Dendritic vectors for gene transfection†

Marine Guillot-Nieckowski, Sara Eisler and François Diederich*

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The wonderful versatility of dendritic structures is demonstrated by their ability to act as efficient gene transfection vectors. This review will tell the story of how dendrimers came to be exploited for gene delivery, outline the different types of structures that have proven to be successful vectors and demonstrate how structural variation is being used as a tool to increase efficiency and probe structure–activity relationships.

Gene therapy

With the conclusion of the human genome-sequencing project, the medical research community has an unparalleled opportunity to understand and cure diseases on a genetic level. Gene therapy aims to deliver DNA, RNA, or antisense sequences that alter gene expression within a specific cell population, thereby manipulating cellular processes and responses. A number of genetic deficiencies as well as acute diseases, in particular cancer, are therefore targeted. Just recently, the first commercial gene therapy was approved for the treatment of patients suffering from head and neck cancers.[1] A number of on-going projects are also currently in the first phases of clinical trials. Gene therapy has thus become a realistic opportunity, though many limitations still need to be overcome.

The main issue of gene therapy is not the cellular expression of an exogenous gene itself, but the development of safe and efficient gene delivery systems. "Cell transfection" refers precisely to the process of delivering genetic material to the cell nucleus. Therapeutic approaches focus on two delivery modes.[2,3] The *in situ* strategy refers to the direct injection of genetic material into a localized body part, *e.g.*, a tumor mass, whereas the *ex vivo* strategy involves harvesting targeted cells from the patient, transfecting them *in vitro*, and after successful genetically modification, re-implanting them in the body.

In the midst of such challenging biomedical technologies, *in vitro* transfection is being explored as a powerful tool for basic research achievements. Stable transfected cell lines provide essential insight into gene function, and transient gene expression in cultured cells is a straightforward process to evaluate the efficacy of new potential gene carriers.

Initial research efforts focused on delivering naked DNA, but its huge size and its quick degradation, *e.g.*, by nucleases, are major obstacles. Naked DNA delivery and expression is only possible by physical methods such as gene gun, hydrostatic pressure, electroporation, continuous infusion and sonication.[4] However, these techniques are not applicable to every cell line and often reduce cell viability, as they compromise the integrity of the cell membrane. Several alternative approaches have therefore been developed to ensure compaction and protection of DNA until it reaches the cell nucleus.

The vast majority of efficient DNA carriers, especially those used for clinical applications or first phase clinical trials, are engineered viral or adenoviral vectors. Viruses have sections of

Laboratorium für Organische Chemie, ETH-Zurich, Hönggerberg, HCI, 8093 Zürich, Switzerland. E-mail: diederich@org.chem.ethz.ch; Fax: +41 44 63 21109; Tel: +41 44 63 22992

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Sara Eisler received her PhD in 2004 at the University of Alberta under the supervision of Prof. Rik R. Tykwinski. Her doctoral thesis focused on the synthesis of conjugated polyynes and the study of their non-linear optical properties. She was subsequently awarded a fellowship from the National Science and Engineering Research Council for postdoctoral research and joined the research group of Prof. Diederich at ETH Zürich to investigate gene transfection mediated by dendritic vectors. She is currently designing supramolecular materials in the group of Prof. Jim Wuest at the University of Montreal.

Marine Guillot-Nieckowski graduated in 2002 from the Ecole Nationale Supérieure de Chimie in Paris, France. In 2006 she completed a PhD in natural sciences under the supervision of Prof. F. Diederich at ETH Zürich, Switzerland. This work focused on optimization of amphiphilic dendritic vectors for gene transfection. Her postdoctoral studies in physical organic chemistry are currently being conducted at the University of Tokyo under the supervision of Prof. E. Nakamura.

their genome removed to make them replication deficient, allowing the insertion of genes encoding for therapeutic proteins. The limited space available in the viral genome combined with expensive production requirements and major safety issues related to over-reaction of the immune system have inspired the development of alternative non-viral strategies.[5,6]

Synthetic vector systems have much to offer. They can be structurally varied, are relatively safe to produce, and are able to carry large and diverse genetic material into cells. In comparison with sophisticated and adaptable viruses, the main drawback with these relatively simple systems remains the low level of gene delivery. Studying their biological and physico-chemical properties by structural modification represents an exciting challenge for chemists as this approach constantly provides new and valuable information for the design of more complex systems. A general overview of gene transfection mechanisms for synthetic vectors will be presented first, and the second part of this review will focus on the synthetic strategies developed by chemists to optimize gene transfection efficiency mediated by dendrimers.

Synthetic vectors

The two major approaches to non-viral gene delivery involve the combination of nucleic acids either with cationic lipids (lipoplexes) or with cationic polymers (polyplexes). Lipoplexes were pioneered by Behr[7,8] and Felgner *et al.*[9] and are extensively used for nucleic acid transfection, even *in vivo*. Many formulations of cationic lipids contain a zwitterionic or neutral colipid, such as DOPE[10] or cholesterol, to enhance transfection. More than thirty such products are commercially available.[11] Chemists have successfully varied the structure of small cationic lipids leading to the establishment of numerous structure–activity relationships.[12–14]

Cationic polymers also vary widely in their structures, ranging from linear to highly branched molecules that can be specifically tailored by choosing the appropriate molecular size or by adding cell- or tissue-specific targeting moieties.[15] Among the best studied examples are polyethyleneimine (PEI) and poly(L-lysine) polymers, which have high densities of protonatable amines in their periphery that allows for the efficient condensation of nucleic acids.

Dendrimers, also known as arborols, cascade or starburst polymers, have attracted increasing attention in biomedical fields because of their well-defined structures, which offer unique multivalency and a convenient scaffold for functionalization, not to mention a lower toxicity than traditional cationic polymers.[16–18] Whereas linear polymers often adopt random-coil structures, the three-dimensional structure of a dendrimer is characterized by radial symmetry. Dendrimers of lower generation number tend to exist in relatively open forms but as successive layers are added, a spherical structure is adopted (often at the fifth-generation). Biomedical applications of dendrimers today range from biological mimics,[19,20] to imaging agents,[21] to drug delivery agents or gene transfection vectors,[22] due the great diversity of structures available to this class of molecules.[23,24] For the purpose of gene delivery, functionalisation and variation of the dendrimer structure[25,26] is an important tool for the purposes of maneuvering through the many cellular obstacles.[27]

The cell transfection mechanism

Any synthetic agent designed to deliver genetic material specifically will be exposed to biological mechanisms that limit its trafficking both outside and inside cells. Extracellular barriers to the systemic delivery of nucleic acids are hurdles that can be encountered from the point of injection up to the surface of the targeted cell.[28] Non-viral gene delivery systems are colloidal suspensions of compacted nucleic acids that must be sterically stabilized to remain as discrete particles in the blood and prolong circulation time. They must show low toxicity, evade the adaptive immune system and minimize interactions with plasma proteins, extracellular matrices and non-targeted cell surfaces. Specific cell types can be targeted by exploiting the affinity between ligands connected to the non-viral vectors and cell surface receptors; such interactions can also facilitate cell entry.

Cell surfaces are negatively charged due to their content of glycoproteins, proteoglycans and glycerolphosphates. Thus, efficient gene/vector complexes often exhibit a positively charged surface. Cellular uptake of synthetic DNA shuttles generally proceeds *via* adsorptive endocytosis (Fig. 1),[29] as demonstrated by confocal microscopy studies.[30]

Once internalised, the complexes follow the endolysosomal pathway where they must escape degradation (Fig. 1).[31] The lysosomal environment contains aggressive nucleases and has an acidic pH of approximately 5. At this stage, polyplexes made of protonatable amines are able to buffer the on-going endosomal acidification, resulting in osmotic swelling and bursting of the endosome, according to the "proton sponge

Born in the Grand-Duchy of Luxemburg (1952), François Diederich studied chemistry at the University of Heidelberg (1971–1977). He joined the group of Prof. Heinz A. Staab for his diploma and doctoral theses which he completed in 1979 with the synthesis of kekulene. Following postdoctoral studies with Prof. Orville L. Chapman at UCLA (1979–1981), investigating arynes in Argon matrices, he returned to Heidelberg for his Habilitation at the Max-Planck-Institut für Medizinische Forschung (1981–1985). Subsequently, he joined the faculty in the Department of Chemistry and Biochemistry at UCLA where he became Full Professor of Organic and Bioorganic Chemistry in 1989. In 1992, he returned to Europe, joining the Department of Chemistry and Applied Biosciences at the ETH Zürich. His research interests, documented in more than 500 publications, are in the field of supramolecular chemistry, spanning from dendritic mimics of globular proteins, to molecular recognition studies including structure-based drug design, and to fullerene and acetylenic networks.

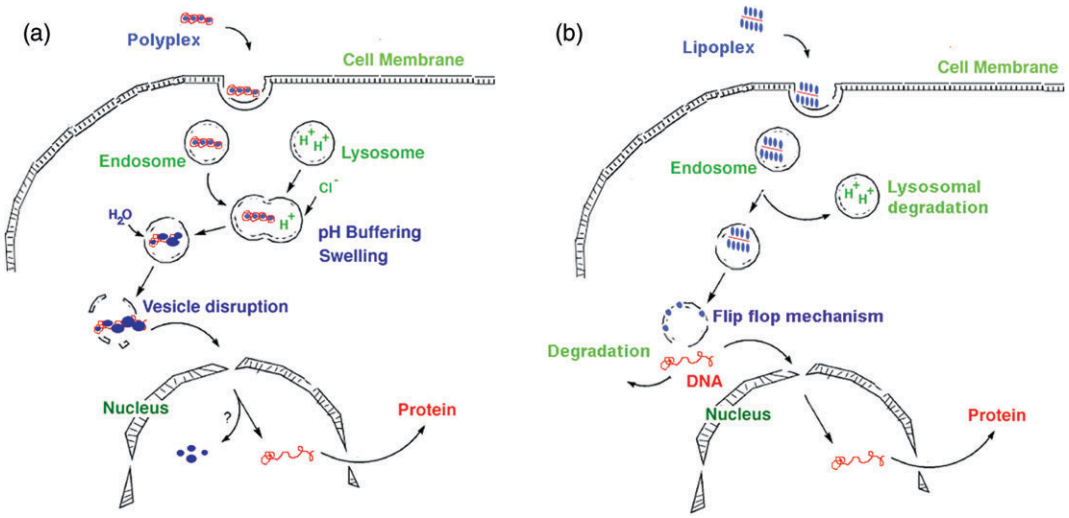


Fig. 1 Subcellular trafficking of DNA delivery systems. (a) Progression of a polyplex exhibiting H^+-buffering ability, according to the "proton sponge hypothesis". (b) Fate of a lipoplex carrying out a flip-flop mechanism with the endosomal membrane to release DNA. Adapted from ref. 31.

hypothesis" (Fig. 1(a)). Direct evidence correlating polyplex escape from the endosome with this theory have now been described.[32,33] Lipoplexes have no H^+-buffering ability but their intrinsic membrane affinity plays a part in releasing the genetic material in the cytoplasm (Fig. 1(b)). The complex destabilises the endosome membrane by a flip-flop mechanism where the nucleic acid is exchanged for anionic membrane lipids.[34]

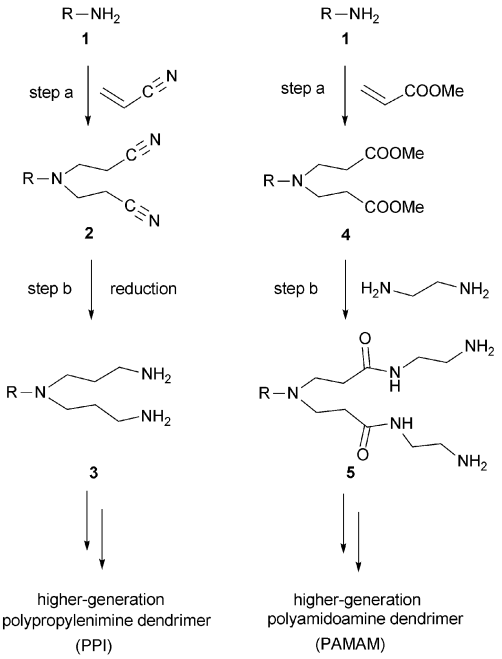
After tricky endosomal escape in the cytosol, the diffusion of large aggregates towards the nucleus is extremely limited by the dense cytoskeleton mesh.[35] Surprisingly, complex dissociation does not always appear to be a prerequisite for nuclear uptake. Nuclear import by a passive diffusion process is possible with small molecules but limited for macromolecules. Some synthetic vectors have indeed been localised in the nucleus, which addresses the question of possible damage to endogenous genes and encourages the design of vectors promoting cytoplasmic self-disassembly of the complex.[36–38] Generally, nuclear uptake of foreign genetic material occurs during cell mitosis, and transfection is thus favored in rapidly dividing cell lines. Alternatively, active transport is an energy-dependent mechanism that can occur when nucleic acids pass through nuclear pore complexes. The attachment of nuclear targeting ligands, such as nuclear localisation signals (NLS), sometimes facilitates the uptake process.[39]

In summary, an ideal synthetic vector should tightly compact the foreign nucleic acid, transport it through cellular membranes while ensuring its protection from degradation and allow its recognition and activation by the cell machinery. For *in vivo* therapeutic applications, high colloidal stability in the bloodstream and organ targeting ability is required. Low production cost, storage stability and biocompatibility are also highly desirable. Of course, the synthesis of a "magic bullet" affording all these skills together remains idealistic, though the level of complexity in delivering genes differs widely between biological systems, *e.g.* between cultured cell lines and a whole body. Many synthetic vectors have been active enough to justify concentrated research effort and sometimes, even commercialisation. More specifically, dendrimers constitute an attractive variety of synthetic vectors for transfection today. The different dendritic structures that have already been tested in biological systems will be presented here by systematically linking their design to biological issues.

Polyamidoamine dendrimers (PAMAM)

Emergence of the polyamidoamine dendritic molecules. Among all dendritic vectors applied so far in gene delivery, polyamidoamine (PAMAM) molecules clearly represent the workhorses of transfection studies. This predominance can be linked to the fact that PAMAM dendrimers were one of the earliest dendritic systems synthesised at high generation numbers and commercialised. Dendrimer chemistry emerged in the early 1980s when innovative macromolecular architectures possessing regular branching and radial symmetry were developed. The first example of "cascade" synthesis was described by following a divergent approach where growth emanates from a central core.[40] Repetitive sequences of (a) double Michael addition of primary amines to acrylonitrile and (b) reduction of the nitrile afforded polypropylenimine (PPI) dendrimers (Scheme 1(left)). At that time however, low yields for the reduction step prevented the development of higher generations than $G = 3$.

To circumvent the limiting reduction step and allow the synthesis of higher generation numbers, Tomalia *et al.* explored and optimized reaction conditions by using a methyl acrylate subunit instead of acrylonitrile (Scheme 1(right)).[41] Satisfyingly, starting with a nucleophilic core, the repetition of a two-step sequence involving (a) quantitative Michael addition to methyl acrylate and (b) quantitative addition of the resulting ester intermediate to ethylenediamine afforded regular (also called "starburst") PAMAM dendrimers up to generation seven. The challenge was the precise control of symmetry, branching and versatility to ensure monodispersity, defined topology and functionalisation of the macromolecules, respectively.[42] The generation number became limiting, the so-



Scheme 1 Synthesis of PPI and PAMAM dendrimers.

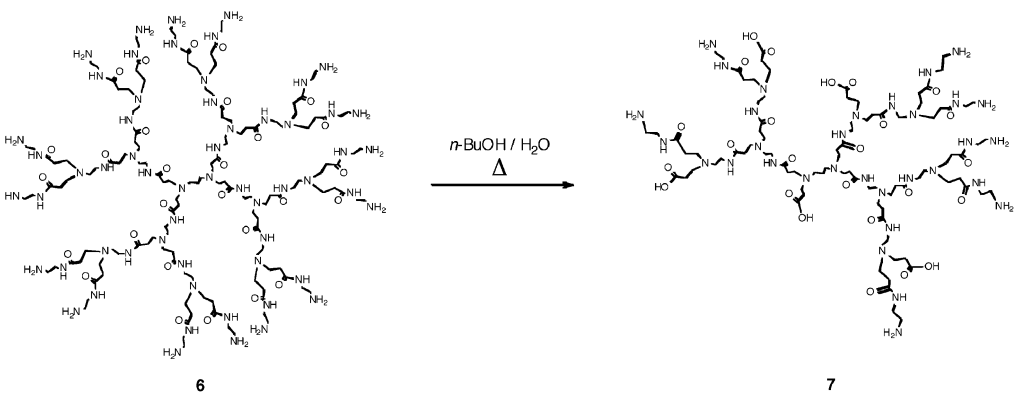
called "starburst" effect, when steric congestion prevented further addition to the terminal groups.

This breakthrough in the synthesis of large and well defined macromolecules rapidly gained the interest of biological, physical and organic chemists. At first, biomedical applications of dendrimers mainly focused on drug delivery and imaging, although gene delivery was envisaged. PAMAM dendrimers have a spheroidal shape and a high density of primary amines on the surface. Under physiological conditions, such structural features therefore hold great promises to tightly compact DNA. In 1993, Haensler and Szoka assayed the commercial "starburst" PAMAM dendrimers for their ability to mediate transfection in cultured cells and observed high levels of activity.[43] These results represented the first successful attempt at gene transfection using dendrimers and initiated a cascade of more refined biological studies. When designing original dendritic transfection agents, the vast majority of research groups still carry out structural variations of the wonderfully successful polyamidoamine branching motif. Starburst PAMAM dendrimers have therefore become a reference vector.

Seminal studies on PAMAM dendritic vectors for gene transfection. Szoka and co-workers performed the first transfection assays with dendritic vectors using a series of regular, commercial PAMAM dendrimers (generation $G = 2$–10) in a broad range of cell lines.[43] Gene delivery efficacy of the new vectors was found to be promising and reached a maximum with the sixth-generation dendrimer. The authors were able to relate activity with the molecular size and the shape of the vectors. Like the histone core in chromatin around which DNA is wrapped,[44] the 68 nm diameter of the spherical sixth-generation PAMAM was found to be optimal to mediate transfection. Moreover, the authors linked transfection ability with the presence of tertiary amino groups in the dendritic interior. Localised at the precisely defined branching points, these mildly acidic ammonium centres ($pK_a = 3$–7)[45] were assumed to cause a lysomotrophic effect as explained by the "proton sponge hypothesis".

Further assays, however, could not reproduce such high levels of transfection. In fact, monodisperse PAMAM dendrimers were synthesised and indeed found to be 100-fold less active than partially degraded dendrimers. The most active vectors were identified in degraded commercial batches where partial alteration of the dendritic branches was found to increase transfection efficiency.[46] When heat activation was properly controlled as depicted in Scheme 2, high molecular weight fractions of the resulting degraded mixture proved to be the most active. The few defective branches played a key role as they rendered the polymer more flexible and increased interior void space. This enabled tighter DNA compaction, which facilitated cellular uptake and improved the buffering ability of the interior amines in the endosome. Experimentation with core substitution and complex morphology was found to have much less influence on the biological activity. The conclusions garnered from these studies triggered the commercialisation of a dendrimer-like polymer, Superfect™ (Qiagen), now a reference for gene transfection vectors.

Baker and co-workers also synthesised and assayed regular PAMAM dendrimers for their transfection ability with plasmid DNA in a variety of cell lines.[47,48] Much higher transfection efficiencies were recorded than previously reported. Generally, DNA binding ability was found to increase with



Scheme 2 Synthesis of fractured PAMAM.

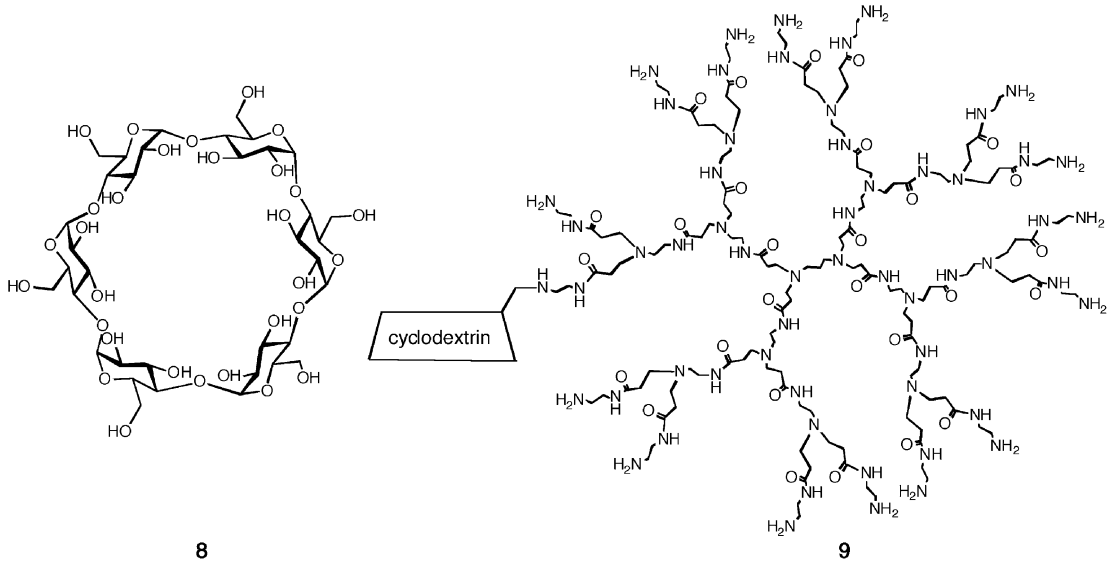


Fig. 2 α-Cyclodextrin (**8**) and schematic representation of a cyclodextrin–PAMAM conjugate.

generation number as measured by agarose gel electrophoresis and the best performing generation (G = 5–10) for cell transfection was strongly dependent on the cell type. Nevertheless, the presence of an additive, DEAE-dextran,[49] was necessary to enhance the biological activity of starburst PAMAM dendrimers by several orders of magnitude in numerous cell lines. DEAE-dextran is a highly positively charged polymer with low transfection ability on its own, no buffering ability and marked cytotoxicity. Its ability to disperse large DNA/dendrimer aggregates (~100–300 nm, G = 10) into small particles (<20 nm, G = 10) was observed by electron microscopy and directly correlated to better transfection efficiency of the resulting mixtures.[50,51]

While controlled degradation of PAMAM dendrimers was shown to enhance transfection activity, many successful examples have steadily promoted the idea of functionalising regular PAMAM dendrimers.[52] For example, the specific targeting of cancer cells was accomplished by tailored modification of the dendrimer surface groups. This new research area has focused mainly on low-generation (G = 2–4) PAMAM dendrimers. These non-spheroidal but starfish shaped, flexible carriers are more interesting due to their easy synthetic production, low cost and reduced cytotoxicity. Their activity, however, needs to be improved. Three different strategies have been developed to modify the PAMAM structure: (a) rendering the dendrimer amphiphilic, (b) making it biocompatible and (c) enhancing the flexibility of its branches. These strategies are presented below.

Lipophilic residues attached to alter membrane affinity. Membrane transfer represents a rate-determining step for transfection vectors, and regular PAMAM vectors have some intrinsic membrane disruption activity. Provided that the dendrimers exhibit surface amino groups at a high generation number, they are capable of inducing leakage in anionic vesicles by "bending" the membrane and by forming holes in lipid bilayers.[53,54] However, such membrane affinity is not particularly pronounced and does not apply to low-generation PAMAM molecules. A number of studies have established that lipophilic moieties in synthetic gene delivery systems favor fusion of the complex particles with the endosomal membrane.[33] Thus, Szoka and co-workers noticed that the covalent linkage of PAMAM dendrimers to GALA (an amphipathic, membrane destabilizing peptide) enhances the transfection efficiency of the DNA/dendrimer complex.[43] As little was known about the fate of the DNA/PAMAM dendrimer complexes after cell internalisation, Yoo and Juliano covalently linked a PAMAM dendrimer (G = 5) to a small hydrophobic fluorescent dye.[55] To their surprise and despite the low dendrimer/dye ratio, all labeled conjugates were more active in delivering antisense oligonucleotide (ODN) *in vitro* than the original dendritic vector. These were the first reported examples of regular amphiphilic dendritic vectors. Combining the polycationic nature of PAMAM dendrimers with lipophilic or amphiphilic units was systematically investigated in some research groups to improve the membrane transfer ability of the transfection shuttle.

In an ingenious approach, a family of PAMAM dendrimers was thus covalently linked with cyclodextrin macrocycles.[56] These cyclic oligomers of glucose feature a hydrophobic interior cavity and a hydrophilic outer surface (molecule **8**) (Fig. 2). The relatively low toxicity and lack of immunogenicity of cyclodextrins has led to numerous applications for the encapsulation of guest molecules or the delivery of nucleic acids.[57] A key feature of cyclodextrins is their ability to induce hemolysis and collapse of liposomes, which allows the permeation of water-soluble guests through biological membranes. Uekama and co-workers covalently linked α-, β- and γ-cyclodextrins, consisting of six, seven and eight α-D-glucopyranosyl rings, to low-generation PAMAM (G = 2) to create a synergistic effect of dendrimer and cyclodextrins (molecule **9**) (Fig. 2).[56] Cyclodextrin functionalisation was clearly necessary to reach good levels of transfection. Detailed investigations of the complexation process and the transfection mechanisms helped point out differences in activity between α-, β- and γ-cyclodextrin–dendrimer conjugates. Significant differences

arose once the DNA/dendrimer complex was internalised and trafficking within the cell. More precisely, α-, β- and γ-cyclodextrins have different cavity sizes and did not extract the same membrane components, so that disruption of the endosomal membranes was more or less pronounced for different dendrimer-cyclodextrin conjugates. Transfection assays highlighted the dendrimer functionalised with an α-cyclodextrin macrocycle as the most efficient agent.

With this lead in hand (Fig. 2), further transfection studies were conducted to determine the optimal generation number (G = 2, 3 or 4),[58] and the best degree of substitution (from 1.1 to 5.4 α-cyclodextrins per PAMAM dendrimer).[59] As expected, higher generation numbers yielded compact, more efficient DNA/dendrimer conjugate complexes, while derivatives with more α-cyclodextrin substituents yielded better membrane disrupting vectors. The latter results, however, had to be balanced by the simultaneous increase in cell death. Optimization of the lead resulted in the choice of the PAMAM (G = 3) conjugate substituted with 2.4 α-cyclodextrins.

Unlike in the case of viruses, one of the biggest issues with synthetic vectors is their lack of cell-specificity. Targeting units are now commonly linked to cationic polymers or peptide sequences for gene transfection to address this problem. The substitution of α-cyclodextrin–PAMAM (G = 2) conjugates by sugars, namely α-D-mannose[60,61] and α-D-galactose[62] has been explored. In both cases, increased sugar substitution on the small conjugates hindered DNA binding as the number of free terminal amines was dramatically reduced. A particular degree of substitution, around three sugar moieties per cyclodextrin-PAMAM conjugate, afforded the highest transfection efficiency values. The benefit of connecting sugars was, however, observed whether sugar-specific receptors were expressed at the cell surface or not, disappointingly pointing to intracellular events rather than to cell-specificity as the basis of the effect.

To enhance PAMAM transport into cells, the approach of Park and co-workers was to design a series of artificial proteins by grafting L-arginines or L-lysines to the surface of regular PAMAM (G = 4) dendrimers (molecules **10** and **11**, respectively) (Fig. 3).[63] These amino acid residues were chosen because they occur frequently in peptidic sequences of protein transduction domains (PTDs) and membrane translocalisation signals (MTS), and have been successfully inserted into peptidic vectors. When cell lines were difficult to transfect with a classical PAMAM vector, derivatisation with arginine proved to be effective, affording enhanced cellular uptake and/or nucleus localisation. One may notice that the biological activity of the functionalised dendritic vectors was also possibly enhanced by the basicity of the terminal groups. Both side chains of the arginine and lysine amino acids have higher pK_a values (pK_a = 12.5 and 10.1, respectively), than the native amino residues (pK_a = 8–10) at the surface of PAMAM dendrimers.[45]

Alternatively, regular PAMAM dendrimers (G = 4) were also substituted with leucine and phenylalanine residues (molecules **12** and **13**, respectively) (Fig. 3).[64] While phenylalanine conjugates appeared promising, the leucine conjugates were significantly less efficient vectors. Dissimilar activities were directly related to the lipophilicity of the amino acid

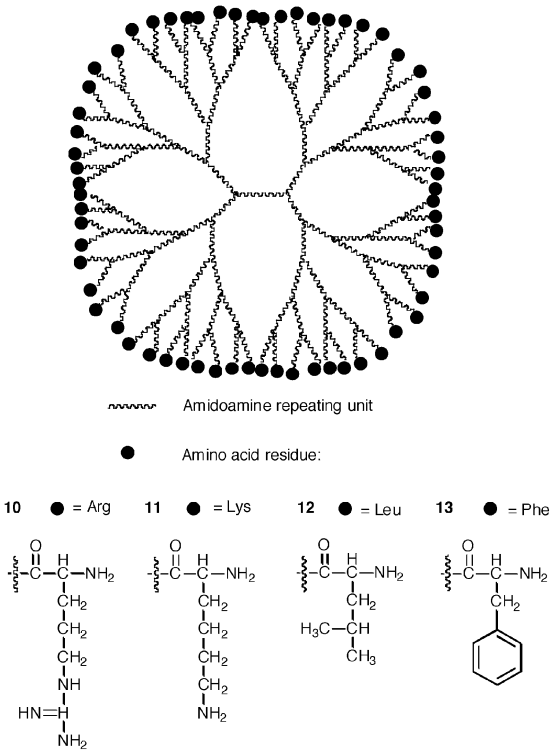


Fig. 3 Conjugation of PAMAM dendrimer surface amines with basic (Arg, Lys) or lipophilic (Leu, Phe) amino acid residues.

residue. The flat, highly polarisable surface of phenylalanine side chains favors dense packing of the conjugates in aqueous media and promotes strong interactions with lipophilic regions of the cellular membrane. Taken together, these features of the phenylalanine-PAMAM conjugates explain the enhanced translocation of genes through the membranes.

In another approach, two long alkyl chains were attached at the focal point of a PAMAM dendron (G = 1–4) to generate a novel amphiphile (molecule **14**) (Fig. 4).[65] However, satisfying levels of gene transfection could only be reached in the presence of the fusogenic lipid DOPE that is traditionally associated with cationic lipids.[66]

Lower cytotoxicity

Toxicity issues represent a major concern when developing nano-objects, especially in the context of therapeutic applications.[67,68] A number of *in vitro* and *in vivo* experiments have assessed the biocompatibility and biodistribution profile for polycationic PAMAM dendrimers.[69–71] PAMAM dendrimers alone (G = 1–7) clearly possess concentration- and generation-dependent toxicities, confirming that a high density of cationic amines is damaging to cellular membranes.[72] However, a prerequisite for gene transfection is the formation of DNA/dendrimer supramolecular assemblies where the protonated amines are complexed. In this respect, biological evaluation in a variety of mammalian cell lines was performed with DNA/PAMAM dendrimer complexes up to generation ten. Even after 48 h incubation time, no evidence of marked cell death could be detected in a concentration range that was appropriate for transfection assays.[47] Moreover, intravenous

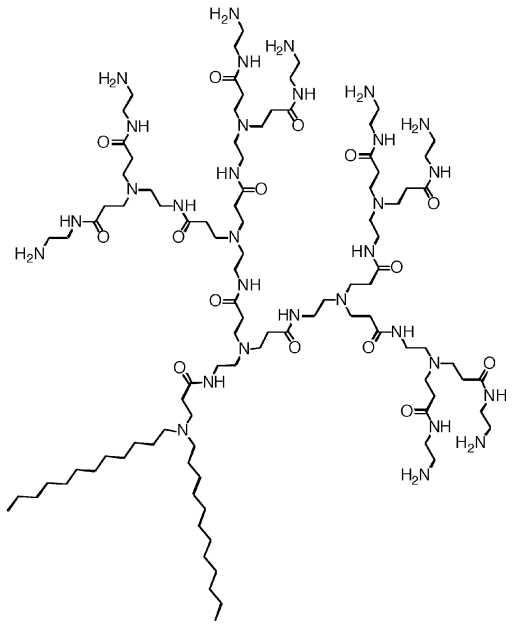


Fig. 4 Lipid-bearing PAMAM dendron.

delivery of the DNA/PAMAM dendrimer ($G = 9$) to mice did not appear to cause acute toxicity.[73]

In a contrasting study, the gene transfer ability and the toxicity of structurally diverse polycationic reference vectors were evaluated. As for cell viability, two points were highlighted: (a) polyplexes are not necessarily less harmful to the cells than the free polycations; and (b) more active polyplexes, such as the PAMAM derivative Superfect™, are also the most toxic.[72]

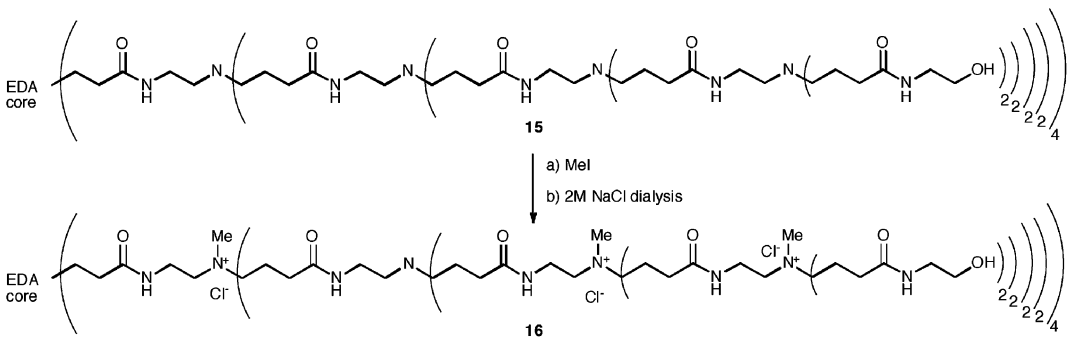
In view of such dissimilar conclusions, Park and co-workers derivatised PAMAM ($G = 4$) dendritic vectors in an "upside-down" fashion, with the aim to reduce their intrinsic cytotoxicity (molecule **15**) (Scheme 3).[74] Terminal hydroxyl groups were utilized instead of primary amines in order to reduce the surface charge density and thus prevent cell membrane alteration processes. The interior amines of **15** were then converted to the quaternary ammonium salts in **16** to ensure permanent cationic charge and DNA compaction ability. The resulting dendrimers showed considerably reduced toxicity, essentially due to the shielding of the interior cationic charges by the surface alcohol groups. Transfection efficiency, however, did not reach the levels obtained with regular PAMAM dendrimers, and no subsequent gene delivery studies with these conjugates have been reported.

In another approach, two PAMAM dendrons were covalently linked at their focal point to both ends of a poly(ethylene glycol) (PEG) chain.[75] The resulting PAMAM-PEG-PAMAM triblock copolymers, **17**, resemble bolaamphiphiles with two hydrophilic moieties connected with either one, two or three elongated chains (Scheme 4).[76] In addition to generating a more flexible and elongated core, Park and co-workers wanted to exploit the biocompatibility and solubilising effect of the hydrophilic chain. Because the DNA/dendrimer complexes **18** were coated with PEG segments, cell viability and complex solubility were effectively improved. On the other side, transfection ability did not significantly surpass those of related globular PAMAM vectors.

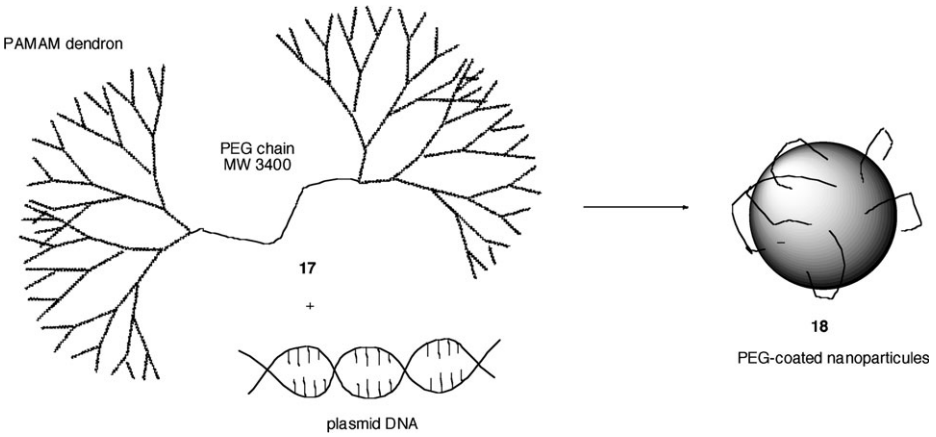
Inorganic nanoparticles show low toxicity as well as promise for delivery processes.[77] Rather exotic for dendritic vectors, an example of regular PAMAM ($G = 2$) dendrimers grafted onto silica nanoparticles was recently reported.[78] Mesoporous silica nanospheres serve traditionally as a biocompatible, universal membrane carrier for drug delivery and imaging. Transfection efficiency with the novel vector was clearly superior to commercial agents, such as Superfect™, and the new carrier was remarkably harmless to the cells. Dense silica nanoparticles, which by themselves do not deliver DNA, concentrate DNA/vector complexes at the cell surface, thus enhancing transfection in a physical mechanism called the "particle sedimentation effect".[79,80] This result is related to another successful nanoparticle-based vector, albeit not a dendritic one: an amino-functionalized silica nanoparticle was shown to surpass viral vectors *in vivo*.[81]

Enhancement of branch flexibility

The plasticity of a transfection vector drives its ability to compact DNA tightly and to cross membranes. Considering high-generation dendrimers, molecular density increases gradually from the core to the surface where it reaches a maximum level. Such a rigid spherical object can be converted to a more flexible structure, if interior void space is expanded, *i.e.* for dendrimers, if some of the dendritic branches are shortened as demonstrated by "fractured" PAMAM polymers. Partial substitution of a PAMAM generation 5 dendrimer with PEG chains was carried out to increase the volume of the vector, while endowing a controlled degree of flexibility (molecule **19**)



Scheme 3 Quaternization of hydroxyl terminated PAMAM dendrimers (EDA is ethylenediamine).



Scheme 4 Nanoparticles of triblock copolymer gene delivery systems.

(Fig. 5).[82] The elongated hydrophilic segments were chosen to mimic the fractured PAMAM chains in Superfect™. Shielding of the surface charged amines by PEG chains not only detoxified the dendritic vector, but also weakened electrostatic interaction thus allowing charge separation within the DNA/dendrimer complex, thereby facilitating DNA release to the cell nucleus. These elegant conjugates were highly efficient transfection vectors with low toxicity, although they required a high charge excess ratio.

Another strategy that was used to alter the flexibility of the PAMAM structure was to vary the core. Originally, regular PAMAM dendrimers were grown from ammonia, tris(2-aminoethyl)amine or ethylenediamine (EDA), (Scheme 2) but none of the variations in structure,[46,47] branching multiplicity or core–branch distances significantly altered the biological activity.[83] Considering the steric hindrance between the dendrons around these three small and therefore readily crowded cores, Zhang *et al*. experimented with trimesyl **20**, pentaerythritol **21** and inositol **22** cores connected to three, four, and six PAMAM dendrons, respectively (Fig. 6). Derivatives up to

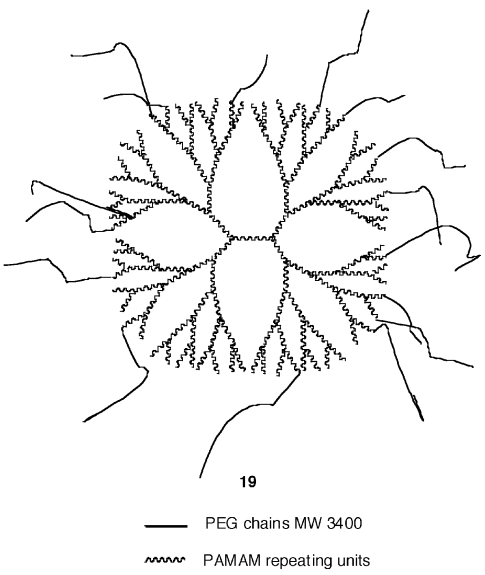


Fig. 5 Functionalization of the PAMAM peripheral amines with hydrophilic PEG chains.

generation eight were realized.[84] Among all the designed cores to date, a trimesyl unit appears to be one of the best choices to connect regular PAMAM dendrons. Due to larger interior void space, structural flexibility enabled the tightest DNA compaction: particle diameter determined by light scattering was 100–300 nm for a trimesyl core *vs*. 600 nm in the case of pentaerythritol and inositol cores. This confirms the idea that the size of the complex is a crucial parameter for crossing biological barriers.

An increasing number of research projects deal with the delivery of RNA or of oligodeoxynucleotides (ODNs). Instead of expressing a therapeutic product, antisense therapy aims to down-regulate the production of disease-causing proteins by inhibiting gene expression at the level of mRNA.[85] The delivered antisense nucleic acids are short DNA or RNA sequences, generally ODNs, ribozymes (RNA enzymes), or DNAzymes (DNA enzymes).[86,87] Now considered as the simplest, most effective gene silencing tool, the recently developed RNA interference (RNAi) strategy is based on small interfering RNAs (*si*RNAs) that induce post-transcriptional gene silencing.[88] Gene delivery does not necessarily require difficult nucleus entry anymore, delivery to the cytosol may be enough.

Curiously, while many detailed transfection studies with modified PAMAM dendritic vectors have been conducted for plasmid DNA, limited research effort has been devoted to structural variations for the improvement of delivering ODNs.[89,90]

Flexible PAMAM dendrimers were specifically developed to investigate the possibility of RNA targeting and RNA delivery. Peng and co-workers designed and synthesised a series of PAMAM dendrimers where the distance between the amino core and first branching units was increased from 7 to 10 bonds *via* the insertion of an ethylene glycol unit (molecule **23**) (Fig. 7).[91] Low dendritic generation (up to 4) was sufficient to bind and thus to inhibit ribozyme RNA activity, according to gel electrophoresis assays. Subsequent cell culture studies demonstrated the ability of the dendrimers bearing primary amine groups on the surface to efficiently deliver *si*RNA to cells. In an interesting structure activity relationship, gene silencing was found to be more effective as generation number of the dendrimer was increased from 1 to 7.[92] Minimal steric

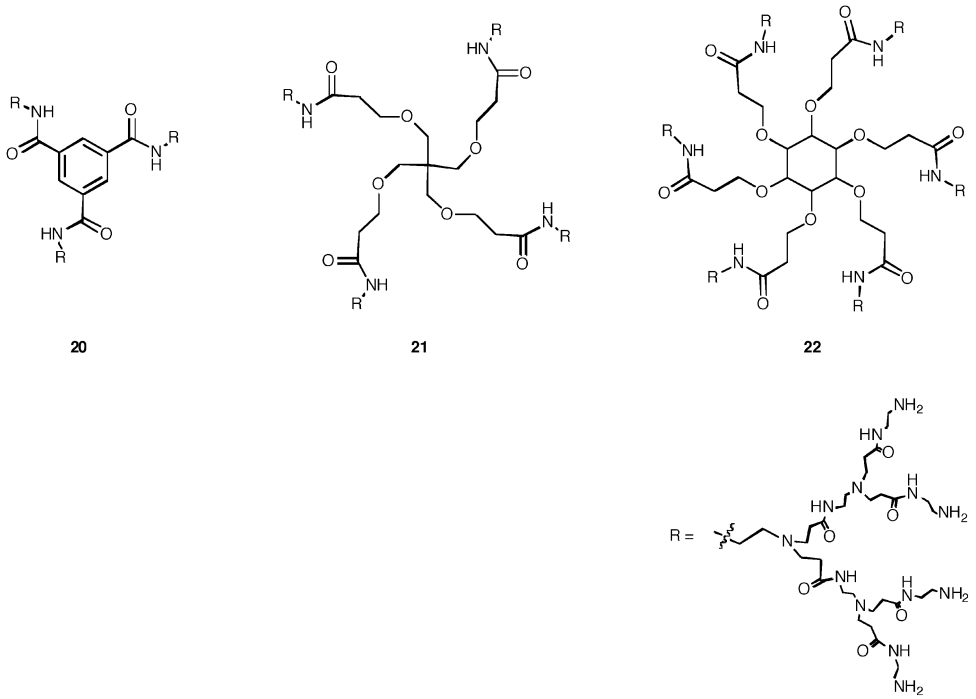


Fig. 6 Trimesyl (left), pentaerythritol (middle) and inositol (right) cores for PAMAM dendritic vectors.

constraints between the branches were later achieved by employing a single PAMAM dendron with a tri(ethylene glycol) chain at the focal point to eventually connect a targeting unit.[93]

The intense study of PAMAM based transfection vectors has resulted in ambitious *ex vivo* experiments. Baker and coworkers have reported successful transfection into murine cardiac transplants,[94,95] while other studies revealed efficient *in vivo* gene delivery to murine lung tissues and epidermal cells.[73,96,97] A fair amount of research on regular PAMAM dendrimers currently focuses on drug delivery, targeting cancer cells by tailored modification of the dendrimer surface groups.[98,99]

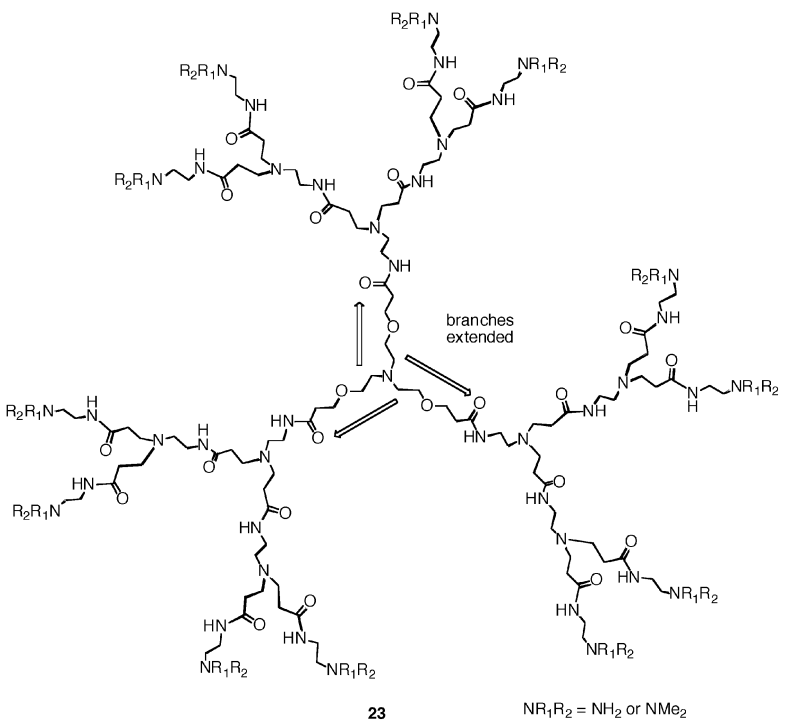


Fig. 7 Maximizing flexibility of PAMAM dendritic vectors for RNA targeting and RNA delivery.

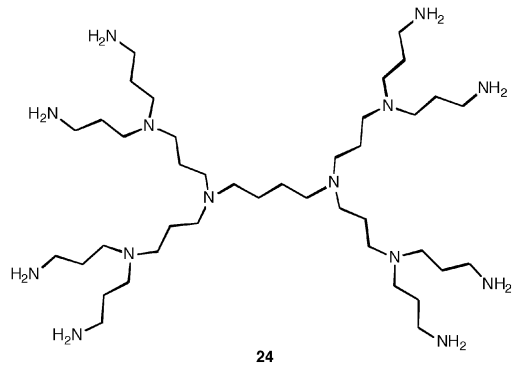


Fig. 8 An efficient, lower-generation polypropylenimine (PPI) dendritic vector.

Polypropylenimine (PPI) dendritic vectors

Like PAMAM dendrimers, high-generation PPI dendrimers were also synthesised on a large scale and commercialised once Wörner and Mühlhaupt[100] and de Brabander-van den Berg and Meijer[101] optimised the pioneering synthetic sequence of Vögtle depicted in Scheme 2(left). By employing a Raney-cobalt catalyst and more controlled reaction conditions, the production of side-products could be prevented, thereby improving the otherwise low-yielding reduction of terminal nitrile groups to primary amines. Acid–base properties of PPI dendrimers are similar to those of PAMAM dendrimers, with peripheral basic amines (pK_a = 9–11) and interior, more acidic tertiary ammonium centres (pK_a = 5–8).[102] Due to these structural similarities to PAMAM vectors, the high-generation PPI dendrimers were assayed for transfection. However, disappointing transfection and toxicity levels of high-generation (G = 8) vectors precluded the use of the whole family of PPI dendrimers for gene delivery.

It was already noticed with PAMAM dendrimers that low-generation vectors, albeit poorly active, were relatively non-toxic compounds due to their small number of cationic amines. For many years, this was assumed to be true for PPI dendrimers as well. In 2002, Uchegbu and co-workers evaluated regular PPI dendrimers up to generation five and demonstrated that the lower-generation dendrimers, specifically generation two, are effective gene transfer agents with a good biocompatibility profile in a human epidermoid cell line (molecule **24**) (Fig. 8).[103]

Molecular modeling and ethidium bromide intercalation studies with the DNA/dendrimer complexes provided some insight into the interactions between DNA and the vectors and allowed the elucidation of strong structure–activity relationships (SARs).[103] As the size of the dendrimer increases, DNA binding is more effective, and past the third generation, DNA begins to wrap around the PPI molecules. The ideal PPI vector contains sufficient cationic amines for DNA binding but not enough to cause cell membrane degradation. Other assays with regular second- and third-generation PPI dendrimers were performed to examine delivery of ODNs, again with a human skin cell line.[104] Both vectors caused gene expression knock-down comparable to Oligofectamine™ (Invitrogen), a traditional cationic lipid vector for ODNs. Cell death was fortunately limited.

Successful *in vitro* transfection assays are a prerequisite for starting *in vivo* studies where the DNA shuttle must escape degradation and clearance from the body. At the level of the entire organism, quaternisation of the surface amines emerged as a powerful tool for improving the colloidal stability and biocompatibility profile of the DNA/PPI dendrimer complexes. Methylated quaternary ammonium derivatives of the low-generation (G = 2–4) PPI vectors were thus synthesised.[105] The addition of a permanent positive charge ensures DNA binding. The expected colloidal stability and biocompatibility of the new derivatives were tested and confirmed *in vitro* and *in vivo* by intravenous administration to mice. The most active PPI dendrimers were the newly designed quaternised second-generation derivatives as well as the regular third-generation PPI dendrimers. Interestingly, the size of the quaternised dendrimers determined which organ was targeted. Second- and third-generation systems transfected only the liver, whereas the fourth-generation vector transfected only the lungs. These two PPI based non-viral vectors do not need to be associated with targeting ligands or shielding groups. Their intrinsic targeting of the liver as opposed to the lungs is unique and holds great promises for suicide gene therapy, where the gene delivered encodes a toxic protein.

In a detailed study of the regular third-generation PPI vector, Dufès *et al.* examined another pharmaceutical approach, namely the delivery of tumor necrosis inducing genes in mice.[106] First, systemic intravenous administration of DNA/dendrimer complexes led to potent retardation of tumor growth. Second, the dendrimer alone possesses an intrinsic antitumor activity at higher levels than other commercial polycationic vectors. In a parallel study, Omidi *et al.* shed light on the potential changes in gene expression that cationic lipids or PPI dendritic vectors can induce.[107,108] In addition to transfection ability and low toxicity, regular PPI dendrimers also alter the expression of the endogenous genes, particularly those involved in cell cycle progression. Such intrinsic gene regulation should be seriously considered when designing new vectors for suicide gene therapy. The synergy of the activities of both nucleic acid and vector could be particularly promising in anticancer gene therapy.

Copolymers

Although block and graft copolymers are structural cousins to the dendrimeric vectors that are being discussed in this review, these interesting molecules deserve a brief mention considering their impact on gene transfection. The majority of studies combine the DNA binding power of linear or hyperbranched PEI with hydrophilic PEG chains.[109,110] When mixed with DNA, this particular combination of building elements self-assembles into a nanoparticle consisting of a water-soluble, nonionic PEG coating protecting the DNA/PEI core. The Kissel group studied the transfection behaviour of a series of PEI-*graft*-PEG copolymers with varying degrees of PEGylation (molecule **25**) (Fig. 9).[111] These complexes were shown to successfully deliver genetic material to a mouse fibroblast cell line, more efficiently than PEI at high N/P ratios (N/P is nitrogen polycation/phosphorus polyanion ratio). Subsequent work by the Kissel group involved modifying the basic

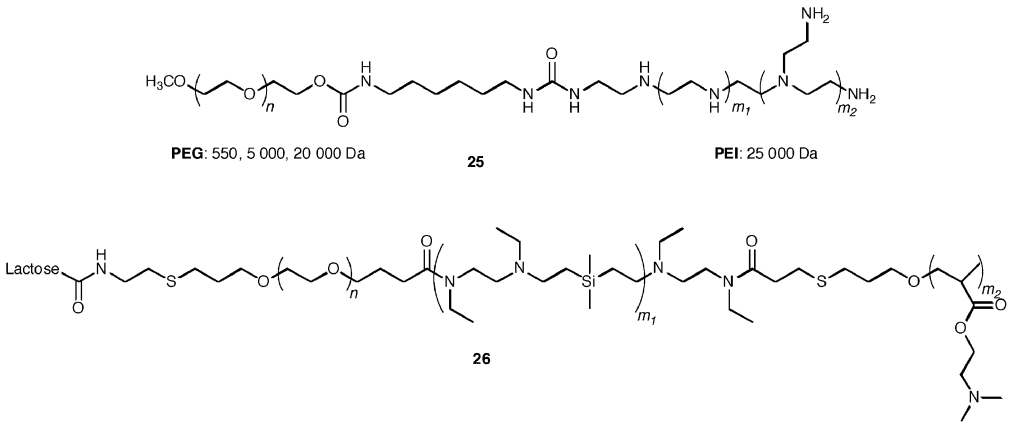


Fig. 9 Block copolymer transfection vectors.

structure to include biodegradable poly(ε-caprolactone) (PCL) in a successful effort to decrease the cytotoxicity.[112]

In an inventive approach, Nagasaki and co-workers targeted the difficult step of endosomal escape by combining lactosylated PEG, pH-sensitive poly(silamine) and poly [2-(*N*,*N*-dimethylamino)ethyl methacrylate)] to form a triblock copolymer (molecule **26**).[113] This strategy was vindicated by the successful increase in transfection efficiency when compared to control molecules that contained only two of the three essential design elements. These are just a few examples of the interesting and effective designs available to this diverse class of molecules.

Another structural cousin to dendrimers are the hyperbranched polymers. In particular, the work of Haag and coworkers is relevant to the current discussion.[114,115] Their approach was to functionalise well-defined, hyperbranched PEI with PPI and PAMAM branches. It was found that molecular weight as well as branch flexibility had an impact on transfection efficiency and toxicity.

Amino acid dendritic vectors

Among early synthetic vectors assayed, polymeric poly (L-lysine) systems have been extensively studied with the aim of developing biodegradable macromolecules.[15] The L-lysine AB_2 monomer is a good choice as it is a natural metabolite. Linear polycationic vectors, however, were found to be relatively cytotoxic and only sufficiently active if associated with helpers such as DEAE-dextran. The idea that the L-lysine unit could be used to generate branched molecules emerged rapidly, and in 1983 Denkewalter *et al*. established a proof of principle for the synthesis of dendritic poly(L-lysines).[116] Following a divergent route, conventional peptide chemistry was applied to the Boc-L-Lys(Boc)-OH monomer and afforded the first lysine dendrimers (molecule **27**) (Fig. 10). Asymmetric L-lysine residues as branching units clearly distinguish the dendritic poly(L-lysine) from the classical, highly symmetric dendrimers. Unlike the "starburst" dendrimers, where molecular density is the highest in the shell, dendritic poly(L-lysine) presents a relatively constant density from the core to the periphery. Nevertheless, controlled synthesis yields the required monodispersity, defined topology and tailored versatility, all the properties that define a dendrimer. The resulting branches exhibit a higher degree of flexibility than conventional dendrons, a definitive asset for gene delivery relative to the successful, partially degraded PAMAM dendritic vectors.

Similarly to PAMAM or PPI dendrimers, several lysine dendrons can be connected to a central core. A single research group envisaged applications of the resulting regular molecules in the field of gene delivery. Aoyagi and co-workers recorded moderate levels of gene delivery with a sixth-generation lysine dendrimer in a series of mammalian cell lines, with minimal toxicity.[117] In contrast to PAMAM, the largest complexes (diameter $>$ 1 μm according to dynamic light scattering analysis) afforded the highest transfection levels.[118] Zeta potentials[119] of the complexes were also measured and kept close to a neutral level. This explained the large aggregate size and why transfection ability was not hampered by the presence of serum (anionic proteins). A precise explanation for the neutrality of the zeta potential, even at up to CE $=$ 8 was not given (the CE ratio is defined as the number of positive charges on the dendrimers divided by the number of negative charges present on the plasmid DNA). Additional *in vivo* studies demonstrated the stealth character of the DNA/dendrimer complexes in the blood as long circulation times were observed. However, no marked expression of the DNA was

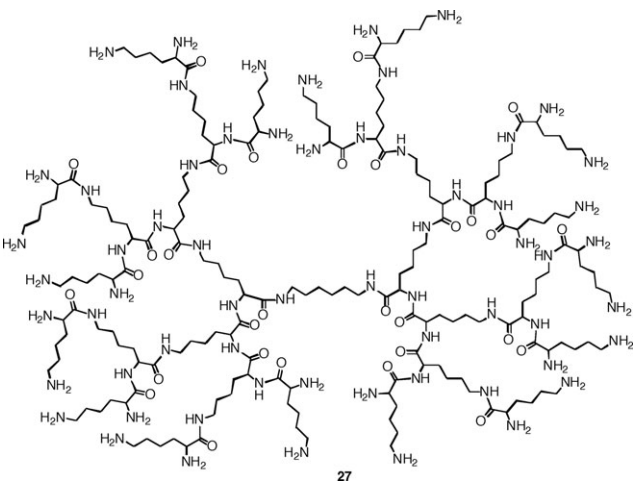


Fig. 10 Fourth-generation regular lysine dendrimer.

detected in the major organs, presumably due to poor cellular uptake and/or random DNA release.[120]

Lysine-based dendrons do not possess interior amines as PAMAM dendrons do and strategies other than "proton-sponge" mechanisms must be developed to facilitate escape from the endosome. To expand membrane affinity and give some buffering ability, the terminal lysine groups in the sixth-generation dendritic vector were thus substituted with arginine or histidine residues.[121] As expected, the presence of terminal guanidinium groups in the arginine-functionalised dendrimer improved gene transfection ability somewhat. In the case of the histidine derivative, little activity was observed. It was only under acidic conditions, where the imidazole moieties are protonated and can bind DNA, that improved cell transfection was detected. This trick, however, precludes exploiting the potential buffering ability of the imidazole to trigger endosome disruption. No further assays have been reported with this system.

In early work, amino acid dendrons (G = 3–4) were combined with a "root" presenting long alkyl side chains to generate an amphiphilic molecule.[122,123] A number of molecules were tested with the basic structure **28** shown in Fig. 11. The most active vectors proved to be ornithine or lysine dendrons connected to three dodecylamine chains (n = 2; n' = 3). However, solubility of the ornithine vectors was low and subsequent work, described below, focused exclusively on the lysine derivative.[124,125]

Extended physical studies were conducted with this system. The size of the most compact DNA/vector complexes was estimated *via* electron microscopy to be less than 20 nm, within the size-exclusion limit for the nuclear pore. Not surprisingly given this result, the *in vitro* transfection assays were also positive. Nevertheless, and similar to amphiphilic PAMAM vectors, addition of the fusogenic lipid DOPE yielded the best activities. *In vitro* delivery of *si*RNA or ODNs was also successful.

For the purpose of eye therapy, a related research project also dealt with the lysine amphiphilic dendrimers depicted in Fig. 11. In this work, finer structural variations afforded a vector that provided optimal delivery efficacy of a specific ODN both *in vitro* and *in vivo* as well as protection against nucleases. Briefly, a library of dendrimers displaying different lengths (n) and numbers (n') of lipid chains and different generation numbers (R) of polycationic lysine dendrons was synthesized using standard solid-phase synthetic methods and assayed for delivery in retinal cells.[126,127] Targeted inhibition was found to increase with (a) the number of cationic charges, (b) the number of lipophilic chains and (c) the decreasing length of the chains. The most effective structural combination consisted of three dodecyl side chains and a second-generation lysine dendron. Subsequent *in vivo* time course studies revealed that the lead dendrimer and the tailored ODN significantly inhibited the development of choroidal neovascularisation in wounded eyes for several months with negligible toxicity, as desired.[128]

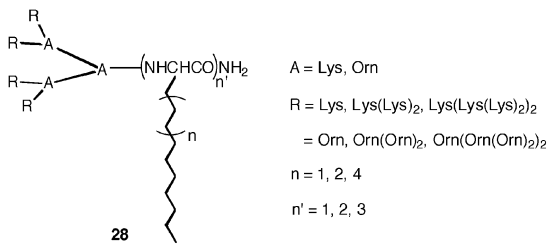


Fig. 11 Amphiphilic vectors based on low-generation amino acid dendrons.

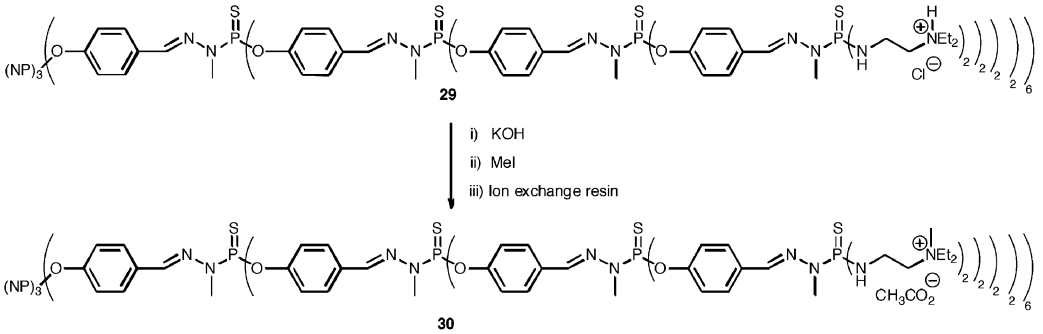
To date, most amino acid dendrimers for gene delivery have been based on lysine branching units. In an original approach, polyproline helices were considered recently as suitable dendritic branches to build biodendrimers[129] for gene delivery as proline rich sequences are present in membrane crossing peptides.[130] Making use of the imidazolidine-2-carboxylic acid two-branching unit, a second-generation proline-based dendrimer was developed *via* solid-phase synthesis and surface-functionalised to generate a small library of potential gene delivery vectors. Ranging from amine rich groups to peptidic sequences or PEG chains, each functionalisation targeted a specific hurdle in the biochemical processes that had already been identified with traditional synthetic vectors. From this library only one dendrimer was capable of forming a complex with DNA and of mediating transfection in cultured cells, highlighting the key role of end groups in dendritic vectors. In the case of the successful vector, the end group was a short peptide chain of arginine residues that is known to favor accumulation of the complex in the cell nucleus. As expected, this lead was also the only dendrimer to be rather harmful to the cells. However, the cytotoxicity profile was not established for the DNA/dendrimer complexes themselves.

Original branch motifs for gene transfection

A further ambitious step with respect to chemical variation consists of designing completely new branch motifs. In the field of gene therapy, structural innovations do not necessarily afford a lead immediately, but at least they push the limits established by traditional non-viral vectors, perhaps more radically than functional derivatives.

Pioneering experience with phosphorus dendrimers led Caminade and Majoral to design new vectors for gene delivery to cultured cells.[131] Two series of water-soluble phosphorus dendrimers were synthesised up to generation five, featuring *N,N*-diethylamine end groups, either protonated as the hydrochloride salt **29** or as the methylated quaternary ammonium form **30** (Scheme 5).[132] Whereas the protonated derivatives afforded transfection levels similar to commercial PEI vectors, even in the presence of serum, quaternised dendrimers were much less active. In both cases, biological activity was amplified with increasing generation of the dendrimer. The authors suggested that the irreversible charge of the quaternised species clearly shuts down the activity, implying a lack of adaptability of such end groups to the microenvironmental pH. On the other hand, the protonated species act as a proton reservoir and are able to modulate their surface charge density *in situ* while trafficking outside or within the cell, possibly explaining also the serum tolerance.

In another approach, eight phosphorus branches of the protonated dendrimer were also connected to a



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Scheme 5 Surface modification of phosphorus dendrimers.

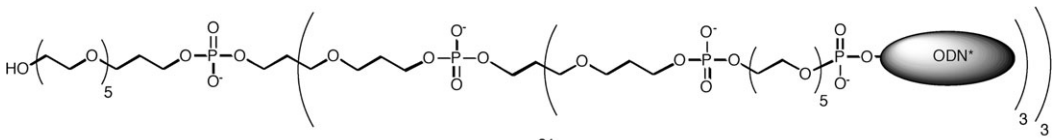

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Fig. 12 Covalent attachment of nucleic acid chains to a dendritic vector. ODN* = ^{32}P labelled short antisense oligonucleotide sequence.

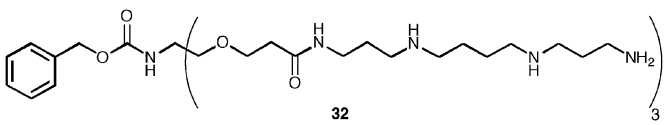
phthalocyanine core to test penetration inside cells.[133] Efficient levels of gene delivery were observed, indicating that the core size does not significantly alter the biological activity of high-generation phosphorus dendritic vectors. The expected red fluorescence of the complex was, however, too weak to allow following of its intracellular fate. In the last reported example, anionic oligomers were mixed with the DNA *prior* to phosphorus dendrimer addition with the aim of altering the morphology of the complex.[134] Enhanced transfection levels were observed and explained by a kinetic competition between plasmid DNA and ODN, which results in less condensed complexes.

Multivalency and low-molecular weights together are crucial assets for therapeutic applications. In comparison with the two-branching dendrons, three-branching units, for example from a quaternary carbon atom, lead to a quicker onset of dense packing in the iterative synthetic process. Consequently, the three-branching dendrimers do not need to be synthesised at high generation numbers to be active. As an added bonus, lower generation dendrimers have a better monodispersity, which is preferable to establish SARs and to control toxicity.

In a truly inventive approach, a three-branching pattern based on the pentaerythritol structure was specifically designed by Hussain *et al.* to develop ODN containing dendrimers (molecule **31**) (Fig. 12).[135] This time, nucleic acids were not complexed by highly charged polycationic, *i.e.*, quite toxic vectors, but directly linked covalently to an anionic, biocompatible dendron containing phosphoramidite units. Cellular uptake of the anionic ODN dendrimer was actually greater than that of free ODNs and proceeded *via* protein-dependent adsorptive endocytosis. As free ODNs and ODNs delivered by other dendritic vectors diffuse to the nucleus, these ODN dendrimer conjugates mainly stayed in the cytosol, a potential advantage for targeting cytosolic mRNAs. Furthermore, this linkage protected the ODNs while allowing their gene silencing activity.

Utilizing the three-branching design, Smith and co-workers exploited the slight DNA binding ability of natural spermine and connected these groups in a dendritic structure to provide the compounds **32** and **33** (Fig. 13).[136] DNA binding and compaction were indeed greatly enhanced by this multivalent approach, however, cell assays with the new vectors were not conclusive, the chloroquine helper being required to enable the endosomal escape mechanism and ensure some gene delivery to cultured cells.[137]

Amphiphilic dendritic vectors were also designed on the basis of a three-branching motif in the Diederich group.[138] Low-generation hydrophilic and lipophilic dendrons were attached to a linear, rigid core to provide molecules **34–37** (Fig. 14). The hydrophilic branches were specifically kept short



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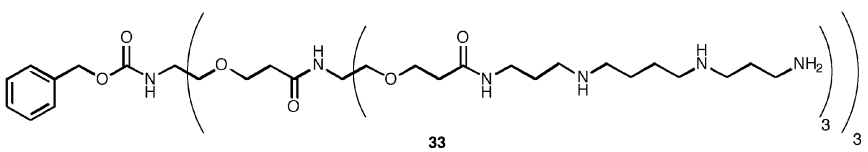

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Fig. 13 First- and second-generation spermine based dendrimers.

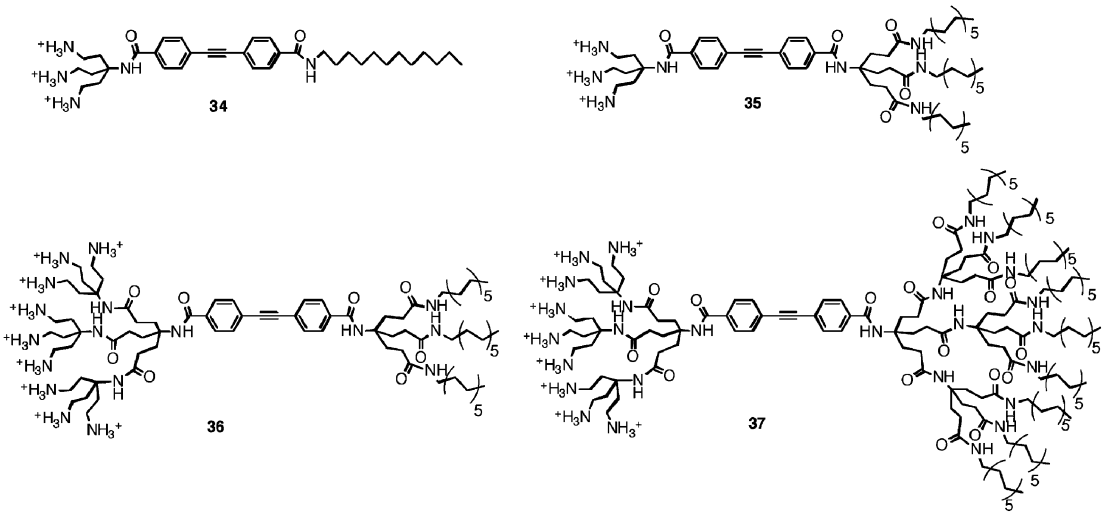

Fig. 14 Amphiphilic dendrimers for gene transfection.

to enhance the acidity of the terminal ammonium centres, and the structures contain no interior tertiary amines.[139] As compensation for the lack of buffering ability, the lipophilic dendron bearing three long aliphatic chains was meant to bring self-assembling properties and membrane-disruption power to the amphiphilic vector. The three-branching mode was chosen to provide enhanced multivalency and to keep the generation number modest. Satisfyingly, compound **35** was identified as a promising transfection vector *in vitro* for plasmid DNA, even in the presence of serum, and with acceptable cytotoxicity.

With this lead in hand, a new library of dendrimers was designed by incorporating fine structural changes to each of the three crucial structural parameters. We were interested in forming correlations between structural modification, physicochemical properties and transfection efficiency.[140] As can be seen in Fig. 15, the lipophilic dendron, the hydrophilic dendron and the core were varied systematically. The importance of the core size and structure was explored by the realisation of the less planar biphenyl core in **38**[141] and the extended phenylacetylenic rod in derivative **39**. The degree of lipophilicity as well as shape and bio-compatibility were altered in vectors **40** to **43**. Structural motifs from PEI, PPI and PAMAM dendrimers were incorporated to provide the series of vectors **44** to **47** with a range of surface charge densities.

Extensive *in vitro* transfection assays were performed on the library using HeLa cells. While many observations could be made, a few trends were highlighted in this amphiphilic design such as the importance of the core and the lipophilic dendron. Full core planarity was not found to be essential for gene delivery, and vector **38** has similar transfection properties to reference vector **35**. However, lengthening of the core as in amphiphile **39** resulted in a substantial decrease in transfection efficiency at low to middle charge excess. Concerning the changes to the lipophilic branches, no direct correlation between lipophilicity of the branches and transfection efficiency could be found. For example, increasing the length of the alkyl chains as in **43** lowers the activity at low concentration. To further illustrate the point, compounds **41** and **42** have quite different degrees of lipophilicity yet both have decreased activity. It is possible that this result is due to the shorter, bulkier branches, which may disturb the self-assembly process. The cholesterol derivative **40** has almost no activity up to medium charge excess ratios, which is surprising as this unit has been successfully used in mixed cationic lipid systems.[142]

For the cationic division, within the first generation changes to the number, length and constitution of the branches have little effect on transfection efficiency. Incorporation of the more flexible, second generation PAMAM dendron, however, had a dramatic effect on the activity. Almost no transfection was observed regardless of dendrimer/DNA ratio. It is possible that the increased flexibility of the design and electrostatic repulsion between the protonated amines combined to increase the size of the dendron, therefore disfavoring self-assembly and transfection.

Complementary physicochemical studies were particularly rewarding. The self-assembling ability of amphiphiles **38–47** in Langmuir films were investigated and offered some insight into the biological results. In brief, the most active vectors in the library, **38** and **44–46**, behave similarly to reference molecule **35**. They form homogeneous monolayers and their isotherms reveal a liquid-like behavior with no phase transitions. These observations and the value of the final molecular areas, between 72 and 96 nm, leads to the conclusion that the amphiphiles most likely form monomolecular films in which the van der Waals interactions between the alkyl chains and the electrostatic interactions of the protonated ammonium groups are dominant. However, it is likely that these molecules dimerise through core–core interactions and behave as a single "supermolecule" with a critical packing factor of ~ 1, meaning that molecules **38** and **44–46** are able to form bilayers and vesicles. In contrast, the least active vectors **39**, **40–43** and **47** show much different activity at the air-water interface such as inability to pack, solid-like behaviour, large molecular area and first-order phase transitions. These results would indicate that dimerization does not occur in these derivatives, resulting in a critical packing parameter lower that 1. As a result,

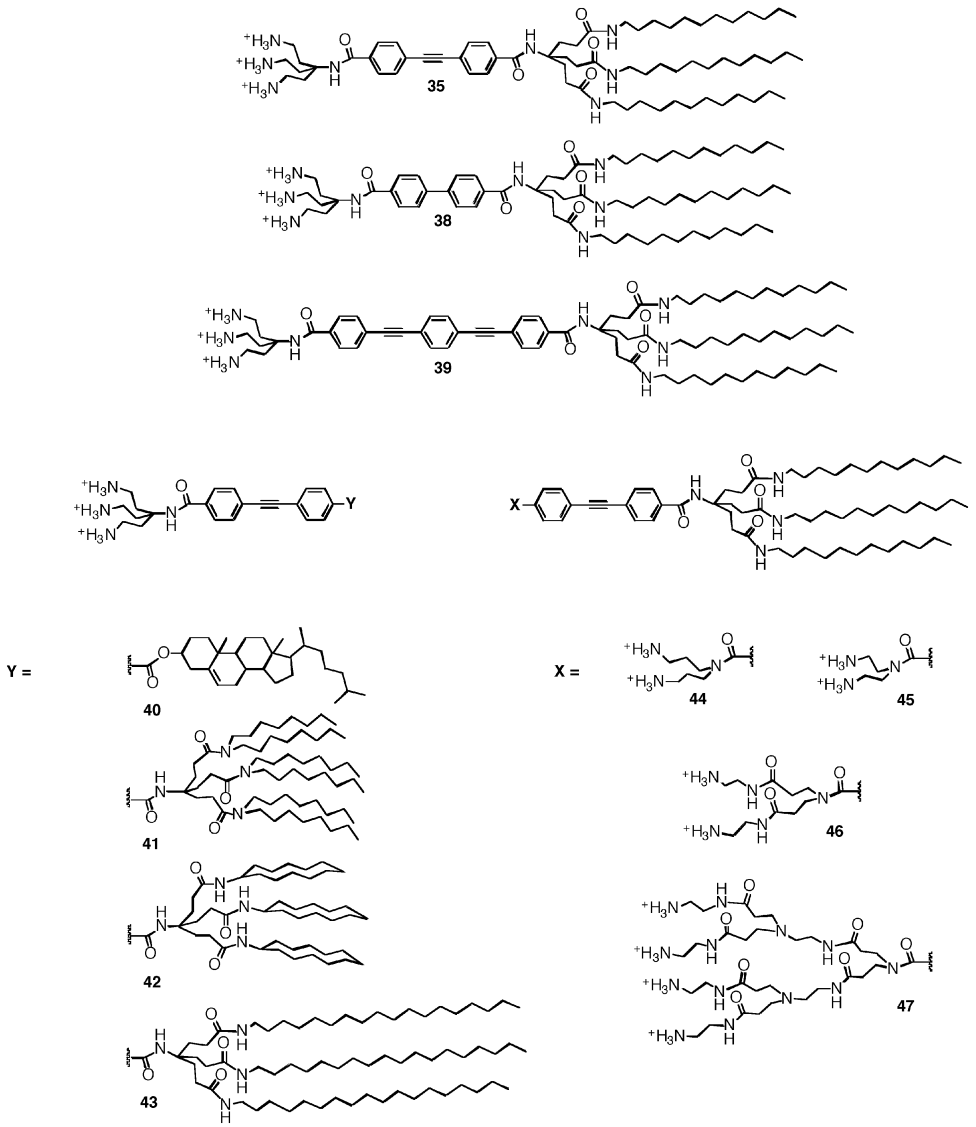

Fig. 15 Library of amphiphilic dendrimers for gene transfection formed by variation of core and hydrophilic and lipophilic dendrons.

vectors **39**, **40–43** and **47** do not favor forming films or membranes, but instead most likely form micelles.

Conclusions and perspectives

The search for an efficient and non-toxic gene transfection vector has led to the design and synthesis of a wonderful variety of dendrimeric structures. It is remarkable how in this area of research, chemical modification has been so imaginatively exploited for optimization toward a specific scientific goal.

The realization of a single "magic bullet" vector that is capable of satisfying all the requirements for transfection is an extremely lofty goal. However, two future directions can be foreseen given the current progress in structural sophistication. First is the formation of "super" functionalized molecules that are capable of carrying out several tasks. The roots of this concept are spread throughout the synthetic vector designs presented in this article. The second possibility is the development of vector systems where two or more different types of molecules work together to perform different tasks in the cell transfection process.

There is much to look forward to as the work reviewed here forms the base for further expansion and evolution of dendrimeric scaffolds for gene therapy.

Acknowledgements

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