Expert Opinion

Gene delivery using functional dendritic polymers

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Objective: This review describes a strategy for the development of multifunctional dendritic polymers for application as gene delivery systems. These polymers can address the low transfection efficiency usually encountered by synthetic non-viral vectors. *Methods*: Employing appropriate, well-characterized and mainly commercially available dendritic polymers, the emphasis is placed primarily on step-wise molecular engineering of their surface for providing gene carriers of low toxicity, specificity to certain cells and transport ability through their membranes, with the ultimate objective of enhanced transfection efficiency. Cationic dendritic polymers interact with appropriate genetic material, affording complexes that are employed for cell transfection. *Conclusion*: Multifunctionalization of dendritic polymers provides gene vectors of low toxicity, significant transfection efficiency, specificity to certain biological cells and transport ability through their membranes.

Keywords: dendrimers, dendritic polymers, dendrons, gene delivery vectors, hyperbranched polymers, nanocarriers

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1. Introduction

The development of safe and efficient gene delivery vectors is a challenging task in gene therapy. Such vectors should protect DNA from enzymatic degradation, bind to target cells, cross cell membranes, escape from the endosome following endocytosis and release therapeutic DNA or RNA resulting in gene expression. In addition, these vectors should be non-toxic, preferably biodegradable and not induce an immune response. For an efficient systemic circulation *in vivo*, the size of the vector should be less than 200 nm in diameter and preferably monodisperse [1].

Extensive investigations into viral [2-5] and non-viral [6-10] gene delivery systems have been conducted in recent years. Viral vectors, although more efficient compared to non-viral vectors, are overwhelmed by safety issues, including virus replication and inflammatory reactions [11]. In contrast, among the advantages of non-viral vectors are facile preparation at a reasonable cost, the superior safety profiles and low immunogenicity, while targeting ligands can also be readily introduced. Furthermore, non-viral vectors can protect DNA from degradation by nucleases in the lysosome and bloodstream. Thus, non-viral systems are promising candidates for becoming commercial products and this fact has triggered extensive research for their development.

The commonly utilized non-viral vectors, or synthetic gene carriers, are cationic lipids, polymers, dendritic polymers and peptides that form complexes with genetic material. Complexation occurs through electrostatic interactions of the anionic phosphate backbone of the nucleic acid with positively charged moieties of the carriers. Among these vectors, the structural features of dendritic polymers [12-16] render these polymers potent for addressing the above problems. Dendritic polymers consist of four subclasses, namely: i) random hyperbranched polymers;

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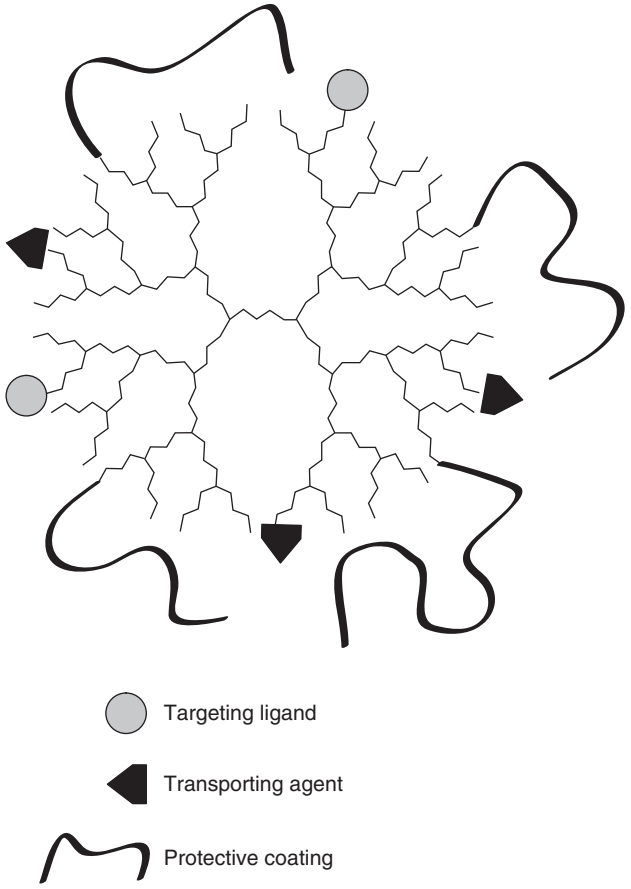


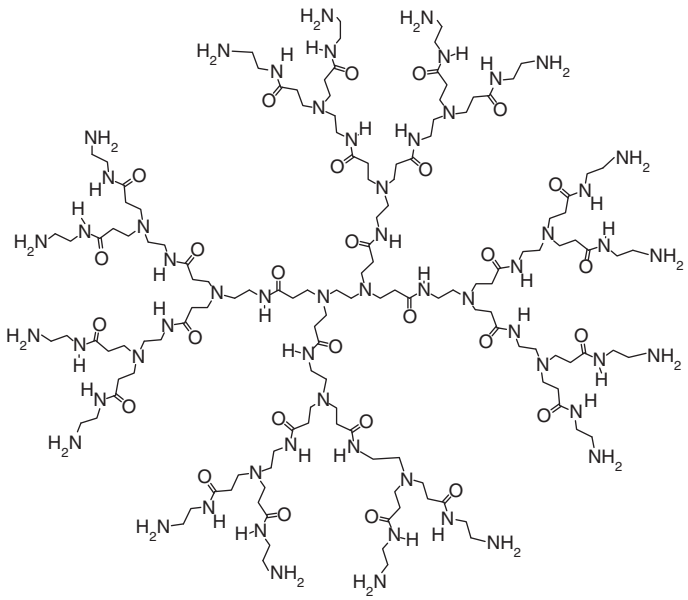
Figure 1. Schematic representation of a multifunctional dendritic system.

ii) dendrigrafts; iii) dendrons; and iv) symmetrical dendrimers; the order of this subset reflects the relative degree of structural control exhibited by these dendritic architectures [17]. All these polymers are highly branched and contain nanocavities, in the interior of which encapsulation can occur. Furthermore, their surface groups are easily functionalized, providing a diversity of polymers.

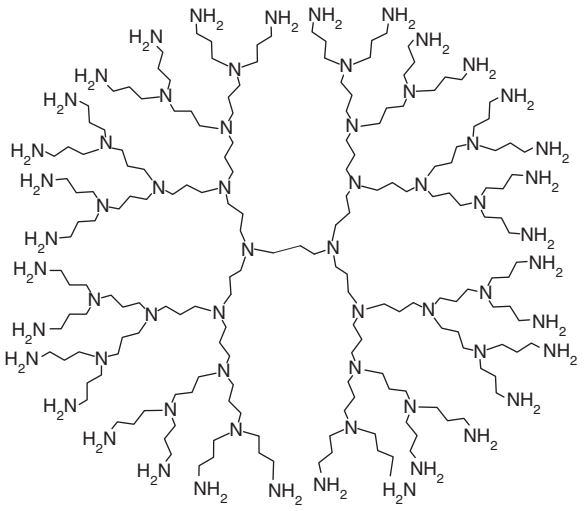
The application of dendritic polymers as non-viral gene vectors fulfilling the above-mentioned requirements was achieved by employing basic dendritic polymers, commercially available or custom-made, which were subjected to consecutive chemical modification affording functional or multifunctional dendritic systems. Such a typical dendrimeric vector is shown in **Figure 1**. Each type of external group has a specific function. Thus, enhanced water solubility, decreased toxicity, biocompatibility, stability and protection in the biological milieu has been achieved by functionalizing the terminal groups of dendrimers with poly(ethylene glycol) chains (PEG). PEG chains are crucial for modifying the behavior of the drugs themselves, such as peptides or proteins [18-20] or those of dendritic drug carriers [21-25] as it has been extensively applied with liposomes [26-29], which are well-established drug delivery systems. Furthermore, as is the case with other targeted nanoparticles [30-35], specificity to certain cells has been achieved by attaching targeting ligands on the surface of dendritic polymers. This binding to cell receptors has further been amplified by taking advantage of the so-called polyvalent interactions [36-38] attributed to the proximity of the targeting ligands on the dendritic surface. Transport through cell membranes, as mentioned above, has been facilitated by attaching molecular transporters on to the nanocarrier. The application of cell penetrating peptides [39-43] and specifically of arginine-rich derivatives, which exhibit enhanced translocation ability, has been the basis for preparing molecular transporters based on guanidinylated dendritic nanoparticles [44]. Finally, cationization, obtained through protonation, quaternization or guanidinylation, of the so-obtained dendritic nanocarriers, followed by their interaction with the negatively charged genetic material, leads to the preparation of complexes which are used as gene vectors.

Following endocytosis, the dendritic/DNA complexes which are now located inside the endosome should have the property to be released from it. For this reason, dendritic polymers bearing many secondary and tertiary amino groups, which become protonated at a weakly acidic environment, are usually selected. These amino groups suppress the lowering of pH in endosomes and lysosomes by interacting with protons and prohibiting, therefore, their degradation in the lysosome. In addition, endosome buffering by these polymers induces osmotic swelling of the endosome interior, engendering rupture of the endosome and subsequent release of DNA into the cytoplasm [45]. The function of these secondary and tertiary amino groups of polymers is called the 'proton sponge effect' [46].

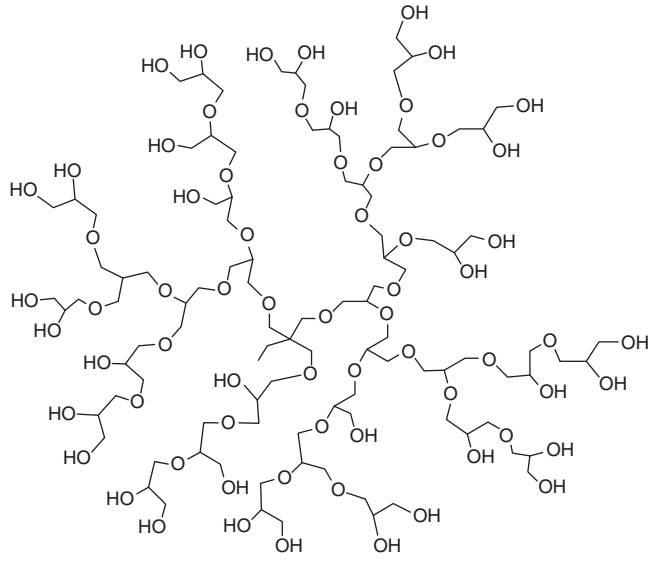
Several extensive recent reviews on dendrimers as gene transfection agents can be found in the literature [47-50]. The present non-exhaustive review will critically deal with the preparation of effective gene delivery systems by adopting the multifunctionalization strategy of modifying basic dendritic scaffolds. The dendritic systems discussed have been mostly evaluated *in vitro*, which is the first step for probing structure–activity relationships, before selecting those complexes that could be promising for *in vivo* testing. Various dendritic polymers have been used as starting materials, such as the symmetrical poly(amidoamine) dendrimer (PAMAM), the poly(propylene imine) dendrimers (PPI), having either the diaminopropane or the diamino-butane core (the latter referred to as DAB in the literature), PAMAM dendrons, and also random hyperbranched polymers such as poly-glycerol, PG and poly(ethylene imine) (PEI), the chemical structures of which are shown below. The examples presented in this review belong to three out of the four subclasses of dendritic polymers mentioned above and will highlight on these dendritic polymers that gene delivery properties have been primarily improved through molecular engineering of their



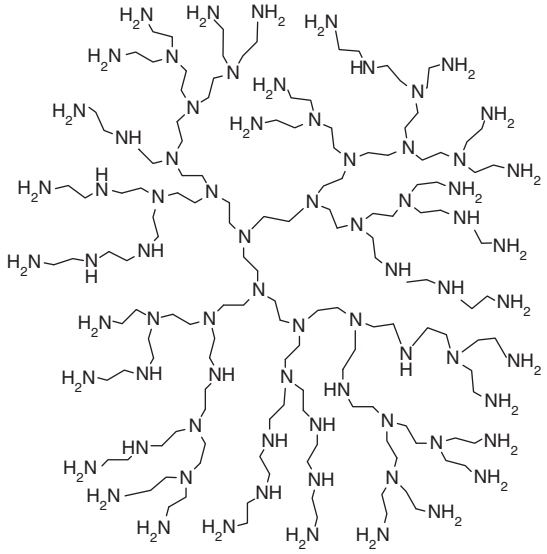
PAMAM



PPI



PG



PEI

surface groups and secondly by proper selection of their dendritic scaffold.

2. Dendrimers as gene carriers

The amino-terminated poly(amidoamine) or poly(propylene imine) dendrimers, bearing tertiary amino groups in their interior, exhibit the so-called 'proton sponge effect', fulfilling to a significant extent the criteria for gene transfection of cells, a topic that has recently been reviewed [49,50]. Transfection complexes result from ionic interactions between the phosphate moieties located on the DNA backbone and the primary and tertiary amine moieties of the dendrimers, which are positively charged under physiological conditions. More recent work deals with simple functionalization or multifunctionalization of these dendrimers for improving their transfection efficiency while simultaneously maintaining low toxicity.

In an attempt to lower the cytotoxicity of PAMAM, hydroxylation of its primary amino groups resulted in the preparation of PAMAM-OH dendrimers. These dendrimers are structurally similar to PAMAM, except that their surface amino groups have been replaced by hydroxyl groups [51]. The absence of surface primary amino groups in PAMAM-OH

renders this polymer nearly neutral, which might be advantageous in terms of cytotoxicity. However, PAMAM-OH is nearly unable to form complexes with DNA because of the low pK_a of interior tertiary amino groups. For this purpose, the synthesis and characterization of internally quaternized PAMAM-OH (QPAMAM-OH), differing in the degree of quaternization, has been conducted.

The internal quaternary ammonium groups of QPAMAM-OH interact with negatively charged DNA, while they preserve a neutral polymer and/or polyplex surface. These QPAMAM-OH/DNA polyplexes were round-shaped, more compact and of small size as the charge ratio increased. Although the transfection efficiency of functional QPAMAM-OH derivatives was lower by one order of magnitude than the parent PAMAM, the QPAMAM-OH/DNA particles exhibited reduced cytotoxicity compared to PAMAM and PEI. The shielding of the interior positive charges by surface hydroxyl groups may be the reason for this behavior.

The lower efficiency of the non-viral gene delivery systems compared to viral vectors has been addressed by the strategy of introducing cell-targeting ligands or cell penetrating peptides to the carrier, for facilitating the intracellular delivery of bioactive molecules [39-43,52]. Arginine-rich peptides have exhibited enhanced translocation ability, which was attributed to the presence of the guanidinium moiety [43,44,52]. This group is capable of forming hydrogen bonds coupled with electrostatic interactions [53] with phosphate, carboxylate or sulfate group located on the surface of cell membranes.

The synthesis of L-arginine functionalized PAMAM dendrimer (PAMAM-Arg) has been reported [54], the surface of which was decorated with L-arginine residues. By the introduction of arginine moieties, gene delivery efficiency was greatly enhanced compared to the starting PAMAM. Its efficiency was comparable to PEI for HepG2 and primary rat vascular smooth muscle cells, and was more efficient in the case of Neuro 2A cells than PEI and lipofectamine. L-Lysine functionalized PAMAM (PAMAM-Lys), which was used as a control, showed slightly better transfection efficiency in HepG2 cells compared to PAMAM, while increased efficiency was not observed in primary cells. In conclusion, arginine functionalized PAMAM is readily prepared and possesses high transfection efficiency with relatively low cytotoxicity. These properties render PAMAM-Arg a promising non-viral vector for both *in vitro* and *in vivo* applications.

Another step towards the preparation of multifunctional dendritic polymeric vectors [55] is the conjugation of arginine moieties to the periphery of PAMAM-PEG-PAMAM block copolymer. In this case, PAMAM stands for half of the ordinary symmetric dendrimer. Due to the introduction of PEG-chain in this fifth generation PAMAM derivative, Arg-PAMAM-PEG-PAMAM-Arg bearing c.a. 36 arginine residues on the external amino groups of the polymer should exhibit good water solubility, while the guanidinium groups should facilitate molecular transport through the cell's membrane [43]. The polymer forms polyplexes with plasmid DNA, the average size of which was about 200 nm. Positive potential values (+22 to +28 mV) of polyplex indicate the formation of positively charged stable particles and suggest that large dendritic blocks with high positive charge may not be fully shielded by PEG chains even after PEG-coated complex formation. As anticipated, the complex shows good water solubility due to the polymer's PEG core and also shows low cytotoxicity. Enhanced transfection efficiency of the polymer was found in comparison to the starting non-arginine bearing polymer on various cell lines. Moreover, in view of various cellular uptake inhibitor treatments during transfection, the cellular uptake of Arg-PAMAM-PEG-PAMAM-Arg, leading to effective transfection, is thought to be independent on one exclusive pathway but has the possibility of using multiple pathways (caveolae-, clathrin- and macropinocytosis-mediated pathways), contrary to the caveolae-dependent uptake of the PAMAM-PEG-PAMAM, which lacks arginine moieties. Moreover, this particular cellular uptake pathway of Arg-PAMAM-PEG-PAMAM-Arg is considered to be one of the important reasons for the enhanced transfection efficiency.

In a recent interrelated study [56], a diaminobutane poly(propylene imine) dendrimer (DAB) with 32 terminal amino groups was completely or partially functionalized with guanidinium groups. For the partially guanidinylated derivatives the remaining toxic primary amino groups of the dendrimers were reacted with propylene oxide affording the corresponding hydroxylated derivatives. Five derivatives were investigated, that is the non-guanidinylated one and four others bearing 6, 12, 24 or 32 guanidinium groups. These guanidinylated dendrimers formed complexes with plasmid DNA affording the corresponding polyplexes. Transfection efficiency was assessed employing HEK 293 and COS-7 cell lines, while the serum effect was studied in HEK 293 cells. It was found that full replacement of primary amino groups with the hydroxylated moieties resulted in complete loss of transfection efficiency. In contrast, guanidinylation of the parent dendrimer resulted in significant enhancement of its transfection efficiency. This enhancement depended on the degree of guanidinylation of the dendrimer, the cell line used and the presence or absence of foetal bovine serum (FBS), as depicted in **Figure 2**. The fully guani-dinylated dendrimer exhibited the highest transfection efficiency under all the conditions investigated. This may be attributed to the fact that the phosphate groups of DNA compete with the phosphates resident on cell membranes for neutralization of the dendrimer guanidinium groups and, therefore, complete or significant guanidinylation is required for effective transfection. It was also found that the derivative with 12 guanidinium groups exhibited the lowest toxicity, however, this may well be attributed to its decreased cell internalization [44] (and hence lower transfection efficiency) because of the smaller number of surface guanidinium groups. In conclusion, guanidinylation leads to dendrimeric derivatives that combine satisfactory transfection efficiency and cytotoxicity.

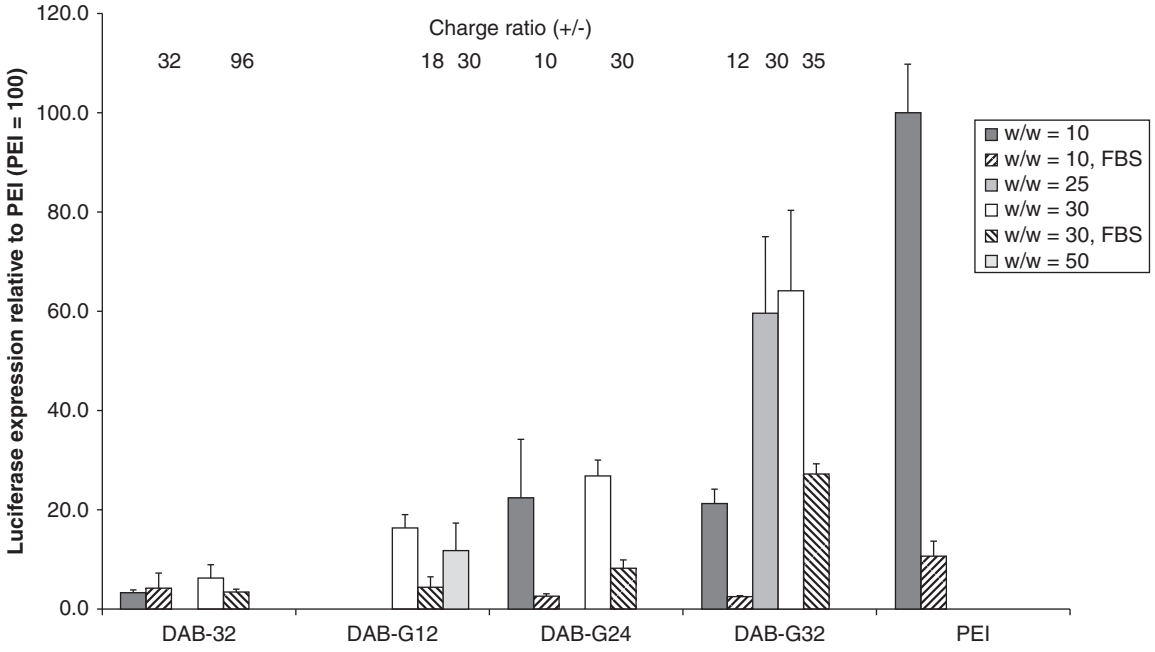


Figure 2. Transfection efficiency of guanidinylated poly (propylene imine) dendrimers against the HEK 293 cell line, in the absence (filled bars) and in the presence (hatched bars) of foetal bovine serum (FBS) at various w/w ratios. The corresponding charge ratios are also shown for each polymer. Luciferase expression was normalized by total cellular protein and a DNA dose of 2 μg/well was used. Data are represented as the mean ± SD (n = 6). Results are presented relative to PEI (RLU/μg protein = 208).
Reproduced from reference [53].

Enhanced gene transfection is also observed when the guanidinium group is not introduced directly in poly(propylene imine) dendrimer with a diaminopropane core (PPI), but through the attachment of arginine on its external amino groups, as recently reported [57]. Specifically, arginine was introduced in poly(propylene imine) dendrimer with eight terminal amino groups affording PPI-G2-Arg, which was applied as gene delivery system. The size of PPI-G2-Arg polyplex was measured to be about 200 nm at a charge ratio of 150. PPI-G2-Arg displayed 80 – 90% cell viability even at a 150 μg/ml concentration. Transfection efficiency of PPI-G2-Arg was found to be high, comparable to that of PEI 25 kD, and to be 8 – 214 times higher than that of unmodified PPI-G2 on HeLa and HEK 293 cells, depending on the charge ratio examined. Moreover, PPI-G2-Arg showed four times higher transfection efficiency than PEI 25 kD, when it formed complexes with 10 μg pDNA due to its low cytotoxicity on HeLa cells. Finally, PPI-G2-Arg showed a transfection efficiency 2 – 3 times higher than PEI 25 kD on HUVECs, showing its potency as a gene delivery carrier for primary cells.

In this context, for enhancing the transfection efficiency of PAMAM dendrimers phenylalanine or leucine moieties have been introduced at the end of their branches [58]. Efficient transfection was obtained through synergy of the proton sponge effect, which is induced by the internal tertiary amino groups of the dendrimer, and the hydrophobic interaction, attributed to hydrophobic amino acid residues on the surface of dendrimer. Specifically, dendrimers with 16, 29, 46 and 64 terminal phenylalanine residues have been prepared by the interaction of fourth generation PAMAM (G4) with L-phenylalanine. Transfection of these phenylalanine-modified dendrimers ([Phe]64-G4) for CV1 cells increased with the increasing number of the terminal phenylalanine residues, except for the dendrimer with 64 phenylalanine residues, which showed poor water solubility and hardly formed a complex with DNA at neutral pH. However, under weakly acidic conditions, the dendrimer with 64 phenylalanine residues formed a complex with DNA, therefore achieving highly efficient transfection (**Figure 3**). In contrast, the attachment of L-leucine residues ([Leu]63-G4) did not improve transfection efficiency compared to the parent dendrimer. This is probably due to the relatively lower hydrophobicity of this amino acid. The phenylalanine-modified dendrimer exhibited low cytotoxicity and a higher transfection activity compared to some widely used transfection reagents.

3. Dendrons as gene carriers

The strategy of multifunctionalization of the dendritic scaffold has also been applied to PAMAM dendrons affording vectors exhibiting high gene transfection efficiency. Thus, Langer *et al.* [59] designed and prepared a series of multifunctional gene delivery polymers based on a PAMAM

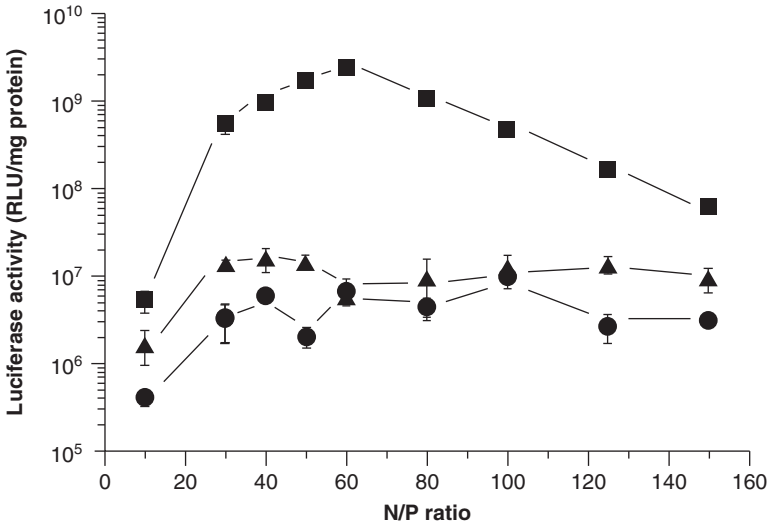


Figure 3. Comparison of transfection efficiency of leucine- and phenylalanine-modified fourth generation PAMAM dendrimers (G4). Luciferase activities of CV_1 cells treated with (Phe)64-G4 polyplex prepared at pH 5.0 (■) and (Leu)63-G4 polyplexes prepared at pH 7.4 (▲) and 5.0 (●) containing 1 μg DNA with varying N/P ratios. Each bar represents the mean ± SD (n = 3).
Reproduced from reference [55].

dendron and linear PEG exhibiting cell targeting, DNA binding and endosomal buffering capacity that can be independently tuned in a modular fashion in order to breach the barriers for an effective gene delivery. In this multi-functional derivative, mannose is the targeting ligand, PEG-chain prevents protein opsonization and plasma clearance, while the internal amino groups promote DNA binding and escape from endosomes to the cytoplasm.

For evaluating the transfection efficiency of DNA complexes, two cell types were transfected: P388D1 murine macrophages bearing a mannose receptor and HepG2 human hepatocytes bearing an asialoglycoprotein receptor (for galactosilated ligands). The levels of luciferase reporter gene expression in macrophages (in the absence of serum) with optimized formulations of ligand-functionalized polyplexes, control polyplexes bearing no ligand, ligand-functionalized polyplexes in the presence of soluble ligand and PEI are shown in **Figure 4A**. The G6.0 mannose-bearing polyplexes demonstrate transfection 1.6- to 1.8-fold higher than PEI, which is the most widely used commercially available polymer for *in vitro* transfection. The G5.0 polyplexes mediate reporter expression levels approximately 1.3-fold higher than PEI, whereas the G4.0 polyplexes (as well as G3.0 and G2.0, data not shown) transfect at low levels comparable to naked DNA. The highest transfection levels were observed in polymer/DNA mass ratios under 50:1 in all systems (under 20:1 in G6.0), presumably because of the effects of toxicity at high concentrations.

Polyplexes non-bearing mannose ligand exhibited significantly lower transfection efficiencies and competitive inhibition of mannose receptors by an excess of soluble ligand virtually silenced reporter-gene expression without affecting expression levels in the positive and negative controls (**Figure 4A**). These data further support the hypothesis of cellular internalization by means of specific receptor-mediated endocytosis. Finally, macrophages were transfected in the presence of a 10% serum-containing medium to probe the serum stability of PEGylated polyplexes (**Figure 4B**). Fourfold transfection enhancements were observed relative to PEI, most likely because of the stealth effect imparted by PEG, which is known to lower particle agglomeration by attenuation of opsonization of serum proteins.

Transfection of HepG2 hepatocytes by linear-dendritic polyplexes bearing the galactose ligand is shown in **Figure 5**. In the absence of serum, optimized formulations of G6.0 and G4.0 ligand-functionalized polyplexes transfect significantly more efficiently ($p < 0.06$) than control polymers with no ligand (**Figure 5A**). Moreover, G6.0-, G5.0- and G4.0-targeted systems mediate transfection levels within one order of magnitude of PEI in the absence of serum and as much as eightfold more than PEI in the presence of serum (**Figure 5**). Optimal polymer/DNA mass ratios were in the range 100:1 – 200:1 for all the systems studied. These data suggest that the hepatocyte-targeted polyplexes are serum-stable and demonstrate enhanced transfection because of a cell-specific receptor-mediated process. Interestingly, expression levels were unaffected by the presence of an excess of soluble galactose, a finding that may arise from the multivalent nature of ligand binding by the asialoglycoprotein receptor. It is, therefore, suggested that multivalent ligand presence through synthetic multimeric galactose ligands may yield enhanced targeting relative to the monomeric species. In all cases, linear–dendritic systems do not show toxicity up to concentrations one to two orders of magnitude greater than those at which PEI was toxic.

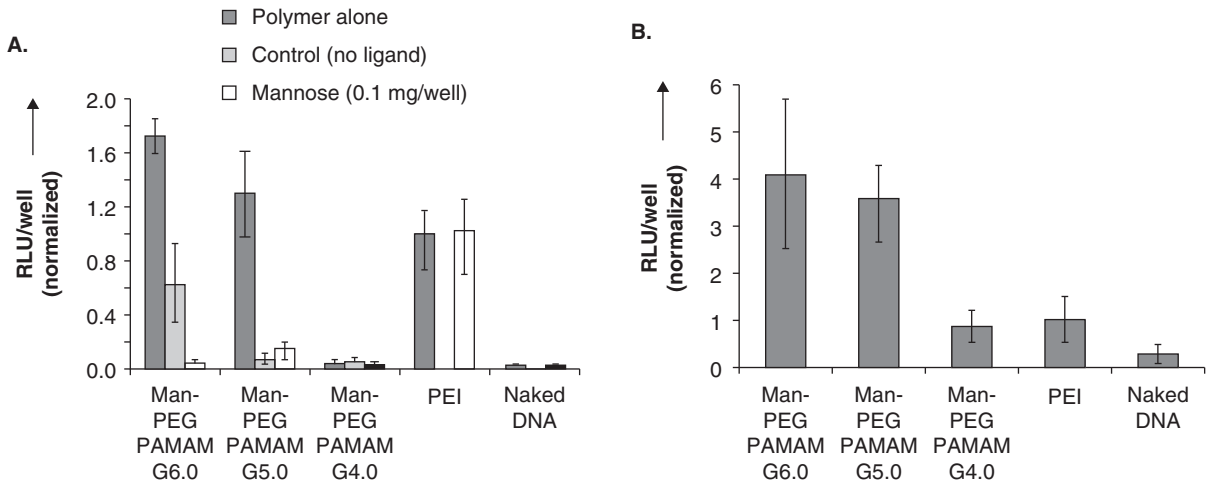


Figure 4. Transfection of P388D1 macrophages bearing the mannose receptor. (**A**) Transfection by linear–dendritic polyplexes with and without the mannose ligand and in the presence of soluble mannose (0.1 mg well^{-1}); Results normalized to an optimized formulation of PEI (2:1 PEI/DNA, serum-free, no free mannose added). (**B**) Serum stability is demonstrated through transfection in the presence of serum proteins. Results normalized to an optimized formulation of PEI (2:1 PEI/DNA, 10% serum, no free mannose added). All results are given as an average ± standard error.
Reproduced from reference [56].

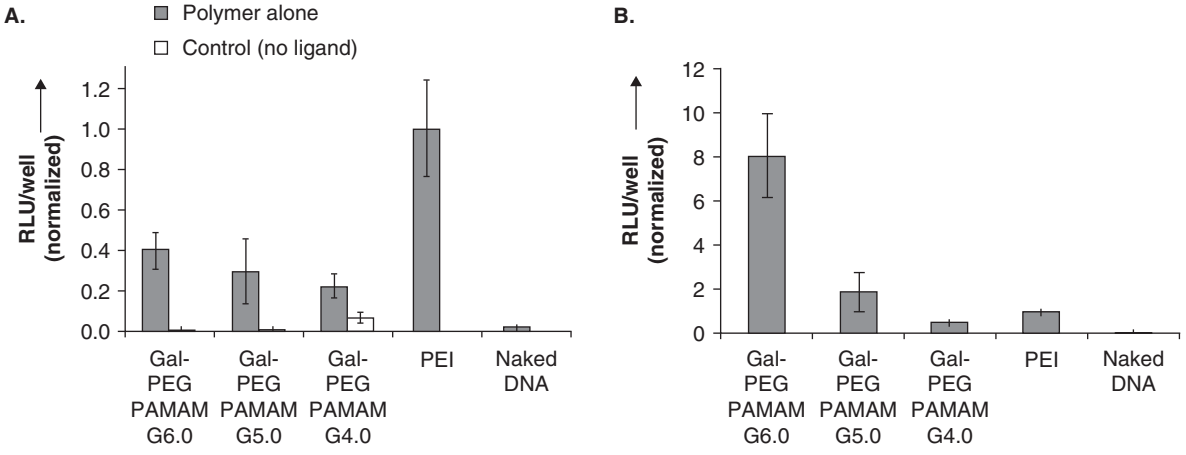


Figure 5. Transfection of HepG2 hepatocytes bearing the asialoglycoprotein receptor. (**A**) Transfection by linear-dendritic polyplexes with and without the galactose ligand. Results normalized to PEI = 1.0 (serum free, no free galactose added). (**B**) Serum stability is demonstrated through transfection in the presence of serum proteins. Results normalized to PEI = 1.0 (10% serum, no free galactose added). All results are given as an average ± standard error.
Reproduced from reference [56].

Furthermore, in order to prepare dendritic multifunctional gene delivery vectors, an analogous system was prepared [60]. This dendron was bearing multiple functional moieties arranged in a linear and modular fashion, such that new functional moieties could be added, modified, or removed affecting, respectively, existing properties. The system, based on a linear dendritic 'hybrid' polymer, consists of a linear PEG and a PAMAM dendron, possesses functional groups for electrostatic DNA condensation, endosomal escape *via* the proton sponge effect, reduction of non-specific tissue interactions and tissue targeting by bearing a peptide ligand (denoted peptide 1, sequence = WIFPWIQL) capable of selectively targeting glucose-regulated protein-78 kDa (GRP-78). GRP-78 is a functional tumor antigen identified in human cancer patients, and peptide 1 has been used to target GRP-78 in tumors *in vitro*, *in vivo* (in mouse models of breast and prostate cancer) and in human patient-derived tumor samples.

To test the ability of these hybrid polymers functionalized with a peptide to target and deliver plasmid DNA to cells expressing GRP-78, DU145 prostate carcinoma cells were

transfected, which have been shown to bind and internalize peptide 1 in a GRP-78-dependent manner. Peptide-functionalized hybrid polymers can transfect DU145 cells at levels nearly 10-fold higher than an optimized formulation of branched PEI (25 kDa). Moreover, this process is receptor-mediated, evidenced by the fact that cells transfected using polymers lacking a peptide ligand, as well as cells transfected with peptide-functionalized polymers in the presence of polyclonal anti-GRP-78 anti-serum (to block receptor–ligand binding), yielded significantly lower levels of transfection.

The inherent asymmetry of the dendron structure facilitates structural modification in a controlled mode, that is either at the surface or at the focal point. This is not possible to the same extent with polymers or spherical dendrimers. The typical 'dendron strategy' is to use a branched molecule with a polycationic surface capable of interacting with the anionic phosphates on DNA and then subsequently functionalize the focal point with a hydrophobic moiety for facilitating membrane fusion or a polar group assisting water solubility and biocompatibility, for example PEG. In this context, a series of dendrons based on the Newkome dendritic scaffold that displays a naturally occurring polyamine (spermine) on their surface have been prepared [61]. These dendrons have been shown to interact with DNA in a generation-dependent manner; the more highly branched dendrons exhibit a strong multivalency effect for the spermine surface groups. In a more recent work [62], the ability of these dendrons to transfect DNA into human breast carcinoma cells, MDA-MB-231, and murine myoblast cells, C2C12, was investigated. Although the dendrons are unable to transfect DNA in their own right, they are capable of delivering DNA *in vitro* when administered with chloroquine, which facilitates the escape of complexed DNA from the endosome. The cytotoxicity of the dendrons was determined using the XTT assay, and it was found that the dendrons were non-toxic either alone or in the presence of DNA. However, when administered with DNA and chloroquine, the most highly branched dendron did exhibit some cytotoxicity. The low toxicity of these dendrons, although they exhibit modest transfection efficiency, renders the systems promising for cell transfection. It should be noted that even in the presence of chloroquine, the transfection efficiencies observed are too low for these structures to have clinically significant activity *in vivo*. In fact, transfection efficiency would need to be improved by several orders of magnitude. The observed efficiencies are in line with low molecular weight PAMAM dendrimers, which only form complexes with DNA at high dendrimer nitrogen/DNA phosphate (N/P) ratios. In general, an increase in molecular weight of PAMAM yields a significant increase in transfection efficiency, and the same trend may be expected for higher molecular weight analogs of these dendrons. However, it is also possible to make other structural modifications to the structure of dendrons G1 and G2, which might be expected to enhance transfection efficiency and would avoid the tedious and time-consuming syntheses required for higher generation dendritic molecules [62]. To this objective, that is of preparing synthetically modified versions of these non-toxic dendrons, work should be pursued.

4. Random hyperbranched polymers as gene carriers

In early experiments [50,63] employing PAMAM as a gene vector it was found that its partially fragmented dendrimers are more appropriate for gene delivery than the intact dendrimers and a fragmentation (activation step) consisting of hydrolytic cleavage of the amide bonds was performed to enhance the transfection. It has been concluded from several investigations that the spherical shape of dendrimers is not advantageous in gene delivery. Partially degraded dendrimers have a more flexible structure and form a more compact complex with DNA than the starting dendrimer; therefore, the partially degraded polymer is preferable for gene delivery by the endocytic pathway [64]. If one excludes the disadvantage of polydispersity exhibited by hyperbranched polymers, which in fact can be controlled, the possibility of forming compact complexes bearing a great number of functional groups, in analogy with degraded dendrimers, may become advantageous in their application as gene delivery systems. Furthermore, low toxicity and biocompatibility of random hyperbranched polyether polyols [65] are incentives for developing novel functional derivatives based on these polymers that could be employed as gene delivery systems following proper functionalization of their surface groups.

Within the context of developing new gene delivery systems, functional random hyperbranched polymers were prepared [66] based on polyether polyols. Thus, partial functionalization of the hyperbranched polyether polyols with either 4, 8 and 12 quaternary (6, 11 and 17% molar coverage) or 4 and 21 tertiary ammonium groups (6 and 31% molar coverage) was achieved through the interaction with glycidyltrimethylammonium chloride, or 2,3-epoxypropyl-diethylamine, respectively. The introduction of the quaternary or tertiary groups renders polyglycerol cationic under physiological pH for interacting with negatively charged DNA and leading to the formation of complexes.

The resulting dendritic complexes with DNA, following a physicochemical characterization, were investigated *in vitro* as far as their transfection properties were concerned on human embryonic kidney (HEK) 293 cell line, which is widely employed for transfection studies, and also on monkey kidney fibroplast COS-7 cell line. These complexes showed marginal toxicity when tested on HEK 293 cells.

Quaternized derivatives yield large polyplexes that exhibit similar or even better (for PG-Q-2) transfection efficiency compared to that of PEI, while limited cytotoxicity in mammalian cells rendered these dendritic derivatives more attractive gene delivery carriers. Variation in the degree of quaternization of the parent dendrimer affects the transfection efficiency and cytotoxicity of the derivatives obtained while

the introduction of tertiary amino groups did not result in any increase in the transfection efficiency of the parent polymer. The observed transfection efficiency of the quaternized polymers has been attributed to the destabilization of the lysosomal membrane originating from the interaction of these cationic polymers with the anionic moieties located at the membrane.

For upgrading PEI's gene transfection properties, a novel water soluble lipopolymer was synthesized [67] by reacting cholesteryl chloroformate with the secondary amino groups of branched PEI of 1800 and 10000 Da. Conjugation through PEI's secondary amino groups affords this novel lipopolymer (PEI-Chol). The advantage over previously synthesized lipopolymers is that they leave intact the primary amino groups for conjugation, as the latter play a significant role in DNA condensation. Interestingly, only one cholesterol moiety was grafted onto each PEI molecule, leaving enough space for the interaction of the PEI's primary amino groups with the DNA. PEI-Chol lipopolymers were characterized, including their buffering capacity, DNA condensation, *in vitro* transfection efficiency and cell viability. The acid-base titration indicated high buffering capacity of the polymers around the pH range of 5 – 7, which indicated their potential for buffering in the acidic pH environment of the endosomes. The *in vitro* transfection of PEI-Chol/pCMS-EGFP complexes in Jurkat cells showed high levels of expressed Green Fluorescent Protein (GFP), with little toxicity as determined by flow cytometry. These novel water soluble lipopolymers provided good transfection efficiency with other desirable characteristics such as water solubility, free primary amino groups for efficient DNA condensation and high buffering capacity that indicated the possibility of efficient endosomal release.

The structural features required for an effective dendritic gene vector can, in principle, be satisfied in a PEI-poly(ethylene glycol)-folate derivative, PEI-PEG-folate, which was recently synthesized [68] and its efficiency as a gene carrier was tested. This multifunctional random hyperbranched PEI of MW = 25000, simultaneously combines protective and targeting properties. In this study, the PEI-PEG-folate nanocarrier was tested for its capacity to form complexes with plasmid DNA and be transfected to folate receptor overexpressing cells (GFP-KB cells) that produce exogenous GFP. A special plasmid system (pSUPER-siGFP) was prepared, that carried a siRNA-expressing sequence, used for inhibiting the expression of exogenous GFP in mammalian cells. The pSUPER-siGFP/PEI-PEG-FOL complexes inhibited GFP expression of KB cells more effectively than pSUPER-siGFP/PEI. These results indicate that folate receptor-mediated endocytosis is a major pathway in the process of cellular uptake.

Vascular endothelial growth factor (VEGF), a potent angiogenic molecule specific for vascular endothelial cells, is overexpressed in most tumors and closely associated with tumor growth and metastasis. In contrast, it has been shown that a soluble fragment of VEGF receptor Flt-1 (sFlt-1) has anti-angiogenic properties by way of its antagonist activity against VEGF. For anti-angiogenesis, a targeted polymeric gene delivery system comprising of PEI-g-PEG-RGD was developed [69] by incorporating the $\alpha_v\beta_3/\alpha_v\beta_5$ integrin-binding RGD peptide, ACDCRGDCFC, into the cationic PEI *via* a hydrophilic PEG spacer (PEI-g-PEG). The complex of sFlt-1 gene with PEI-*g*-PEG-RGD conjugate efficiently inhibited, *in vitro*, the proliferation of cultured endothelial cells by blocking the binding of VEGF to the membrane bound Flt-1 receptor. These findings suggest that the combination of targeted gene carrier and sFlt-1 has the potential of being an efficient tool for the anti-angiogenic gene therapy in the treatment of cancer.

In further studies [70], PEI-g-PEG-RGD/pCMV-sFlt-1 complexes were evaluated in terms of tumor growth inhibition *in vivo*. Complexes were repeatedly injected systemically *via* the tail vein into subcutaneous tumor-bearing mice. As a result, tumor growth was inhibited in the PEI-g-PEG-RGD/pCMV-sFlt-1 injected group. However, this effect was not observed in the PEI-g-PEG/pCMVsFlt-1 or PEI-g-PEG-RGD/pCMV-GFP control groups. Moreover, the survival rate increased in the PEI-g-PEG-RGD/pCMV-sFlt-1 group compared to the control group. These results suggest that delivery of pCMV-sFlt-1 using PEG-g-PEG-RGD may be effective for anti-angiogenic gene therapy.

5. Conclusion

Designed functionalization of dendrimers, dendrons and random hyperbranched polymer scaffolds results in the preparation of nanocarriers of low toxicity, the ability to form complexes with genetic material, specificity to certain biological cells and transport ability through their membranes. Depending on the degree and type of functionalization, gene delivery systems that fulfil one or more of the above characteristics were prepared. Polyvalent interactions attributed to the proximity of the functional groups on the external surface of the dendritic polymers are in all cases crucial for efficient gene delivery of these nanoparticles.

6. Expert opinion

In the era of genetic information and identification of target genes for the treatment of various diseases, the development of efficient gene delivery vectors has emerged. Although nucleic acid therapeutics consist of a new generation of highly effective drugs; their high susceptibility to degradation restrains their wide usage. Therefore, the development of safe and efficient gene delivery vectors is a challenging task in gene therapy. Such vectors should protect DNA from enzymatic degradation, bind to target cells, have the property to be transported through the cell membrane, escape from the endosome following endocytosis and release the therapeutic DNA or RNA resulting in gene expression. In addition, vectors should be non-toxic or induce an immune response.

Viral and non-viral vectors have primarily been employed; viral vectors, although more efficient compared to non-viral vectors, are plagued by safety concerns, including virus replication and inflammatory reactions. However, the transfection efficiency of non-viral vectors is generally lower compared to that of viral vectors, primarily due to the lack of receptor recognition, endosome escape and poor nuclear targeting. However, non-viral vectors share several advantages such as the facile preparation at a reasonable cost, superior safety profiles and low immunogenicity, while it is possible to enhance specificity through the introduction of targeting ligands. Furthermore, non-viral vectors can protect DNA from degradation by nucleases in the lysosome and bloodstream.

Following multifunctionalization of commercially available or custom-made dendritic polymers, their complexes with DNA fulfil to a significant degree the above-mentioned requirements. Thus, by functionalizing the terminal groups of dendritic polymers with PEG chains, they acquire enhanced water solubility, decreased toxicity, biocompatibility, stability and protection in the biological milieu. Furthermore, specificity for certain cells has been achieved by attaching targeting ligands on the surface of dendritic polymers. The binding to cell receptors has further been amplified by taking advantage of the so-called polyvalent interactions, which are attributed to the proximity of the targeting ligands on the dendritic surface. Transport through cell membranes has also been achieved by attaching molecular transporting moieties on the nanocarrier. In fact, the application of cell penetrating peptides and specifically of arginine-rich derivatives, which exhibit enhanced translocation ability, has been the basis for preparing molecular transporting guanidinylated dendritic nanoparticles. Also, following endocytosis, the dendritic/DNA complexes located inside the endosome potentially have the property to be released. This was achieved by employing dendritic polymers which bear many secondary and tertiary amino groups that are protonated under weakly acidic conditions. These amino groups suppress the lowering of pH in endosomes and lysosomes by adsorbing protons and prohibiting their degradation in the lysosome. In addition, endosome buffering by these polymers induces osmotic swelling of the endosome interior, engendering rupture of the endosome and the subsequent release of DNA into cytoplasm. Detailed investigations on the uptake and intracellular fate of the genetic material and on factors that *ex vivo* and *in vivo* affect these processes, would allow a deeper understanding of the prenuclear stage of transfection, and help to increase the efficiency of this process.

Taking into consideration the reported work and primarily the recent highlights in the development of efficient non-viral gene vectors obtained through multifunctionalization of dendritic polymers, one is led to believe that this strategy will be the method of choice in the next five years. The facile and reproducible synthetic procedures and further structural elaboration of dendritic polymers secure the development of even more efficient systems, which are certainly a prerequisite for *in vivo* applications. The framework of research has been set and, therefore, what is needed is the design of optimal dendritic polymers fulfilling most or rather all of the requirements needed in order to prepare effective gene delivery systems.

Declaration of interest

The authors state no conflicts of interests and have received no payment in the preparation of this manuscript.

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