) as an emulsifier [68]. As the NP comes in direct contact with the cell membranes, the surface properties of NPs are critical in determining its intracellular fate.

It has been demonstrated that PLGA NPs are internalised into cells through a concentration- and time-dependent endocytic process [69]. Cellular internalisation of PLGA NPs is partly through fluid-phase pinocytosis and in part through clathrin-coated pits in vascular smooth muscle cells. It was further demonstrated that NPs rapidly escape the endolysosomes and enter the cytoplasm within 10 min of incubation with cells. Selective reversal of surface charge of NPs in the acidic pH of endosomes is responsible for the escape into the cytosol [70]. The authors hypothesised that protonation of PLGA NPs in acidic pH of endosomes results in their interaction with the vesicular membranes, leading to transient and localised destabilisation of the membrane; thus allowing the escape of

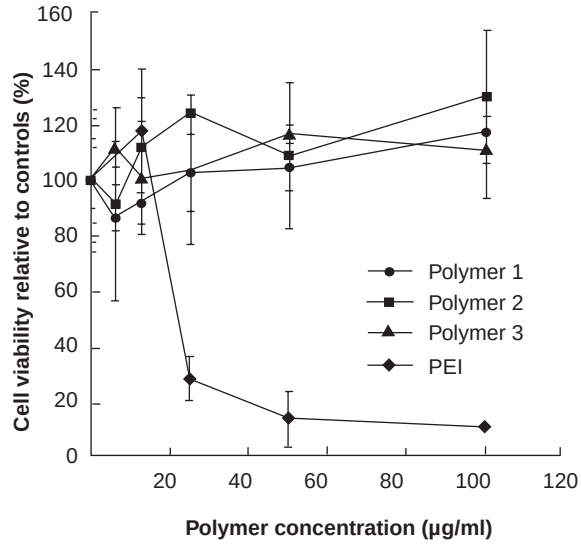

Figure 6. Development of new cationic polymers with relatively low cytotoxicity as compared with PEI. Cytotoxicity profiles of poly(β-amino ester)s (polymers 1 – 3) and PEI. Viability of NIH 3T3 cells is expressed as a function of polymer concentration. The molecular weights of polymers 1, 2 and 3 were 5800, 11,300 and 22,500, respectively. The molecular weight of the PEI employed was 25,000. Reprinted with permission from LYNN DM, LANGER R: Degradable poly(β-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. *J. Am. Chem. Soc.* (2000) **122**:10761-10768 [63]. Copyright (2000) American Chemical Society.
PEI: Polyethylenimine.

NPs into the cytosol. NPs that are present in the cytoplasm release DNA at a slow rate for the subsequent nuclear localisation of plasmid DNA. Successive studies have shown that a significant fraction of NPs also undergo exocytosis in the *in vitro* cell culture conditions [69]. Thus, the surface properties of NPs play an important role in their intracellular trafficking and can potentially influence the gene transfection efficiencies. These include the generally overlooked aspect of surface-associated PVA in NPs, hydrophilicity and the surface charge (zeta potential) of the NPs. It has been shown that a fraction of PVA that is used in the formulation of NPs remains associated with the NP surface and cannot be removed even by multiple washing [71]. This residual PVA on the NP surface can alter its physical properties and affect the cellular uptake of NPs. NPs with lower amounts of surface-associated PVA show about a threefold higher cellular uptake in vascular smooth muscle cells than the NPs with higher residual PVA [72]. This could be due to shielding of the surface-charge reversal of NPs by the presence of the higher amount of surface-associated PVA, which could affect the endosomal escape of NPs. Furthermore, the amount of PVA that is associated with the NP surface depends on the amount of PVA, the molecular weight and the degree of hydroxylation of PVA that is used as an emulsifier in the formulation [68].

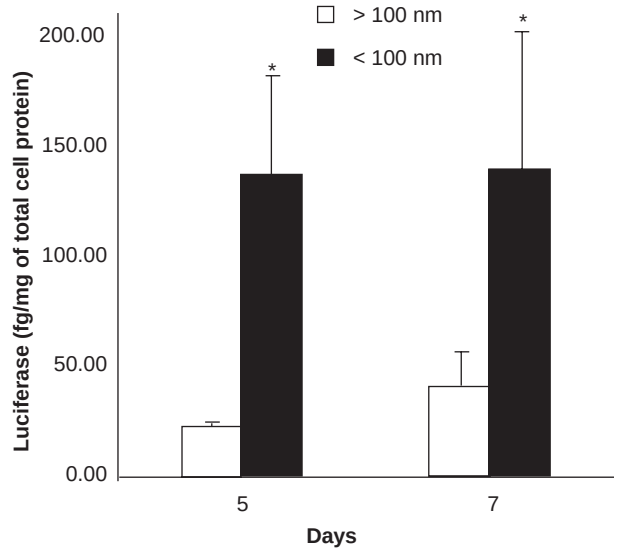

Figure 7. Transfection with fractionated smaller- and larger-sized nanoparticles in HEK-293 cell line. The luciferase protein levels were measured at 5 and 7 days post-transfection. Results are expressed as mean ± standard error of mean (n = 6, *p < 0.05 compared with larger-sized nanoparticles). Reprinted from PRABHA S, ZHOU WZ, PANYAM J, LABHASETWAR V: Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. *Int. J. Pharm*. (2002) **244**(1-2):105-115 [73]. Copyright (2002) with permission from Elsevier.
HEK: Human kidney epithelial.

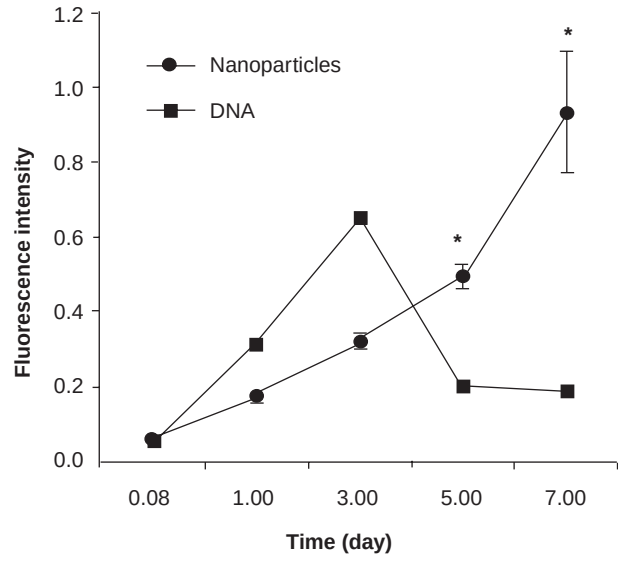

Figure 8. Quantitative determination of intracellular DNA levels. Cells transfected with YOYO-labelled DNA-loaded nanoparticles demonstrated sustained and increased intracellular DNA levels, as opposed to transient DNA levels in the cells transfected with naked DNA. Data are represented as the mean ± the standard error of the mean (n = 6; p < 0.001 for points marked with asterisks). Reprinted with permission from PRABHA S, LABHASETWAR V: Nanoparticle-mediated wild type p53 gene delivery results in sustained antiproliferative activity in breast cancer cells. *Mol. Pharm.* (2004) **1**(3):211-219 [74]. Copyright (2004) American Chemical Society.

Cellular internalisation of NPs also depends on their particle size and has been shown to affect the gene transfection (Figure 7). The smaller size (< 100 nm) of NPs showed a 27-fold higher gene transfection than the larger size (> 100 nm) NPs [73]. However, this difference in gene transfection was not related to the surface properties, cellular uptake or the release of DNA from the NPs. Thus, the smaller size with a uniform particle size distribution is expected to increase the gene transfection efficiency of NPs. Other important formulation parameters that influence the gene transfection ability of NPs include the molecular weight of the polymer and the molecular weight and degree of hydrolysis of PVA [68]. NPs that are formulated with a higher molecular weight polymer showed enhanced gene transfection. This may be attributed to the relatively higher DNA loading and its release from NPs prepared with high molecular weight polymer. Higher viscosity and better emulsifying properties of the polymer solution facilitate a higher loading of DNA in NPs and also leads to a lower particle size of NPs. Polymer composition can affect the hydrophobicity of the polymer and can thus affect the DNA loading and release of DNA from the NPs. These NPs that are prepared using more hydrophobic polymers (polylactides) demonstrated lower transfection than those that are formulated using copolymers of polylactide and glycolide [68]. The slow rate of release of DNA from the relatively more hydrophobic polymeric matrix may be responsible for the lower levels of gene transfection.

One of the key features of PLGA NP-mediated gene delivery is the ability to achieve sustained gene expression. Although the levels of gene expression with NPs are lower than that achieved with lipid-based gene delivery, they are sustained for a prolonged period of time [74]. Further NP-mediated gene transfection is not affected by the presence of serum in the cell culture media and, thus, PLGA NPs constitute a potential gene delivery vector for *in vivo* gene delivery. Prabha and Labhasetwar have shown slow intracellular release of plasmid DNA from the PLGA NPs, which results in sustained levels of DNA inside the cells (Figure 8) [74]. This could be easily related to the greater gene expression, quantitated by mRNA levels that are determined by using reverse transcription PCR. *wt-p53* gene-loaded NPs showed higher mRNA levels for p53, as compared with the liposomal formulation, even 5 days after transfection of the MDA-MB-435S breast cancer cells [74]. The sustained p53 expression levels resulted in greater and sustained inhibition of cell proliferation as compared with plasmid DNA alone. Cohen *et al.* has shown that despite the lower transfection levels that are observed *in vitro* with NPs as compared with liposomal formulations, the *in vivo* gene transfection with

NPs was one to two orders of magnitude greater than the liposomes, 7 days after an intramuscular injection in rats (Figure 9) [75]. Their studies demonstrated gene expression that was sustained for > 28 days *in vivo* with a single dose of intramuscular injection of NPs. Such sustained gene expression is advantageous, especially if the half-life of the expressed protein is very short and/or a chronic gene delivery is required for better therapeutic efficacy.

Furthermore, the polymeric NPs that are based on PLGA/PLA can be surface modified and functionalised to improve their biodistribution and also to conjugate targeting ligands, which can direct NPs to specific cells/tissues where gene delivery is desired. Surface modification of NPs is achieved either by adsorbing amphiphilic excipients onto preformed NPs or by covalently linking excipients to the core-forming polymer prior to NP formulation. Incorporation of additional excipients such as polyethylene oxide (PEO) has been attempted to prevent the generation of an extremely acidic microenvironment inside the NPs on polymer degradation [76].

PEO–PEG has been used to coat the polymeric NPs to provide a protective hydrophilic sheath, which prevents the rapid opsonisation of the otherwise hydrophobic NPs by RES, and thus prolong the circulation time of NPs in the bloodstream [77]. The hydrophobic part of PEO–PEG polymers can adsorb to the NP surface whilst the hydrophilic chains protrude towards the aqueous medium. PEG coats on the NP surface also provide an attractive opportunity to chemically conjugate active-targeting ligands to the NP surface [78,79]. These coatings can modify the biodistribution of NPs when injected into the systemic circulation. However, it has been argued that some of these polymers can be easily displaced by serum proteins, which can lead to the aggregation of NPs [80]. Thus, alternative approaches of synthesising copolymers of PLA–PLGA with PEG [81,82] and co-encapsulation of PEG with plasmid DNA inside PLGA NPs have been tried [83].

4.3 Complexing DNA on the surface of cationic nanoparticles

4.3.1 Polyalkylcyanoacrylate

Polyalkylcyanoacrylate (PACA) NPs were first prepared by Couvreur *et al.* in 1979 [84]. PACA NPs have been used for the intracellular delivery of various ODN sequences. ODNs can be associated with the PACA NPs either by covalently linking to a hydrophobic molecule (cholesterol), which can then be anchored at the NP surface [85]. Alternatively, NPs can be coated with cationic surfactants (cetyltrimethylammonium bromide) or polymers (diethylaminoethyl-dextran), which can be used for complexing ODNs via electrostatic interactions [86]. PACA NPs are capable of entering the cells by an endocytic phenomenon but remain trapped in the lysosomal vesicles of the cell, thus limiting the efficiency of intracellular delivery of ODNs. Hence, many compounds that are known to destabilise the lysosomal membranes have been associated with the PACA NPs to facilitate release from endosomes into the cytoplasm

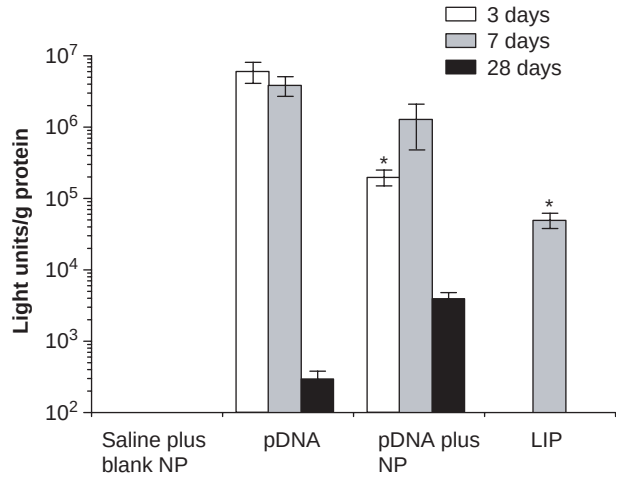

Figure 9. AP expression in rat tibialis muscle 3, 7 and 28 days after a single intramuscular injection of pDNA-NP (pDNA 25 µg) in comparison to saline, blank NP, naked pDNA (25 µg/200 µl) and liposome-pDNA (25 µg). Shown mean ± standard error of mean, $7 < n < 13$, $p < 0.05$ for points marked with asterisks. Reprinted from: COHEN H, LEVY RJ, GAO J *et al.*: Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. *Gene Ther.* (2000) **7**(22):1896-1905 [75]. Copyright (2000), with permission from Macmillan Publishers Ltd.
AP: Alkaline phosphatase; LIP: Liposome; NP: Nanoparticle; pDNA: Plasmid DNA.

[86,87]. In earlier studies, it has been shown that the PACA NPs are taken up by the RES system of the body and can, therefore, be used for targeting DNA delivery to the RES organs such as the liver, spleen, lungs and bone marrow [88].

4.3.2 Bio-inspired polymers

Most viruses and toxins have evolved well-defined machinery to infect the cells and to integrate with the host cell genome. At present, efforts are focused on designing artificial viruses or synthesising bio-inspired polymers, which can mimic some of the viral mechanisms of cellular entry. These include transfection studies that are conducted in the presence of membrane disruptive or viral fusogenic peptides. Wagner has listed some of these membrane active peptides, which are under extensive investigation for enhancing gene transfection by non-viral methods [89]. Synthetic peptides that are derived from the N terminus of the influenza virus haemagglutinin or artificial amphipathic peptides (e.g., GALA and KALA) have been used for improving the intracellular delivery of DNA polyplexes [90]. Specific interaction of these peptides with the endosomal membranes is due to the presence of acidic residues (aspartic and glutamic acid). At neutral pH, the negatively charged carboxylic groups destabilise the α-helical structure of the peptide; however, acidic endosomal pH promotes the formation of amphipathic helices of peptide, which further allows the multimerisation and membrane interaction of peptides. Peptides can be incorporated into PLL–DNA complexes by covalent linkage to PLL

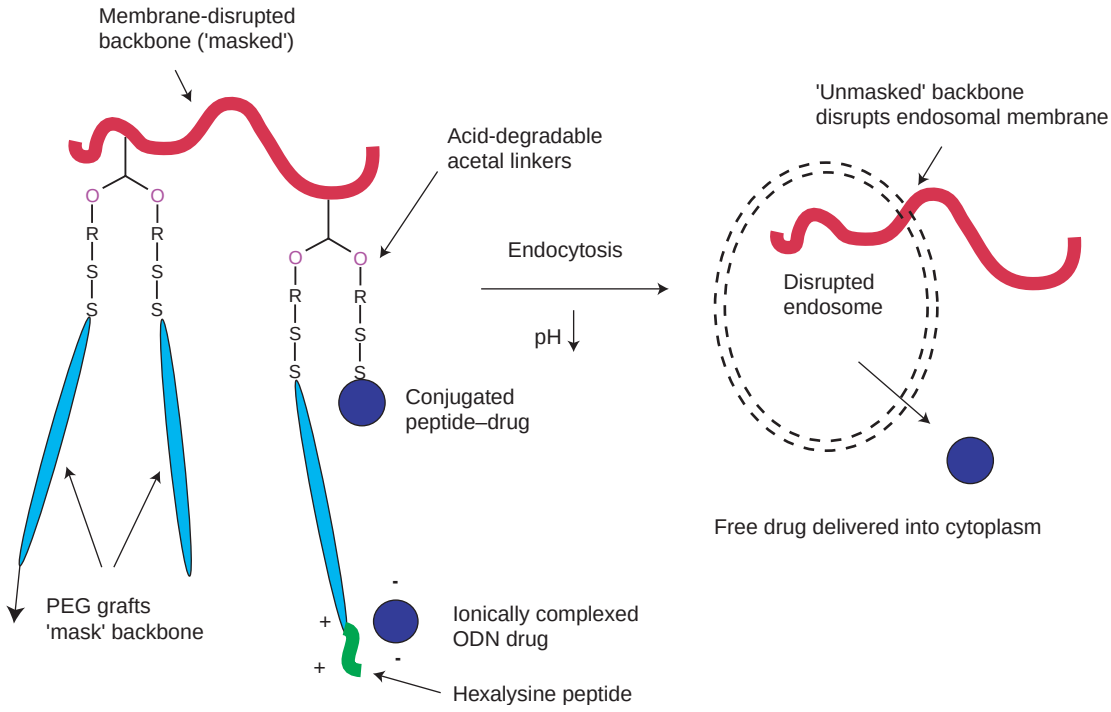

Figure 10. Schematic diagram of the encrypted polymer design. The polymers are designed to be pegylated and serum stable at pH 7.4 but to be disruptive to the endosomal membrane at the acidic pH within the endosome. The polymers have the following components: a membrane-disruptive backbone (red line), acid-degradable linkers (purple circle), PEG grafts (aqua ellipsoid), conjugated or ionically complexed drug molecules (blue circle), hexalysine peptide (green line) and targeting ligands (black arrow). At pH 7.4, the polymers are pegylated ('masked'); however, after endocytosis, the acid-degradable linker hydrolyses and the polymer backbone becomes de-pegylated ('unmasked') and membrane disruptive, causing endosomal disruption. The PEGs may be conjugated to the backbone via both acid-degradable linkages and disulfide bonds. The latter are reduced in the cytoplasm to release the free drug. In the study reported here, two examples are presented. In the first case, a model peptide drug with a terminal cysteine is conjugated to the backbone via S-S bonds, and in the second case an antisense ODN is ionically complexed to cationic lysine groups that have been linked to the terminal ends of the PEG molecules. In the latter case, mannose groups have been linked along with the lysine groups to the ends of the PEGs for targeting the ODN to the macrophages (RAW cells). Reprinted with permission from: MURTHY N, CAMPBELL J, FAUSTO N, HOFFMAN AS, STAYTON PS: Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs. *Bioconjug. Chem.* (2003) **14**(2):412-419 [95]. Copyright (2003) American Chemical Society.
ODN: Oligonucleotide; PEG: Poly(ethylene glycol).

[91] or by noncovalent ionic interaction of negatively charged peptides with positively charged polyplexes [92].

A cationic fusogenic peptide, KALA, has been coated on the surface of polyplexes to enhance gene transfection. Poly(2-[dimethylamino]ethyl methacrylate)-co-*N*-vinyl-2-pyrrolidone was synthesised as a copolymer, and conjugated to PEG, which was further used to couple galactose as a targeting ligand [93]. This polymer construct was used to complex plasmid DNA encoding for luciferase to produce NPs with a negative surface charge. The cationic KALA peptide was then ionically coated on the surface of these polyplexes. Introduction of the KALA peptide allowed transfection efficiencies as high as that of Lipofectamine Plus™ (Invitrogen) in HepG2 cells. Cationic fusogenic peptides have also been used for the intracellular delivery of AS-ODNs. An AS-ODN was conjugated to PEG via an acid-cleavable linkage, phosphoramidate, to form a diblock copolymer structure [94]. The cationic KALA peptide, which interacts with the oppositely charged species of ODN segments, can induce self-assembly into polyelectrolyte micelles (ODN–PEG/KALA). An inner polyeletrolyte complex core was formed by KALA and ODN whilst PEG chains formed a surrounding corona on the core. The presence of an acid-labile linker in ODN–PEG can trigger the cleavage of ODN in the acidic endosomal pH and the KALA peptide promotes the endosomal escape of the ODNs. Such a hybrid micelle system was used for the intracellular delivery of antisense ODN to *c-myb*. These *c-myb* ODN micelles showed ~ 70% inhibition of the proliferation of smooth muscle cells, as a result of efficient intracellular delivery of the therapeutic ODN [94].

Murthy *et al.* have described the design and synthesis of multi-functional pH-responsive polymers to enhance the cytosolic delivery of DNA/drugs [95]. The polymer backbone is a relatively hydrophobic, membrane-disruptive polymer that is masked by grafted PEG chains at neutral pH (Figure 10). The PEG chains are grafted to the polymer backbone using two linkers: disulfide groups and acid-degradable acetal groups. As

the polymer formulations enter the cell, acetal groups are designed to degrade in the acidic pH of the endosome, thereby unmasking the membrane-disruptive backbone. This leads to the disruption of endosomal membrane and release into cytosol. Due to the reductive environment in the cytosol, the disulfide groups are reduced, thereby releasing the conjugated drug/plasmid DNA. The authors have shown successful intracellular delivery of AS-ODNs in cell culture using these polymers.

5. Conclusions

Polymeric NPs can potentially solve the problems of gene delivery, although some more progress is required for improving their delivery efficiency *in vivo*. Cationic polymers such as PLL and PEI, which can effectively condense DNA, often have limitations for *in vivo* applications due to their cytotoxicity, nonbiodegradable nature and the possibility of aggregation in physiological conditions. Suitable formulation conditions (such as N/P ratios, molecular weight of the polymer) can be selected to modify the particle size and surface charge of these NPs, and to potentially reduce the toxicity and stability concerns for *in vivo* use. Moreover, these polymers can be modified chemically to add functional elements (histidylated PLL or endosomolytic peptides) to prevent sequestration of NPs in endosomes, thus improving their transfection efficiency. On the other hand, NPs that are prepared with biodegradable polymers such as PLGA/PLA are stable in the bloodstream, but have a lower transfection efficiency as compared with cationic polymers such as PEI. Nonetheless, one major advantage of PLGA/PLA NPs that cannot be ignored is their ability to release DNA slowly inside the cells, thus sustaining gene expression levels for a prolonged period of time. Recent efforts for the synthesis of degradable, cationic polymers with low cytotoxicity and high transfection efficiency can provide new polymers to formulate NPs for efficient gene delivery. Furthermore, approaches such as the use of cell-specific targeting moieties can target polymeric NPs to particular cells and have helped to overcome some of the cellular barriers to gene delivery. However, the nuclear membrane remains as an invincible barrier. The use of nuclear localisation signalling peptides and the tissue-specific promoters, although in early stages of development, constitute promising efforts at improving the nuclear import of DNA. At the pace at which these developments are progressing, we are not too far from the point where we can present polymeric NPs as promising gene delivery vectors for human gene therapy.

6. Expert opinion

Nature has its own ways of creating things and altering its own creations. Such is the case with viruses, which have remarkably evolved to possess unique mechanisms to infect human cells, to integrate into their genome and thus, alter the normal biology of the cell. Indeed, the wisdom of nature far exceeds man's possibilities but we can always learn from its creations. We have been trying our best to mimic viruses in order to develop polymers for gene delivery, but we have not been completely successful. To an extent, we have been able to comprehend the complexities of the gene transfection process but for the development of a successful approach for *in vivo* gene delivery, it is important to understand the molecular mechanisms by which the polymeric NPs interact with the cell, and undergo intracellular sorting [96]. With the use of polymeric NPs we have been able to overcome the barriers that are posed by the cell membrane and have delivered DNA inside the cell. However, for these systems, the nuclear membrane stands as a rate-limiting barrier for gene expression [97]. The incorporation of cationic peptides such as nuclear localisation signalling peptides, protein transduction domain from the transactivator of transcription (TAT) and protamine have been shown to enable nuclear transport of the DNA delivered by polymeric NPs [98]. Such persistent attempts are indeed a positive sign for the ultimate success of polymeric NPs as gene-expression vectors. Despite these extensive attempts at increasing the gene transfection ability of polymeric NPs in the *in vitro* conditions, often what is ignored is the toxicity profile of the polymers for *in vivo* purposes. The challenge is to develop polymeric NPs from cationic polymers, which are efficient at gene transfection and are, at the same time, non-toxic and stable in the presence of blood and other body fluids. Thus, taking the polymeric NPs from being laboratory reagents for gene transfection to the clinics for gene therapy, requires a comprehensive understanding of not only the polymeric systems but also the complex pathophysiology of the disease condition.

For all these years, the scientific community is in search of an 'omnipotent' vector that would solve all of the problems of delivering genes; this seems to have dogged the field of gene therapy since its inception. Such a universal vector has not yet been found and without being pessimistic (or being realistic), the authors consider that the 'one for all' approach is perhaps not suitable for devising a single gene-delivery vector for all kinds of disease conditions. Such an approach would be beneficial only if we are successful in developing a flexible gene expression vector that would meet the needs of all the disease conditions. The pathophysiology of each disease is quite disparate from others, and so is the need for different levels or duration of the gene to be expressed, for therapeutic efficacy. Another important factor that should be considered is the nature of target cells that are being transfected. For example, for gene therapy approaches for neurological disorders, the vector must be able to transfect the nondividing cells (neurons), whereas even a lower efficiency vector would work for transfection into stem cells, as they divide rapidly. For a therapeutic gene of interest, which expresses a secretory protein that can diffuse through the tissue, therapeutic effect can be observed even if a small percentage of cells gets transfected. In the case of cancer gene therapy, even a low transfection efficiency vector in combination with bystander cell–cell interaction can effectively limit the cell proliferation and the tumour growth.

Gene therapy is an approach for treating diseases at the genetic level; modifying the biology of the cells for a therapeutic

benefit. This makes it all the more important for the researchers who are developing gene-delivery vectors to be very familiar with the biology of the cells. For instance, in the case of gene therapy approaches for correcting gene deficiency, it may be required to maintain the physiological concentrations of the expressed protein for a sustained period of time. Gene therapy strategies to correct hormone deficiencies would require not only the restoration of normal gene expression, but also strict control of the physiological, pulsatile nature of hormone secretion [99]. A common biological effect of cell proliferation can also be different in different diseases. The rate and kinetics of cell proliferation in cancers may be quite different from those in other proliferative disorders such as restenosis; and this demands a consideration when selecting the gene delivery vectors. It has been shown that even a 50% reduction in the proliferating cells can reduce the neointimal volume to 90% in postangioplasty patients; this equates to a substantial therapeutic benefit [100]. Thus, clinically relevant reduction of neointima formation can be attained, with realistic transfection rates. In contrast, it is necessary for all of the the cancer cells to be killed in order to achieve a complete remission in patients presenting a disseminated disease.

Most often, emphasis is laid on the level of gene transfection; however, as indicated below, in certain disease conditions, the low level, but sustained, gene expression could be more effective. Therapeutic angiogenesis is one such example, where a sustained but low level of expression of genes encoding the angiogenic factors (e.g., VEGF or fibroblast growth factor) is required. High levels of growth factors are known to form leaky vasculature, which are nonfunctional and regress with time. Therefore, it is considered that a low level of sustained gene expression could be more effective in forming mature and functional blood vessels [101]. Furthermore, for levels of gene expression, how much is enough needs to be decided before the selection of an appropriate vector. For example, in patients suffering from haemophilia B, which is caused by the deficiency of a blood-clotting protein (Factor IX), giving just 5% of the normal circulating levels of the protein can substantially improve their quality of life [102]. Thus, the selection of a gene delivery vector must be dictated by the particular needs of gene transfer in a disease condition.

Therefore, the question is how one gene expression vector would fit the needs of all of the diseases. At the same time, it may not be practical to design a separate vector for each disease condition. A vector that offers a greater degree of flexibility in terms of modulating the level and duration of gene expression could provide a solution to the above issue, or perhaps we could custom-design disease-specific gene expression vectors, especially for those diseases for which there are no therapies or for such diseases where gene therapy would make a significant difference in terms of the therapeutic outcome as compared with the conventional therapies. Targeted polymeric NPs stand somewhere in proximity to such an idealistic perspective because these can be developed with different composition and properties, and hence can be custom designed for a particular disease condition. However, for becoming a therapeutic reality in clinics, a maze of confounding questions still needs to be solved, with the main one being the efficiency of gene transfection *in vivo*. Advances in rational design of polymers (to improve efficiency of gene delivery and targeting polymeric NPs to specific cells), with an ability to deliver therapeutic DNA to the nucleus, can bring the polymeric NPs on par with viral vectors for gene delivery (Table 1). The preparation of combinatorial libraries for synthetic polymers and screening the same for efficiency of gene transfection signifies a leap towards the successful roadmap of gene therapy using polymeric NPs. However, some caution with respect to the long-term safety in humans must be exercised to select the polymer systems for *in vivo* applications. It has recently been shown that some polymeric excipients may be biologically inert by themselves but show significantly different pharmacological and toxicological responses when formulated with biologically active agents [103]. Therefore, thorough assessments of the polymeric formulations must be made in order to establish their long-term safety in humans.

As with other drug therapies, there can also be adverse effects with gene therapy, due to the undesirable gene transfection in non-target tissues. Thus, targeting can potentially increase the efficacy and reduce the toxicity of gene therapies by restricting the gene transfection to the treated (target) tissue. The surface of polymeric NPs can be decorated with cell-specific ligands to target the NPs to specific tissues or organs for targeted gene delivery. Targeting to specific cells has been explored using the presence of various receptors, antigens/proteins on the plasma membrane of cells and also by virtue of the lipid components of the cell membranes. The receptors and surface-bound antigens may be expressed uniquely in diseased cells only, or may exhibit differentially higher expression in diseased cells as compared with the normal cells. Active agents, such as ligands for the receptors and antibodies to the surface proteins have been used extensively to target polymeric NPs to specific cells. However, the success of this approach is contingent on the use of very specific surface antigens that are present on selective cells in the human body. This problem has in part been overcome by the extensive efforts going into the discovery of cell-specific surface antigens/proteins and the use of ligands that are specific for these surface receptors. For diseases, for which no specific receptors have been reported to be present on the cell membranes, targeting may be achieved by the use of tissue-specific promoter/enhancers in concert with the gene of interest. This involves the use of specific tissue, tumour, or induced promoters that can limit gene expression to target cells, which express a particular transcription factor. This can specifically restrict the transgene expression in the target tissue [104]. Moreover, it allows the flexibility to regulate the duration and level of expression exogenously, by using promoters that are preferentially activated under certain conditions. The promoter for the telomerase gene can be genuinely classified as tumour specific and is being used to drive transgene expression in a variety of cancer cells [105]. Other examples include the α-fetoprotein

Table 1. Strategies to improve gene-transfection efficiency of polymeric nanoparticles.

Strategies	Rationale	Effort/attempt	Ref.
Rational design of new polymers	Biodegradable, non-cytotoxic, cationic polymers	Combinatorial synthesis of polymers and high-throughput cell-based screening assays	[62,65]
	Polymers with inherent features for allowing endosomal escape and thus improved intracellular delivery of DNA	pH-responsive polymers	[63,67,95]
Endosomal escape	To allow polymeric NPs that are internalised into the cells to escape from endosomes into cytoplasm	Inclusion of chloroquine Synthetic pH-dependent endosomolytic peptides Viral-derived fusogenic peptides	[58,59,89,90, 93,94,110]
Nuclear targeting	To enhance nuclear uptake of DNA To improve gene expression levels	Nuclear localisation signalling peptides Transactivating transcriptional activator domains Protamine Strong promoter or enhancer sequences Tissue-specific promoters	[98,104-106, 111]
Cell-specific targeting	To enhance NP internalisation into cells by means of receptor-mediated endocytosis To express the gene of interest only in target tissues in order to avoid undesired nonspecific adverse effects	Cell-specific ligands	[60,112-115]

gene, which is transcriptionally silent in the adult liver but expressed in foetal and hepatocellular carcinoma cells [106]. Thus, specific gene delivery can be achieved either by using active targeting approaches (ligand-mediated targeting) or via biological approaches (tissue-specific promoters). Therapeutic benefits from such specific delivery can only be reaped by attaining sufficient concentrations of the gene delivery vector in the target tissue. It has been previously discussed that these targeting modalities are often insufficient for rapid vector accumulation at the target tissue [107]. The use of alternative approaches such as magnetic field in concert with the above mentioned targeting strategies, can overcome some of these fundamental limitations to gene delivery. Gene delivery vectors (polyplexes) that are associated with super-paramagnetic NPs can be guided to accumulate selectively in the target tissue, by the use of external magnetic field that is focused at the tissue [108]. This tool of magnetofection has been shown to reduce the vector dose and incubation time with the vector that is required to achieve a high transfection efficiency *in vitro*. Magnetic-field guided local transfection in the gastrointestinal tract and blood vessels has also been reported [108]. Another physical method to enhance gene delivery involves the localised administration of the vectors, or enhancing the transdermal gene delivery using microneedles [109].

To conclude, work still needs to be carried out towards solving the jigsaw puzzle of gene transfection and we should continue with our persistent efforts in this direction. Perhaps a more holistic approach needs to be taken when developing polymeric gene expression vectors. For this, an important variable that needs to be included into the equation is the gene expression profile that is desired for therapeutic benefit in specific disease conditions. In order to solve this equation, it is essential to build on our fundamental knowledge and basic understanding of the cellular barriers, mechanisms of intracellular sorting of polymeric NPs and ultimately defining rational strategies to overcome these barriers.

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Website

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• Website describing important statistics regarding gene therapy clinical trials, classifying them by disease, type of vector and geographical locations.

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