



Dendrimers in gene delivery [☆]

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Abstract

Dendrimers have unique molecular architectures and properties that make them attractive materials for the development of nanomedicines. Key properties such as defined architecture and a high ratio of multivalent surface moieties to molecular volume also make these nanoscaled materials highly interesting for the development of synthetic (non-viral) vectors for therapeutic nucleic acids. Rational development of such vectors requires the link to be made between dendrimer structure and the morphology and physicochemistry of the respective nucleic acid complexes and, furthermore, to the biological performance of these systems at the cellular and systemic level. The review focuses on the current understanding of the role of dendrimers in those aspects of synthetic vector development. Dendrimer-based transfection agents have become routine tools for many molecular and cell biologists but therapeutic delivery of nucleic acids remains a challenge.

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1. Gene therapy

The initial promise that the combination of understanding of the molecular pathways of disease and the complete human genome sequence would yield safer and more efficient medicines and revolutionise the way we treat patients, has not been fulfilled to date. Nevertheless there is little doubt that genetic therapies will make an important contribution to our therapeutic armamentarium once some of the key challenges, such as specific and efficient delivery, have been solved. The fact that an adenoviral therapy for direct injection into head and neck cancer has been licensed recently in China [1] is just one indication of this, while highly publicised reports of the risks of some genetic therapies serve as a reminder of some of the potential risks of this emerging therapeutic approach [2,3].

1.1. Gene therapy strategies

The delivery of therapeutic nucleic acids (NA), normally in the form of plasmids, but increasingly also as smaller oligomers, remains one of the major obstacles currently hampering the further exploitation of genetic therapies. Specific and efficient delivery of genetic material to diseased sites and to particular cell populations is the challenge that is being addressed

using a variety of viral and non-viral delivery systems, which all have distinct advantages and disadvantages [4,5]. Compared to viral vectors the synthetic (non-viral) systems are in general reputed to lack of efficiency while offering flexibility and safety. However, this simplistic view ignores the fact that the suitability of any gene delivery system will always have to be matched with the clinical situation, the specific disease and the chosen therapeutic strategy [6].

Nucleic acid based therapies take two conceptually different approaches: firstly the delivery of plasmid DNA or related constructs (e.g. [7,8]) to express the gene of interest under the control of a suitable promoter which will result in the *increased activity of the target*, i.e. by production of a therapeutic protein. In contrast, the expression of oligomeric genetic material such as antisense oligonucleotides (ON), siRNA or DNAzyme which in general will lead to a *reduction of target activity*. In deciding on the appropriate genetic therapy for a given clinical problem, key factors to be taken into consideration include the number of genes involved in the pathogenesis (monogenetic/polygenetic), the required duration of therapy (temporary vs. permanent), potency of the therapeutic product, or the need for targeting or regulation of the genetic war-head.

Clearly none of the current vector systems is able to satisfy these potentially disparate needs and it is therefore important to appreciate the strengths and

weaknesses of synthetic vector systems in the appropriate therapeutic context [9,10].

1.2. Barriers to gene delivery

The observation that free plasmid DNA is able to transfect the skeletal muscle [11], the liver [12] or tumour [13] when given in the appropriate way, but will normally be degraded in the systemic circulation [14] provides the rationale for ‘packaging’ of the plasmid DNA. This packaging occurs with the help of a delivery system which tends to compact and protect the NA. Furthermore, the delivery system should help to target the therapeutic nucleic acid to the desired site of action and facilitate efficient intracellular trafficking, typically to the nucleus [6].

The most common strategy employed for the ‘packaging’ of DNA is based on electrostatic interac-

tion between the anionic nucleic acid and the positive charges of the synthetic vector which will complex and condense the NA into nanoparticles. Commonly used classes of synthetic vectors are based on various cationic lipids or polymers and, depending on the synthetic vector material used, the resulting particles have also been termed lipoplex, polyplex, or dendriplex, when dendrimers are being used [15] (Fig. 1).

Suspensions of such particles only tend to be colloiddally stable if the particles are charged, that is, the cationic carrier will be present in excess to create particles which repel one another. This positive charge is also important because it facilitates cell adsorption and mediates efficient endosomal uptake into cells [16]. However, its non-specific nature is thought to contribute to the discrepancies commonly observed between *in vivo* and *in vitro* experiments. While promiscuous binding may be advantageous in the

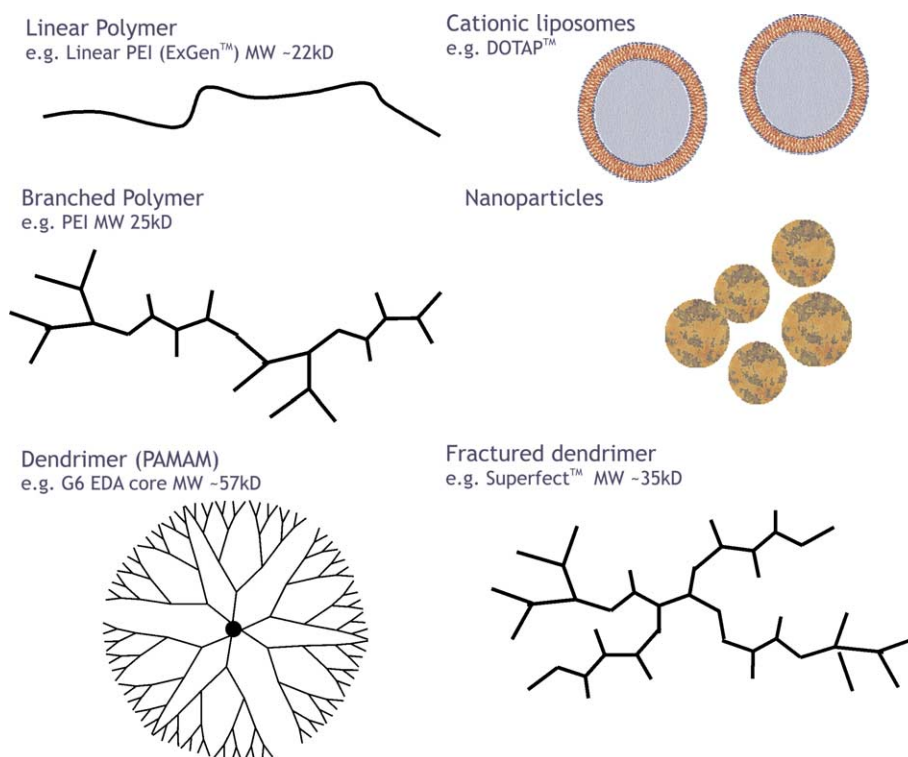


Fig. 1. Examples of synthetic vectors. Synthetic gene delivery vectors are based on a number of different, normally cationic materials which support the packaging of the DNA into nanoparticles. The most important classes of materials are cationic polymers and cationic lipids. The lipids are based on self-aggregating small cationic amphiphiles whereas the cationic polymers form complexes through multivalent electrostatic interactions. The polymers have a variety of structures but overall tend to have molecular weights of 20 kDa and above; by contrast the PPI dendrimers that our group has developed are relatively small (PPIG3, MW ~1.5 kDa).

simplified in vitro environment it translates into extensive non-specific binding to cells, biological surfaces and blood components when the charged particles are administered in vivo. This non-specific binding is thought to modify the complexes and thus make them less stable and difficult to target to organs and remote sites [17,18].

Once the complexes have reached the target cells they need to be taken up efficiently and then processed in the appropriate fashion to allow efficient transfer from the endosome to the cytoplasm and, finally, the nucleus. This requires effective traversing of intracel-

lular compartments and the lack of efficiency for these steps probably represents one of the key limitations of synthetic gene delivery systems (Fig. 2).

2. Dendrimers

Since their conception in the late 1970s and early 1980s the unique properties of dendrimers have spawned a whole range of new research areas ranging from drug and gene delivery applications to processing, diagnostics, and nanoengineering [19].

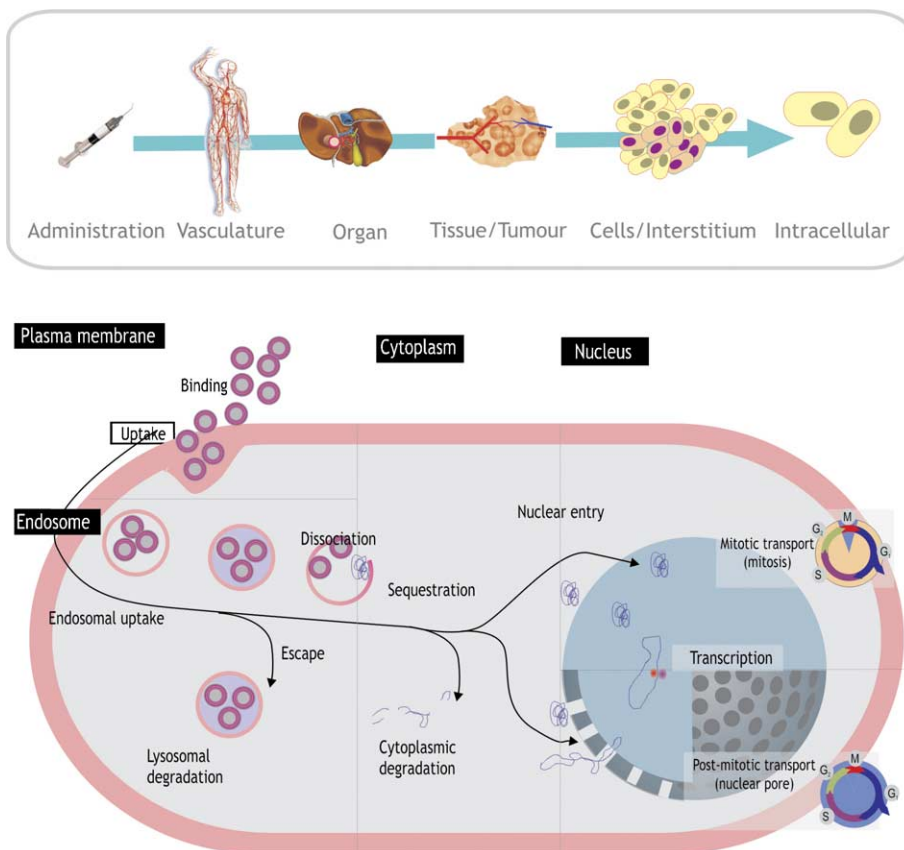


Fig. 2. The systemic delivery of gene medicines to organs and disease sites which are not directly accessible is particularly challenging (top panel). In order to reach a remote target cell, in this example in a liver tumour, the synthetic vector system needs to travel in the blood stream to the organ, and the tumour. Within the tumour the nanoparticles need to extravasate and distribute throughout the tumour interstitium to finally reach the target cells where they need to be taken up efficiently. Current systems are invariably taken up into endosomes where they would eventually be degraded. Therefore a mechanism that allows endosomal escape, e.g. by disruption after osmotic swelling (proton sponge) is required. After escaping into the cytoplasm the nucleic acid (plasmid DNA) needs to gain entry into the nucleus to be able to utilise the nuclear transcription machinery and initiate gene expression. Access to the nuclear machinery can in principle occur during cell division when the nuclear envelope disappears through the nuclear pores which allow shuffling of suitable molecules between nucleus and cytoplasm.

In comparison to the traditional polymers that initiated a materials revolution in the second half of the last century, dendrimers are relative newcomers. Nevertheless, their special properties have made these highly branched three dimensional macromolecules the focus of much research over the last one to two decades.

Dendrimers (from the Greek “dendron”: tree, and “meros”: part) consist of a central core molecule which acts as the root from which a number of highly branched, tree-like arms originate in an ordered and symmetric fashion (Fig. 3).

Dendritic structures emerged from a new class of polymers named “cascade molecules”, initially reported by Vögtle and his group at the end of the 1970's [20] and developed further by Tomalia, Newkome, and others to give rise to the larger dendritic structures [21–24]. These hyper-branched molecules were called “dendrimers” or “arborols” (from the Latin “arbor” also meaning tree, for a historical view see review [25–32]).

Their unique molecular architecture means that dendrimers have a number of distinctive properties which differentiate them from other polymers; specifically the gradual stepwise method of synthesis means that they have in general a well defined size and structure with a comparatively low polydispersity index. Furthermore, dendrimer chemistry is quite adaptable thus facilitating synthesis of a broad range of molecules with different functionality. Key properties in terms of the potential use of these materials in drug and gene delivery are defined by the high density of terminal groups. These contribute to the molecules surface characteristics, offer multiple attachment sites e.g. for conjugation of drugs or targeting moieties, and determine the molecular volume which is important for the ability to sequester other molecules within the core of the dendrimer.

Dendrimers lend themselves to nanoengineering of these key properties in order to fashion materials for applications in drug and gene delivery, imaging, boron neutron capture therapy, but also various

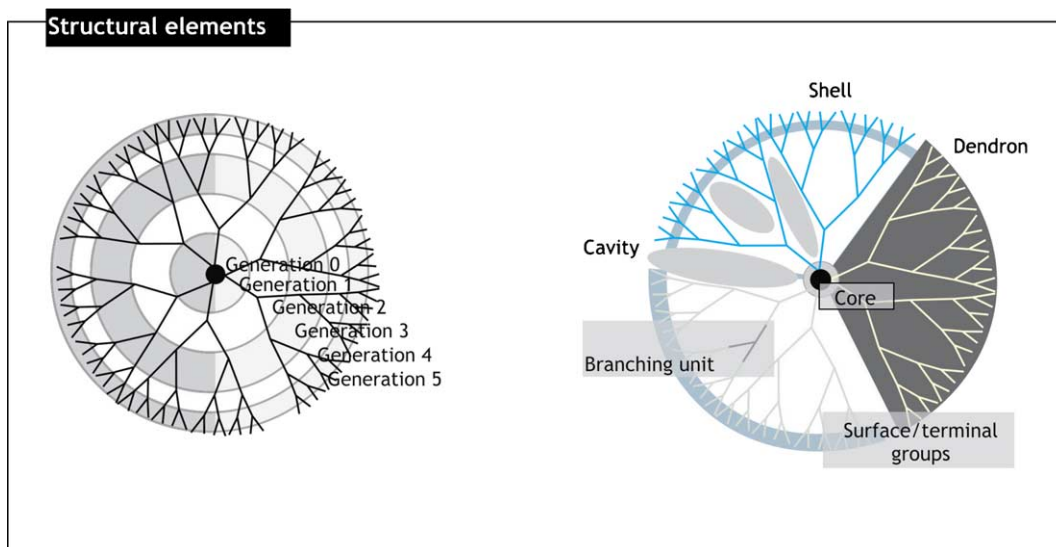


Fig. 3. Dendrimer structure. The stepwise synthesis of dendrimers means that they have a well defined hierarchical structure. This hypothetical dendrimer is based on a core with three covalent root attachment points but other common cores have di- or tetravalent cores. The valency of the core dictates the number of linked dendrons and the overall symmetry of the molecule. The dendrons are synthesised by covalent coupling of the branch units. For each additional layer or *generation* that is being added to the structure the reaction sequence is repeated. In this case the units have two new branching points at which additional units can be attached. (The generation count is not always consistent: normally *generation 0* refers to the core while sometimes it is used to describe the dendrimer after the first reaction cycle.) The number of branching points, branching angles, and the length of the branching units determine to what extent each generation increases molecular volume vs. surface area. For the higher generations the density of the terminal groups reaches a point where for steric reasons no groups can be added (starburst effect). Dendrimers of higher generation also have a typical molecular density profile under favourable conditions; the high peripheral molecular density establishes a steric outer shell and the lower density at the centre creates cavities which can accommodate guest molecules.

biotechnological diagnostics and sensing functions [22,33]. This review will give an overview of the specific use of dendrimers in gene delivery and how it relates to the specific properties of these materials.

2.1. Structures and synthesis

Dendrimers are generally characterised by a well defined molecular architecture based on a stepwise synthesis using either a divergent or a convergent method (Fig. 4) [21,22].

The use of dendrimers in gene delivery draws on a much more narrow range of chemical architectures, predominantly those with a cationic net surface charge, of which the PAMAM and PPI dendrimers are commercially available.

The first exploration of dendrimers as molecules for gene delivery focused on the PAMAM dendrimers [21]. The PAMAM dendrimers are normally based on an ethylenediamine or ammonia core with four and three branching points, respectively [34–36]. Using a divergent approach the molecule is built up iteratively from the core through addition of methylacrylate followed by amidation of the resulting ester with ethylenediamine. Each complete reaction sequence results in a new ‘full’ dendrimer generation (e.g. G3, G4. . .) with terminal amine functionality, whereas the intermediate ‘half’ generations (e.g. G2.5, G3.5. . .) terminate in anionic carboxylate groups (Fig. 5).

The other commercially available dendrimer with relevance for drug and gene delivery is based on polypropylenimine (PPI) units with butylenediamine (DAB) used as the core molecule. The repetitive reaction sequence involves Michael addition of acrylonitrile to a primary amino group followed by hydrogenation of nitrile groups to primary amino groups [37]. These dendrimers are frequently referred to as DAB- x , or DAB-Am- x , with x giving the number of surface amines (Fig. 5).

The commercial availability and relative efficiency of PAMAM and PPI dendrimers have meant that these materials and their derivatives currently dominate the area of gene delivery with dendritic polymers but a number of other alternative systems have been developed:

Phosphorous containing dendrimers (P-dendrimers) of varying generation (G2–G5) terminated with protonated or methylated tertiary amines were

tested as transfection agents [38]. At N/P ratios of 5:1 the larger P-dendrimers (G3–5) with protonable amines were of a similar efficiency as linear PEI 22 kDa (ExGen 500™).

A dendrimer in which the oligonucleotide to be delivered becomes part of an anionic dendrimer has recently been reported [39]. The covalently attached ODNs were protected from degradation and showed improved uptake while still being able to hybridise with their target.

Interestingly, DNA itself has been shown to be able to support assembly into dendritic structures although it is unclear whether this will have any application for nucleic acid based therapies [40].

While the lower generation of dendrimers in general tend not to complex DNA efficiently, PAMAM_{G2} can effectively bind DNA when covalently linked to a mesoporous silica bead (250 nm, 2.5 nm pores) [41–43]. These systems have been shown to transfect mammalian cells and could potentially be used to simultaneously act as carriers for drugs encapsulated within the porous bead.

While most dendritic polymers currently used are based on symmetric structure with multiple branches emanating from a central core, work by Florence and colleagues demonstrates that this is not obligatory: water soluble amphiphilic dendritic polylysine or poly-ornithine peptides with a hydrophobic root ($3 \times \alpha$ -amino myristic acid) are asymmetric and have a relatively lower charge density than PAMAM dendrimers but can still achieve transfection [44,45].

Another polylysine ‘branch’ structure was synthesised as an asymmetric methoxy(ethylene glycol)-*block*-PLL dendrimer or a symmetric ‘barbell shaped’ PLL dendrimer-*block*-methoxy(ethylene glycol)-*block*-PLL dendrimer triblock copolymer which efficiently condenses DNA [46,47].

Many other interesting dendrimer chemistries have been developed and are reviewed elsewhere [48].

2.2. Physicochemical properties

Because of their molecular architecture, dendrimers show some unique physical and chemical properties which make them particularly interesting for drug and gene delivery applications. Direct comparison with linear or branched conventional polymers is however not trivial because of the

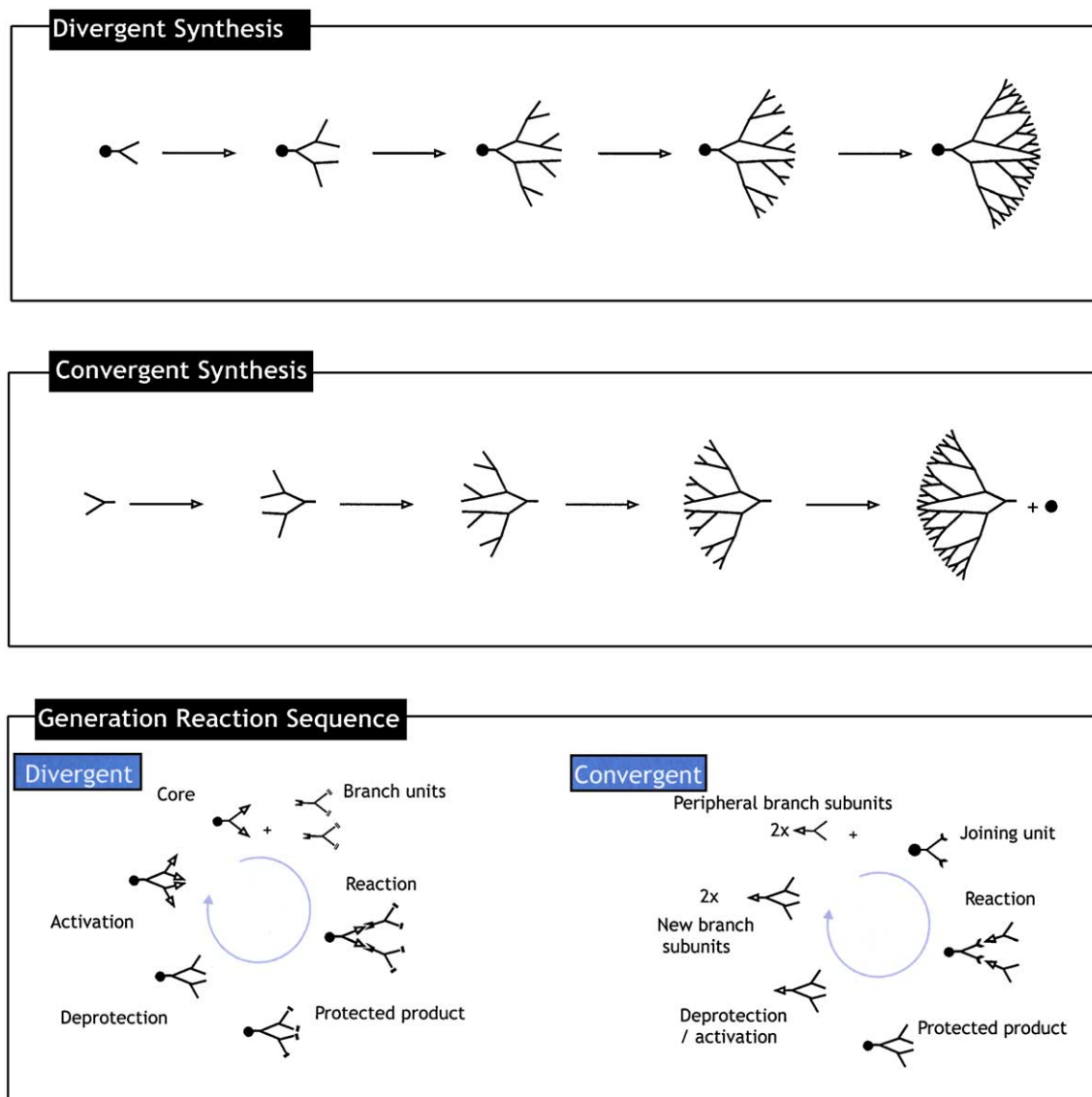
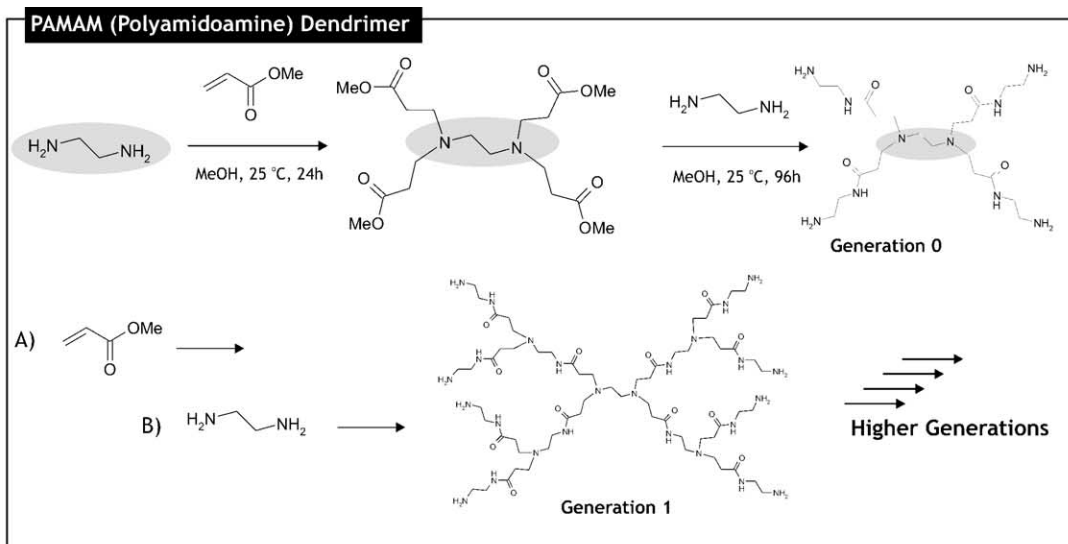


Fig. 4. Synthetic strategy. Synthetic strategies for dendrimers are based on two conceptually different strategies, the divergent and the convergent approaches. *Divergent approach*: in the divergent method, the dendrimer is synthesised starting from the multifunctional core and build up one monomer layer, or “generation”, at the time [33]. The core molecule reacts with monomer molecules containing one reactive group and two (or more) inactive groups. The reactive group reacts with one of the roots of the core molecule giving the first generation dendrimer. After activation of the inactive groups at the periphery of the molecule the reaction sequence is repeated with the next generation of monomers. The process is repeated for several generations until steric effects prevent further reactions of the end groups (starburst effect). *Convergent approach*: in the convergent approach, the dendrimer is also built up layer after layer, but this time starting from the end groups and terminating at the core [21]. Here two (or more) peripheral branch subunits are reacted with a single joining unit which has two (or more) corresponding active sites and a distal inactive site. The reaction is a new larger branch subunit that is again reacted with a joining. When the growing branched polymeric dendrons have reached target size they are in turn attached to a core molecule to yield the dendrimer, generally highly symmetric.

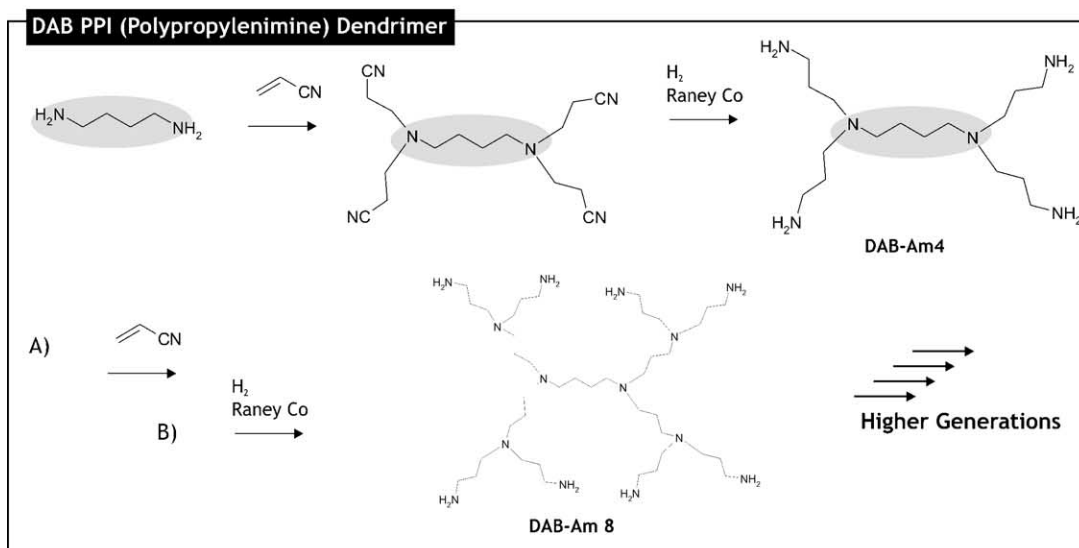


Number of surface groups $(Z) = N_c N_b^G$

Number of branched cells $(BC) = N_c \left[\frac{N_b^G - 1}{N_b - 1} \right] = \text{Number of bonds/generation}$

Molecular Weight $(MW) = M_c + N_c \left[M_{RU} \left(\frac{N_b^G - 1}{N_b - 1} \right) + M_t N_b^G \right]$

for a PAMAM dendrimer with EDA core;
 where N_c = core, N_b = branch cell multiplicity, G = generation;
 from [32]



difficulty of changing polymer architecture without affecting other parameters. Nevertheless, a comparative study of the properties exhibited by dendrimers and linear macromolecules of the same repeating unit ($\text{OC}_6\text{H}_4\text{P}(\text{Ph})_2\text{N}-\text{PS}$ (including both PN and PS double bonds, and P–O and P–C single bonds) provides an acute insight into how their molecular features affect the structure–property relationship (i.e. solubility of phosphorus-based dendrimers in organic solvents, contrary to linear polymers) [49,50].

In contrast to linear polymers the intrinsic viscosity of dendrimer solutions does not increase linearly with mass but shows a maximum at a specific generation [46,51]. This is likely to be because of the way in which dendrimer shape changes with generation, i.e. lower generations adopt a more open planar–elliptical shape with transition to a more compact spherical shape for higher generations. The compact shape also reduces the likelihood of entanglement which affects larger classical polymers.

Because of the dendrimer structure (Fig. 6) the molecular density is theoretically highest in the periphery of the dendrimers. This stylised picture does not necessarily reflect the true shape and it has been suggested that back folding of the terminal branches towards leads to a more uniform or even reverse density profile [52] (Fig. 6). The actual confirmation of dendrimers in solution will ultimately also depend on the interaction with the solvent: for example, the PPI_{G_3} (DAB-Am16) NMR takes on an extended conformation in a “good” solvent (chloroform), but a folded conformation when exposed to a “poor” solvent (benzene) [53,54]. Other factors that influence solubility and conformation include salt conditions, changes in pH, dissolved ions [30].

The higher generation dendrimers have an “outer shell” of high molecular density (starburst effect) just

beneath the surface which provides a barrier that can create a distinct microenvironment within the dendrimer core which potentially also would allow the encapsulation of guest molecules [55].

In nature tree-like structures have evolved to maximise the exposed surface area, e.g. to maximise the light exposure/number of leaves of a tree. In a similar fashion dendritic architecture creates molecules where a large proportion of the groups are exposed at the surface and which can have very high molecular surface to volume ratios (up to $1000 \text{ m}^2 \text{ g}^{-1}$) [49]. The presence of numerous terminal groups in dendrimers facilitates multiple simultaneous interactions of surface groups with the solvent, surfaces or other molecules and, as a consequence, dendrimers tend to show high solubility, reactivity, and binding [56].

This multivalency is of general importance for biological interactions but may be of particular importance for biomedical applications, as the multimeric binding through statistical and/or cooperative effects can increase affinity, avidity and specificity of binding [57]. The multiple interactions between surface amines and nucleic acid phosphates are also important for the formation of dendrimers and DNA complexes (vide infra).

Furthermore, the multiple surfaces groups can be derivatised simultaneously with a number of groups to modify properties, for example targeting ligands or hydrophilic copolymers (PEG) for steric stabilisation.

2.3. Biological properties

When considering the general biocompatibility of dendrimer-based gene delivery systems, one needs to be careful to distinguish between interactions and effects of the free dendrimer and those related to the

Fig. 5. Synthesis of commercially available dendrimers. PAMAM dendrimer (top panel). The PAMAM dendrimers are normally based on an ethylenediamine or ammonia core with four and three branching points, respectively [149]. Using a divergent approach the molecule is built up iteratively from the core through addition of methylacrylate followed by amidation of the resulting ester with ethylenediamine. Each complete reaction sequence results in a new ‘full’ dendrimer generation (e.g. G3, G4. . .) with terminal amine functionality, whereas the intermediate ‘half’ generations (e.g. G2.5, G3.5. . .) terminate in anionic carboxylate groups. PAMAM dendrimer structure (middle panel). The well defined structure of dendrimers means that their properties are not statistical but can be calculated using straightforward formulas based on known parameters such as core root points, branching units structure and number of generations [34–36]. PPI dendrimer (bottom panel). The other commercially available dendrimer with relevance for drug and gene delivery is based on polypropylenimine (PPI) units with butylenediamine (DAB) used as the core molecule. The repetitive reaction sequence involves Michael addition of acrylonitrile to a primary amino group followed by hydrogenation of nitrile groups to primary amino groups [87]. These dendrimers are frequently referred to as DAB-*x*, or DAB-Am-*x*, with *x* giving the number of surface amines; depending on the source DAB-Am4 is sometimes referred to as G0 or G1.

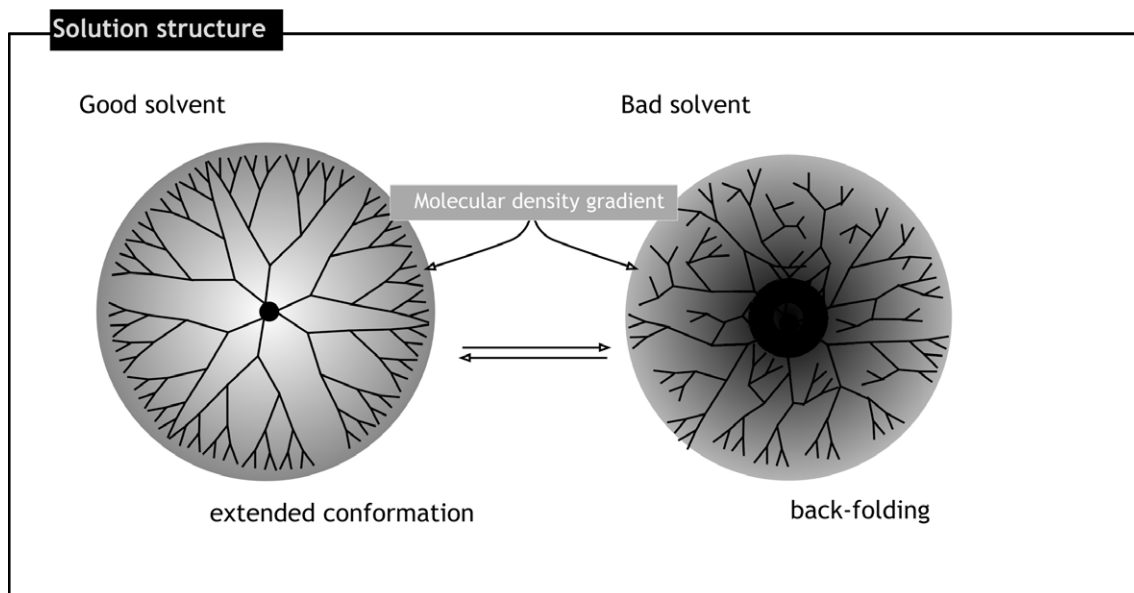


Fig. 6. Dendrimer conformation. The equilibrium between structures with dense shell vs. dense core depends on dendrimer chemistry and solvent properties (polarity, pH, salt, etc.); back-folding of peripheral groups into the centre modifies molecular density away from the outer shell and leads to a more even distribution of molecular density or a 'dense core' dendrimer structure.

delivery system as a whole, i.e. when the dendrimer is part of a supramolecular assembly. When used to deliver NA based therapeutic, molecules dendrimers are typically part of a supramolecular assembly, i.e. nanoparticles. The biological properties of such supramolecular structures may differ considerably from that of the free molecule. In general, complexation with DNA tends to reduce toxicity but particulate materials may show distinct biodistribution or cellular trafficking characteristics which can create unique effects with a different toxicity profile.

2.3.1. *In vitro*

The initial evaluation of biological properties of PAMAM dendrimer *in vitro* found them to be relatively non-toxic [58]. In cytotoxicity assays they compare favourably with some of the other transfection agents, in particular cationic polymers of higher molecular weight such as PEI (600–1000 kDa), PLL (36.6 kDa), or DEAE–dextran (500 kDa) which in these assays are around 3 orders of magnitude more toxic [58]. In contrast to the large MW PEI and PLL polymers, PAMAM dendrimer toxicity did not seem to stem from membrane damage as assayed by LDH release or haemolysis [59]. Nevertheless, dendrimers

interact effectively with cell membranes and the electrostatic interactions of cationic polymer and anionic cell surfaces are highly important for the cellular uptake of charged DNA complexes [60]. Studies of membrane interactions of PAMAM dendrimers with DMPC/DMPA vesicles suggested that vesicles can in fact wrap around larger dendrimers [61]. Membrane interactions are thought to be important for toxicity because of the direct damage to the target cells but, furthermore, such interactions may also pose a problem on systemic injection when erythrocyte lysis or aggregation could lead to toxicity.

Size is a key determinant of dendrimer cytotoxicity for both PAMAM [62] and PPI dendrimers [63,64]. Cytotoxicity of PAMAM dendrimers increases with generation, independent of surface charge, for both full generation cationic dendrimers (G2–G4) and the 'half-generation' anionic intermediates (G2.5, G3.5) [64,65].

The nature and density of charged groups are other factors that determine dendrimer toxicity [66]. Cationic (surface) charges are in general more toxic but details depend on the specific groups involved, that is, for amines it has been proposed that primary amines are relatively more toxic than secondary or tertiary

amines. A concentration dependent tendency to cause haemolysis and changes in erythrocyte morphology has been linked to the presence of $-\text{NH}_2$ groups [66]. In contrast to PAMAM dendrimers PPI dendrimers with DAB and DAE cores did not show generation dependence for the haemolytic effect. In general dendrimers were found to interact significantly less with erythrocytes than PEI but were nevertheless haemolytic at concentrations above 1 mg mL^{-1} [67–70].

Quaternisation has previously been used as a strategy to reduce toxicity of polymers [71]. The approach also seems to be beneficial for higher generation PPI dendrimers but for complexes the effects of quaternisation are complex and can include changes of complex morphology and physical chemistry which are difficult to deconvolute [72].

By contrast anionic dendrimers, e.g. those bearing a carboxylate surface, have been reported to be non-cytotoxic over a broad concentration range [64], although even for anionic dendrimers (e.g. ‘half’ generation PAMAM) a correlation exists between toxicity and molecular weight [73].

Shielding of surface groups has also been used successfully to reduce toxicity e.g. through covalent attachment of C_{12} lauroyl groups or PEG 2000 [66]. The modification of terminal groups has been suggested to be more efficient for higher generations dendrimers, as the relatively higher density of non-toxic surface groups may also be more effective in preventing access to a potentially toxic core [72].

2.3.2. *In vivo*

On intravenous injection ^{125}I labelled cationic PAMAM dendrimers (G_3 , G_4) are rapidly eliminated from the circulation (around 99% in 1h) and accumulate in the liver (more than 60%) [66]. A similar pattern was found for the anionic ‘half-generation’ PAMAM dendrimers (2.5, 3.5, 5.5), although clearing was somewhat slower and accumulation in the liver less pronounced [63]. An earlier study by Roberts and colleagues [74] reported kidney accumulation for $\text{PAMAM}_{\text{G}_3}$ and accumulation in the pancreas for the $\text{PAMAM}_{\text{G}_5}$ and $\text{PAMAM}_{\text{G}_7}$. A high level of kidney excretion was observed for G_7 but studies with PAMAM dendrimers with varying degrees of terminal biotinylation suggest that retention may increase with size and charge density [75].

Clearly these observations do not necessarily hold true for complexes made from DNA and dendrimers. In general the toxicity of cationic polymers bound to DNA decreases in *in vitro* assays but the particulate nature of complexes is likely to have a major influence on their biodistribution, e.g. their involvement with enhanced permeation and retention effect to target tumours [76]. Macromolecules are also expected to be able to utilise this effect [77] which has been exploited for the targeting of drug loaded dendrimers [78,79].

3. Dendrimers as synthetic vectors

3.1. *Dendrimer–nucleic acid interaction*

The complexation process between dendrimers and nucleic acids does not seem to differ fundamentally from other cationic polymers with high charge density: dendrimers interact with various forms of nucleic acids, such as plasmid DNA or antisense oligonucleotides, to form complexes which protect the nucleic acid from degradation [57,78,80]. The interaction between dendrimer and nucleic acids is based on electrostatic interactions [81] and lacks any sequence specificity [80].

During the complexation the extended configuration of plasmid DNA is changed and a more compact configuration achieved, with the cationic dendrimer amines and the anionic NA phosphate reaching local charge neutralisation and the formation of NA–dendrimer complexes (“dendriplexes”).

The nature of the complex is not only dependent on the stoichiometry and concentration of the DNA phosphates and dendrimer amines but also on the bulk solvent properties (e.g. pH, salt concentration, buffer strength) and even the dynamics of mixing. High ionic strength, i.e. increased amounts of NaCl, interferes with the binding process [82] but also appears to help to establish equilibrium [83,84]. The medium in which complexes are formed not only affects their morphology but also modifies other properties and even stability *in vivo* (e.g. PEI [80]).

PAMAM dendrimers bind DNA at a 1:1 stoichiometry of primary amine to phosphate [85,86] but these dendrimer to DNA ratios are not necessarily ideal; as with other polymeric systems, more stable

and efficient complexes tend to be formed only at higher polymer to DNA ratios.

With each increasing dendrimer generation the number of surface amine groups, which are most likely to bind DNA, doubles [81]. This also affects the nature of complexes formed by the different generations; a model for the binding of PAMAM dendrimers to DNA has been put forward which explains the observation of increased binding with higher generation dendrimers (G7 vs. G4, G2) [81]. The model postulates the presence of regions of tightly bound DNA interspersed with ‘linker’ DNA (Fig. 7). Based on the observation of binding of EthBr to DNA–dendrimer complexes the authors postulate that the higher generation dendrimers achieve a higher proportion of tightly bound DNA by ‘wrap around’ of DNA [87].

A recent study suggests that smaller dendrimers PAMAM_{G2} which do not induce a ‘wrap around’ may in fact bind DNA relatively better than the larger PAMAM_{G6}, potentially because of the more fluid structure of these smaller dendrimers [82].

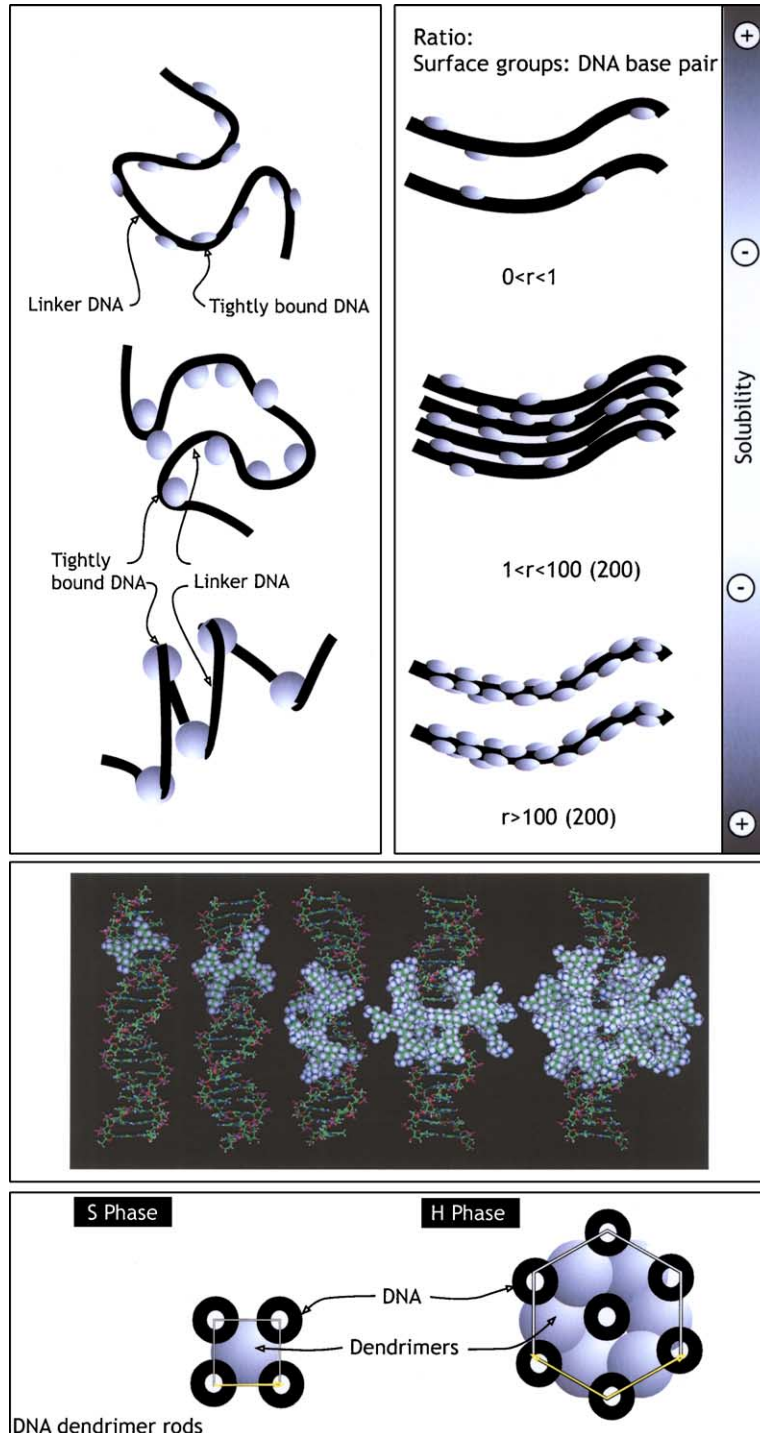
In the same study a model is proposed to account for the observation of distinctive phases at low, medium, and high ratios of PAMAM dendrimer to DNA (expressed as surface groups to DNA base pairs) (Fig. 7). Measurable interaction with moderate DNA stabilisation is already observed at low ratios <1. At ratios > 1 the dendrimers and DNA form the familiar complexes most relevant for gene delivery. Interestingly a saturation of binding occurs at ratios greater than 100 (PAMAM_{G2}) and 200 (PAMAM_{G6}), respectively, above which a resolubilisation of the DNA by ‘salting in’ occurs.

PPI dendrimers of all generations when added in sufficient amounts form water insoluble DNA com-

plexes [82]. While the G1–G2 PPI dendrimers lead to the formation of electroneutral complexes even at dendrimer:DNA charge ratios >1, the higher generation dendrimers were able to produce charged soluble complexes because of the ability to form over-stoichiometric complexes with a net positive charge [88]. Interestingly the authors also demonstrated that the complexation behaviour of DNA itself differed significantly from that of other poly-cationic polymers: the flexible linear polymers (‘single strand’) were able to interact with all dendrimer amines including those ‘inside’ the dendrimer, whereas the more rigid DNA (‘double strand’) was only able to interact with surface amines [62]. This configuration would leave a number of anionic and cationic residues unable to interact and thus retain some charge with the complex. Molecular modelling studies with PPI dendrimers of G1–G5 also suggest that for generations higher than 2–3 a significant proportion of the dendrimer molecule would not interact directly with the same DNA strand (Fig. 7) [62]. The PPI_{G1} appears to bind across the major groove but the larger PPI_{G3} is sufficiently large to bind across an entire helical turn, spanning major as well as minor groove. In the above model of dendrimer binding there is not necessarily a distinction between core and surface amines for DNA binding but, analogous to other models of DNA–dendrimer interaction, uncomplexed groups remain in the case of the higher generation dendrimers. This study also supported the notion of a minimum size requirement for optimal DNA binding, although in this case the optimum is reached earlier than had been suggested for PAMAM dendrimers, i.e. around G3 to G4 [89].

With regard to the longer range arrangement of the dendrimers along the DNA a recent X-ray diffraction

Fig. 7. Dendrimer DNA interaction. The supramolecular structures formed by dendrimers and DNA differ with dendrimer–DNA ratio. A model that is trying to explain generation dependent differences in DNA binding suggests the existence of more loosely bound regions of DNA (‘linker DNA’) and tighter bound DNA, with the ability to induce DNA wrap around for higher generation dendrimers (top left panel, after [81]). Depending on the ratio of DNA and dendrimer various supramolecular aggregates of DNA and dendrimers exist. The observation of distinctive phases of high and low solubility aggregates at low, medium, and high ratios of PAMAM dendrimer to DNA (expressed as surface groups to DNA base pairs) can be explained as illustrated. Soluble aggregates exist at ratios below 1 (PAMAM) and at high ratios of greater than 100 (PAMAM_{G2}) or 200 (PAMAM_{G6}). The intermediate ratios are dominated by insoluble complexes (top right panel, after [87]). Molecular modelling studies with the smaller PPI dendrimers (PPI_{G1–5}) suggest different dendrimer generations interact with DNA to a varying extent: the first generation dendrimer only binds across the major groove whereas the larger G3 binds across an entire helical turn. With increase in size a larger proportion of the dendrimer appears not to directly interact with the neighbouring DNA (middle panel, from [62]). Longer range forces organise the structures formed through DNA–dendrimer interaction. An X-ray diffraction based model of PPI_{G4} and PPI_{G5}–DNA aggregates shows the formation of columnar mesophases when condensed with high molecular weight DNA. The cartoons depict cross sections through extended fibrils with square (S phase) or hexagonal (H phase) arrangement around the dendrimer core (bottom panel, after [89]).



study using PPI_{G4} and PPI_{G5} suggests that these complexes form hexagonal mesophases when condensed with the high molecular weight DNA. These phases show a square or hexagonal arrangement around the dendrimer core [78,80] with a tendency to form extended fibrils. Furthermore the exact structure was shown to be changeable in response to changes in ion concentration and DNA/dendrimer ratio.

Initial studies of DNA complexes formed by PAMAM dendrimers found that their morphology was quite similar to complexes formed with other cationic polymers such as polylysine or polyethylenimine [80]. In all cases the formation of toroidal structures of around 50 nm was observed. Polylysine and intact PAMAM dendrimer-based complexes, in particular with higher generation dendrimers, were found to have a tendency to form clusters rather than distinct units, in contrast to those complexes observed for the PEI and fractured PAMAM. Complex size tended to decrease with increasing polymer:DNA ratio for the fractured PAMAM dendrimer [71,79,90].

The morphology of PAMAM and PPI dendrimer DNA complexes has recently elucidated further using atomic force microscopy [91].

Interestingly there is some evidence to suggest that at least for some of the systems there is considerable heterogeneity among the complexes formed under specific conditions. For a specific formulation of PAMAM_{G7} DNA complexes more than 90% of transfection resulted from only 10–20% of complexes which were of lower density and solubility [92].

3.2. Mechanistic aspects of dendrimer transfection

In mechanistic terms it appears that dendrimers are in fact quite comparable to other polymeric transfection agents. The specific structure of a dendrimer influences its physicochemical properties and thus the properties of the resulting complex, but any such difference seems to result only in gradual (rather than categorical) or qualitative differences between the different formulations.

The binding of cationic DNA complexes to the cell membrane is in general based on an initial electrostatic attraction between the cationic complex and the negatively charged cell surface groups. The complexes are then taken up by endocytosis and depend on efficient endosomal escape mechanisms to be able

to reach the cytosol and finally the nucleus. Recent research suggests that binding and uptake of dendrimer (Superfect™) depend on cholesterol [93], as had been reported for lipoplexes [94].

The G6 and G7 PAMAM dendrimers are highly effective in inducing leaky fusion of model vesicles probably by induction of an inverted hexagonal phase [95]. This tendency for strong interaction with membranes was confirmed in fibroblasts; PAMAM dendrimer binding to single fibroblasts was quantified using confocal microscopy and was found to correlate with dendrimer generation [96]. A recent report suggests that the dendrimers interact with artificial and cellular membranes in a way that facilitates formation of small (15–40 nm), transient pores [61,97,98]. This effect was dependent on cationic dendrimer surface groups and correlated with charge density, that is, the PAMAM_{G7} was found to be significantly more active than the PAMAM_{G5}.

There is now good evidence supporting the importance of dendrimer buffering capacity to act as a ‘proton sponge’ and facilitate efficient endosome disruption [98]. The high buffering capacity of polymers such as PEI and PAMAM leads to a decelerated acidification of the endosome, an increased accumulation of osmotically active Cl⁻, and induces a 140% increase in endosome volume [99,100].

The notion that the amount of dendrimer is not only important in creating the excess positive charge which supports cellular association and uptake but also for the intracellular trafficking process is also supported by data for cyclodextrin–dendrimer complexes [101]. Here it was shown that cellular association depends on excess positive charge, but, while the optimum of transfection was achieved with higher charge ratios of 200:1 maximum cellular association was already reached at ratios of around 5:1.

The transfer from the cytoplasm to the nucleus is a critical step in the transfection process. Fluorescence microscopy of dendrimer–AS complexes (Oregon green conjugated PAMAM_{G5} and TAMRA labelled AS oligonucleotide) suggests that the dendrimer itself has the ability to accumulate to some extent in the nucleus [102] similarly as it has been described for PEI [103].

One intriguing difference between polymeric and lipidic delivery systems seems to lie in their intracel-

lular processing. While for lipid based systems dissociation of the complex at the level of the endosome seems to be obligatory, this does not necessarily hold true for polymeric systems which appear to have at least some activity even when still complexed. Specifically PAMAM_{G5} dendrimer antisense ON complexes seemed to be active although a large proportion of AS in the nucleus seemed to be still complexed [78]. However, an early study suggested that PAMAM dendrimers would inhibit the initiation of transcription *in vitro* but not affect the elongation of the RNA transcript [104].

3.3. Dendrimers as cellular transfection agents

The first report of the use of Starburst™ PAMAM dendrimers as transfection agents demonstrated that these agents could efficiently induce expression of reporter genes in adherent and suspension cell cultures with the G6 (NH₃) dendrimer having optimum efficiency [104]. Relatively small dendrimer DNA complexes with a significant excess of positive to negative charge (6:1) were most efficient but strongly affected by the presence of serum [104]. Interestingly it was also demonstrated that these materials, in contrast to poly-L-lysine, were not dependent on the presence of lysosomotropic agents, suggesting that they had an intrinsic ability to escape from the endosome. The authors suggested that this ability may be related to the ability of the dendrimer amine groups to buffer pH changes in the endosome [104–106]. This has been proposed as a general mechanism that facilitates escape from the endosome because of the accumulation of Cl⁻ and subsequent osmotic swelling of the endosome [98]. This hypothesis has also been supported by some recent experiments which studied the effect of various polyamines on endosome swelling [57].

Bielinska and colleagues then demonstrated that the cationic PAMAM dendrimers were not only able to complex and deliver plasmid DNA but also antisense oligonucleotides [107]. Furthermore, they were able to use PAMAM dendrimers to create cell lines which constitutively express a reporter gene.

While PAMAM starburst dendrimers of generation G3 to G10 were found to form stable complexes with DNA their ability to transfect different cell lines varies. Overall the higher generation dendrimers (G5–

G10) were found to be of superior efficiency, showing a near exponential increase of efficiency with generation in Rat2 cells [107]. In some cell lines the ability to create stable clones was also quantified and found to be on the order of 10⁻³ to 10⁻⁵. The nature of the core, ammonia (NH₃) or ethylenediamine (EDA) was found to be less significant, highlighting the greater importance of the surface in the nature of the complex [108]. This may however be less clear for smaller dendrimers where access to the core groups is sterically less restricted. More recently a comparison of PAMAMs derived from pentaerythritol (DP), inositol (GI) and trimesyl (DT) core architectures demonstrated an effect of core structure on both the optimum dendrimer generation for condensation and *in vitro* transfection, with DT having an optimum of G6 rather G5 [109]. A molecule which resembles a ‘pulled apart’ PAMAM dendrimer, i.e. a barbell shape with an extended core, was synthesised as a PAMAM–PEG–PAMAM triblock copolymer [104]. This spatial extension of the core improved cytotoxicity of the dendrimer and colloidal stability of the complexes without major changes to the transfection efficiency.

Density of groups however appears to be of importance for the ability of PAMAM dendrimers to transfect: difficulties in reproducing earlier results with PAMAM_{G3} NH₃ starburst dendrimers [110] led to the suspicion that degradation of the polymer had contributed to its good transfection ability [110]. By heating in solvolytic solvents dendrimers can be fractured or activated to give ‘imperfect’ dendrimers which have clearly improved efficiencies with enhancements in the order of > 50× [111]. These significant differences in biological effect between almost identical compounds highlight the importance of even subtle changes on complex physicochemistry and subsequently transfection efficiency of complexes.

One of the key advantages of synthetic transfection agents is their sequence independence and the ease with which even large DNA constructs can be accommodated. An extreme example is the successful transfection of a 60 Mb artificial mammalian chromosome into cells using a PAMAM dendrimer (Superfect™) [112].

In a comparative evaluation of various polyplexes based on linear, branched, and dendritic polymer structures, Gebhart and colleagues demonstrated that the transfection activity between these polymers

varied by 3 orders of magnitude [112]. The authors ranked the best agents according to their ability to transfect a panel of cell lines. The ranking was 22 kDa linear PEI (ExGen 500™) > activated PAMAM dendrimer (Superfect™) ≫ 25 kDa branched PEI > P123-g-PEI(2 k), a Pluronic PEI graft block copolymer. These polymer based systems were found to be more active than some of the commercial cationic lipid systems [112]. However, transfection activity varied up to 3 orders of magnitude depending on the specific cell line. Interestingly the same study also demonstrated that factors such as incubation time of the complexes with the cells, or cell density will affect different polymers to a varying degree, that is, linear PEI 22 kDa based complexes show a cell density dependence, while the fractionated PAMAM dendrimer complexes (Superfect™) show some time dependence, requiring longer incubation time [112]. A PPI dendrimer with DAB core (DAB-Am64, Astramol™), despite the similar architecture to the PAMAM dendrimer, appeared to be the least efficient agent. Its application was also hampered by signs of toxicity at higher N/P ratios [72], which had been highlighted previously [62].

Our own observations support the notion that for the DAB-PPI dendrimers—as for most other synthetic transfection agents—a balance needs to be struck between the ability to facilitate transfection and cytotoxicity [62]. Both the ability of DAB-PPI dendrimers to bind DNA, as well as their cytotoxicity, are generation dependent. Physicochemical characterisation of complexes and molecular modelling studies support the notion that an optimal size, i.e. dendrimer generation, for DNA binding exists which also shows some correlation with the efficiency of transfection. In this study the lower generations of PPI dendrimers, specifically PPI_{G3} (DAB-AM16), demonstrate a transfection capability similar to that of the cationic lipid DOTAP [113]. More recently this dendrimer was also shown to be able to mediate antisense transfer in vitro at levels comparable to the commercial oligonucleotide transfection agent Oligofectamine™ [114]. PPI dendrimers were also shown to strongly improve cellular delivery of ON in another study focusing on triplex forming ON [114]. The enhancement was reported for various cancer cell lines (MDA-MB-231 14-fold) and found to be strongest for G4 [57,101,115–117].

The ability of PAMAM dendrimers to deliver oligonucleotides has been established previously [115] and a PAMAM_{G3} dendrimer was found to increase cellular uptake of phosphorothioate ON by a factor of 50 compared to ON alone [118]. Yet, for unclear reasons, the *fractured* PAMAM_{G6} (Superfect™) and other polymers such as PEI and PLL were reported to not be able to enhance ON transport in D407 and CV-1 cells [119].

4. In vivo gene expression and experimental therapy

The ability of non-viral systems such as Superfect™ to efficiently transfect various cells in vitro has made synthetic vectors a routine tool in molecular biology. Yet, they have had little impact on the translation of genetic therapies into the clinic to date. It remains a significant challenge to make valid predictions on the in vivo behaviour of synthetic vectors. When one considers the vastly increase complexity of the system that is being introduced by the range of possible interactions between array of biological macromolecules and cells this is not really surprising. The challenge can be significantly reduced by circumventing the vascular compartment. Consequently, many applications of dendrimers in vivo have focused on their use for local or ex vivo administration. Despite of these challenges there is some evidence that dendrimer-based delivery systems have a significant potential for the delivery of genetic therapies in vivo.

4.1. Localised/ex vivo administration

4.1.1. Eye

Direct application of activated PAMAM (Superfect™) complex ex vivo on human and rabbit corneas resulted in 6–10% of cells being transfected (18:1 ratio) [120] and intravitreal injection of complexes with AS-ON and a lipid lysine dendrimer inhibits neovascularisation of the choroidea by down regulation of VEGF over a period of up to 2 months [121].

4.1.2. Tumour

Intratumoural injection of 100 µg HSV-tk suicide vector complexed with Superfect™ PAMAM den-

dimer at a 3:1 ratio (w/w) led to a pronounced growth delay [121]. The plasmid contained EBV sequences with the ability to replicate and persist in the nucleus of the transfected cells (carrying the Epstein–Barr virus nuclear antigen, EBNA1, and oriP). The animals received up to four weekly cycles (single injection of complex followed by 100 mg/kg/day of the prodrug Ganciclovir for 6 days) [122]. Measuring levels of β -gal expression of the plasmid employing the EBNA1/oriP system were eight times higher than in a conventional plasmid and in conjunction with a vector expressing *Fas* ligand the injection of 10 μ g plasmid complexed with dendrimer (Superfect™) at a ratio of 10:1 (w/w) also led to a pronounced tumour growth delay [123,124].

A growth delay was also demonstrated after intratumoural injection of plasmids coding for the anti-angiogenic peptide angiostatin or the tissue inhibitor of metalloproteinase (TIMP)-2 genes in special dendrimer/plasmid/oligonucleotide complexes [125]. The formulation was based on a mixture of 5 μ g of plasmid, 60 μ g of activated PAMAM dendrimer (Superfect™), and 20 μ g of a 36-mer oligonucleotide complexing the plasmid coding for the therapeutic gene.

Efficient local delivery of an ^{111}In labelled ON to tumour cells in an intraperitoneal tumour model has been demonstrated when complexes with PAMAM_{G4} were injected i.p. [126].

4.1.3. Heart

Using direct injection in a murine cardiac transplant model PAMAM_{G5} dendrimer complexes demonstrated more widespread and prolonged expression compared to the naked plasmid and when combined with a viral interleukin 10 gene were able to prolong graft survival [127]. The efficiency of the procedure was improved at a higher charge ratio of 20:1 [128], and in combination with electroporation [129].

On direct local administration to the adventitia of the rabbit aorta Superfect™ was also found to be more efficient (4.4%) than branched PEI 25 kDa (2.8%), branched PEI 800 kDa (1.8%), or naked DNA (0.5%) [130].

4.1.4. Lung

Gene expression after intratracheal instillation of complexes with fractured PAMAM dendrimer (Super-

fect™) at N/P ratio of 4.7 (32.1 μ g dendrimer/20 μ g plasmid, 50 μ L) was found to be 130-fold lower than for the branched PEI 25 kDa formulation (N/P 10:1) [131].

4.2. Systemic administration

Intravascular administration of complexes with PAMAM_{G9} (200 μ g DNA complexed with 650 μ g dendrimer) led to expression mainly in the lung parenchyma but not in other organs [132].

The systemic administration of dendrimers was also investigated for a PAMAM_{G3} and conjugates of α -cyclodextrin (aCD) with the terminal amines of PAMAM_{G3} [132]. After 12 h the spleen was clearly the dominant organ and at least one order of magnitude higher than the next highest organ, the liver [132]. The modified dendrimer led to a shift in the expression pattern depending on the level of substitution and in some organs significantly improved the expression [9,17,18].

The aim of our own work has been the development of delivery systems for the systemic treatment of diseases and specifically of solid tumours. Cancer therapy is currently limited by the difficulty to efficiently deliver therapeutic molecules or genes to remote tumours and metastasis by systemic administration [70,133,134].

We have previously developed a number of systems suitable for in vivo delivery of genes [62]. We have reported that the lower generations of PPI dendrimers are promising delivery systems which strike a good balance between binding/stability and toxicity [71]. We have demonstrated that these systems are also potentially useful for systemic gene therapy: PPI dendrimers of G1–4 (DAB-Am 4/8/16/32) were characterised and compared with their quaternised counterparts [71]. The quaternisation improved DNA binding of lower generations and cytotoxicity of the higher generations tested. In particular for PPI_{G2} quaternisation proved advantageous as it rendered the previously toxic complex safe. In vivo the formulations based on PPI_{G3} (DAB-Am16) and the quaternised PPI_{G2} (QDAB-Am8) efficiently expressed transgenes predominantly in the liver rather than the lung [135].

We have recently been able to demonstrate that an intravenously administered gene medicine consisting

of the PPI_{G3} complexes is able to induce intratumoural transgene expression [135]. When murine xenografts are treated by intravenous injection of PPI_{G3} complexes with a tumour necrosis factor (TNF α) expression plasmid under control of a tumour specific promoter, regression of established tumours has been observed in 100% of the animals. The treatment (5 injections over 10 days) also led to an excellent long-term response (at 17 weeks: 80% complete and 20% partial response). The anti-tumour activity is the result of synergies of the effects of the tumour specific expression of TNF α and an intrinsic anti-proliferative effect of the dendrimer. This novel anti-proliferative effect was also observed with other cationic polymers. The lack of apparent toxicity and significant weight loss compared to untreated controls suggest the treatment to be relatively well tolerated and safe [107].

4.3. Complex modulation through additives and/or conjugation

In one of the first series of experiments that explored PAMAM dendrimers as a gene delivery system the modulation of complexes through the presence of other compounds during the complex formation was described [107]. The addition of the cationic transfection polymer DEAE–dextran to the dendrimer–DNA complexes appears to have an additive or possibly synergistic effect on the transfection efficiency observed with PAMAM_{G9} [136,137]. At concentrations of 0.25–1 μ M the positive effect on transfection was however balanced by a doubling of cell death to 5–8% of cells.

The effect of the DEAE–dextran was ascribed to changes in complex morphology, that is, the admixture counteracted the tendency of the higher generation PAMAM dendrimers to form supramolecular aggregates. Positive effects on the ability of complexes to transfect have also been reported for the combination of low MW PEI with high MW PEI and various lipidic systems but do not seem to be applicable to the fractured PAMAM dendrimer Superfect™ [138].

The enhancement of viral transduction in the presence of cationic lipids as well as the increase of transfection from synthetic systems in the presence of replication defective adenovirus has previously been

established ('adenofection'/'lipoduction') [139] and has then been extended to PEI based systems [140], and finally dendrimers [140]. In the case of addition of synthetic systems to adenovirus the effect is explained by an increased uptake of dendrimer/adenovirus independent of CAR receptor status, whereas in the case of adenovirus addition to a non-viral transfection systems seems to increase the level and duration of expression [141].

The combination of dendriplex with major polyomavirus capsid protein has been tested for the delivery of ON and plasmid DNA [141]. Dendrimer–DNA complexes applied in conjunction with pentamers of the VP1 capsid were reported to have improved transfection compared to the complex alone [142].

Substituted cyclodextrin (CD) can enhance the transfection efficiency of dendrimer-based complexes [142]. The amphoteric and sulfonated β -CDs do not complex DNA by themselves but can modulate the size and distribution of PAMAM_{G5} dendrimer complexes, particularly at relatively low N/P ratios. In this fashion they appear to significantly enhance efficiency, specifically of surface mediated transfection [99,100,132].

4.3.1. Conjugates

Covalent conjugates of CyD–dendrimer have also been used to increase the efficiency of PAMAM systems [99]. Only conjugates of α , β , γ -CD but not mixtures of dendrimer and CyDs were able to significantly increase the transfection observed with the lower generations of PAMAM dendrimers (G2–G4) which are otherwise not able to transfect cells efficiently. The efficiency of CyD–dendrimer mediated transfection increased with N/P charge ratio and reached a plateau at comparatively high values of 200:1 ('charge ratio') with a maximum for the α -CD conjugate [100]. With respect to the different dendrimer generations the G3 conjugate seemed to be superior to both the G2 and G4 conjugates [100]. Fluorescence microscopy showed that a higher proportion of the CD conjugate complexed DNA was delivered to the cytosol thus suggesting a potentially increased endosomal release from the conjugates [132]. A medium degree of substitution (2.4 CyDs/dendrimer) was advantageous in terms of transfection in vitro compared to a low (1.1) and high (5.4) degree of substitution and was also able to

induce luciferase expression in various organs after tail vein administration. The dominant organ in the distribution of gene expression for this conjugate was the liver [101].

When investigating the intracellular distribution of PAMAM_{G5} dendrimer–AS complexes using fluorescently labelled conjugates Yoo et al. discovered that the conjugation of the small hydrophobic dye *Oregon green 488* to the dendrimer significantly increased shuttling efficiency to a level comparable with commercial agents [116]. It remains unclear whether the effect of the dye is exerted through changes in the dendrimer–AS complex physicochemistry or through a biological effect of the conjugate e.g. an increased interaction and disruption of endosomal membranes.

Substitution of PAMAM_{G4} dendrimer by conjugation of the surface amines with L-arginine was reported recently [143]. This approach was able to enhance transfection efficiency in several cell lines compared to unmodified dendrimer or dendrimer modified with terminal lysine [144].

A positive correlation of the level of substitution of terminal amino functions of PAMAM_{G4} with a hydrophobic amino acid residue (phenylalanine) and the ability of the respective complexes to transfect mammalian cells (CV1) was also recently reported [144]. A fully substituted PAMAM dendrimer had relatively low water solubility but was highly efficient in transfection experiments [145].

The addition of the biologically inactive cucurbituril, a large cage compound composed of glycoluril units interconnected by methylene bridges, has also been shown to modulate complex formation between PPI_{G4}/PPI_{G5}–DAB dendrimer and DNA [145]. Depending on the sequence of mixing and concentrations, various ternary complexes with sizes between 150 and 210 nm are formed between all three constituents that were able to transfect with similar efficiency but reduced toxicity. The authors proposed the non-covalent addition of dendrimer–ligand conjugates as a strategy for the non-covalent addition of targeting ligands [123].

The presence of anionic oligomers such as dextran sulphate or oligonucleotides together with plasmid DNA during the complex formation has been demonstrated to significantly influence the morphology of complexes formed [124], although the extent of the enhancement seems to strongly depend on the specific

conditions such as buffer and cell line [37]. Complexes formed from phosphorous containing dendrimers [123] and activated PAMAM dendrimer (Superfect™) were significantly less dense and appeared to be less prone to aggregation when the anionic oligomers were used [123]. Expression levels from complexes formed at N/P of 1.8 were the same when from 0.5 µg of plasmid and 3.0 µg oligonucleotide as from 3.5 µg of plasmid. Oligonucleotides of 36–55 length were found to be optimal and, similar to a 10-mer dextran sulphate, had improved efficiency, which was apparently linked to an increased uptake of the less dense complexes into cells [146].

Another strategy proposed to increase transfection efficiency from dendrimers was based on the hypothesis that a conjugate of PEG (3.4 kDa) to a lower generation PAMAM dendrimer would mimic properties of the fractured PAMAM (Superfect™). The authors report that a PAMAM_{G5} conjugated in this fashion possesses low cytotoxicity and leads to a 20-fold increase in transfection efficiency compared to the activated PAMAM [70,147].

4.3.2. Quaternisation

We have previously used quaternisation of water soluble linear and branched polymers as a strategy to modulate their physicochemistry and cytotoxicity [71] and have recently also explored this strategy in conjunction with PPI dendrimers [71]. The lower generation PPI dendrimers (G1–G4) were modified to the respective methyl quaternary ammonium derivatives. In particular for the quaternisation of the PPI_{G2} this led to an improved DNA binding and complex stability. This was accompanied by a dramatic improvement of in vivo safety, that is, in contrast to complexes formed with the unmodified dendrimer the formulations were now well tolerated on intravenous injection. Furthermore, the modified polymer was able to facilitate transfection in the liver after systemic administration. Quaternisation also increased biocompatibility of G3 and G4 complexes by about 4-fold but did not affect in vitro toxicity for the lower generations G1, G2 [68].

PAMAM–OH dendrimers do not interact sufficiently with DNA to form complexes as their external amines have been replaced with hydroxyl functions and the internal tertiary amines have a diminished tendency to bind DNA because of their relatively low

pKa [68]. Quaternisation of the internal amines with methyl iodide was used to produce PAMAM–OH dendrimers with different levels of quaternary internal amines (0.27–0.97) and their interaction with DNA studied [17]. These experiments demonstrate that both N/P charge ratio and charge density of the dendrimer influence complex formation. Only dendrimers with a degree of quaternisation greater than 0.78 were able to form compact complexes and efficiently exclude ethidium bromide. These complexes, in contrast to those formed with PAMAM_{G4}, had a neutral ζ -potential even at N/P ratios of 10, suggesting that the charge interaction with DNA occurred through the internal quaternised amines while the hydroxyl groups at the dendrimer surface appear to have lead to a shield of the positive complex interior at the surface. The quaternisation of amines was found to be an efficient strategy to modulate polymer toxicity. The transfection capability of these dendrimers was an order of magnitude lower than that from the cationic PAMAM_{G4}, which itself is not the most potent PAMAM dendrimer. This may be predominantly due to the lack of ζ -potential which would eliminate non-specific electrostatic binding to cell surfaces and, consequently, is likely to have reduced uptake dramatically. The lack of charge could conceivably be advantageous for ligand based targeting strategies [68]. It is the comparison of expression per internalised plasmid which in this case would give a more accurate measure of the ability of a complex to transfect. The study also confirms the notion that cytotoxicity of dendrimers is a function of the nature of the amine (1°, 2°, 3°, 4°) [148].

5. Conclusion

The holy grail for the rational design of synthetic gene delivery systems would clearly be to link chemical structure of cationic complexation agents to the morphology and physicochemistry of the respective nucleic acid complexes and then to further link this to the biological properties on a cellular and systemic level. However, our understanding of each of these steps is still very much incomplete and a large proportion of research remains based on empiricism.

Conceptually the way synthetic delivery systems are generally being developed suggests that what is

required is a relatively non-toxic cationic material which can form a complex with DNA of a size on the order of a few hundred nanometres which show retardation in agarose gels, prevent binding of ethidium bromide, and protect the DNA from degrading enzymes. The next step in general involves testing of the ability of complexes formed at various N/P ratios to increase reporter gene expression in a small number of cell lines in the presence or absence of serum components. If the system shows some activity compared to currently used standard reagents one would then consider testing of the system *in vivo*, using reporter gene expression in various organs as the readout.

This development strategy is being widely followed but is probably based as much on the availability of relatively straight forward assays as on empirical success. Even an extension of the analytical armamentarium does not readily create a link between physicochemistry, i.e. the interaction between DNA and dendrimer, and the ability of a dendrimer to facilitate transfection [112]. The research focus on improvement of transfection levels means that there is probably a considerable bias against publications which highlight this critical problem.

If one considers recent data in which a number of polymers and dendrimers were compared in various cell lines the picture that emerges demonstrates how fragile the current approach can be: the differences between the seven tested polymers were as large as the differences seen for one of the polymers in different cell lines, i.e. three orders of magnitude, and the ranking of agents varied accordingly between different cell lines but was also affected by other factors such as e.g. cell density [22].

This variability means that it can be problematic to compare results between *in vitro* studies and the potential implication of such variability for therapeutic delivery has not yet been explored; nevertheless, dendrimer-based agents and specifically the fractured PAMAM dendrimers (Superfect™) are clearly among the best and most widely applicable transfection agents and have become a standard tool for many cell and molecular biologists.

The transition from *in vitro* transfection experiments to *in vivo* studies is the logical next step in the development of gene delivery systems, as ultimately the aim is to use these systems to treat human disease.

Yet one needs to remain sceptical about the extent to which *in vitro* systems are currently able to predict the ability of a formulation to bring about targeted high level transgene expression. When one considers the added levels of complexity *in vivo*, it is not surprising that many promising developments fail to make an impact *in vivo*, most commonly because of a lack of efficacy or concerns about safety and/or toxicity. Nonetheless, dendrimer-based delivery systems have shown considerable promise as tools for the further development of genetic therapies. While most of the applications so far have focused on the use of dendrimer-based vectors for local or *ex vivo* administration, our own recent work has demonstrated that specifically PPI dendrimers may have some properties which appear to make the particularly suited to systemic *in vivo* administration. Undoubtedly the obstacle of safe and efficient delivery of genetic medicine largely remains significant and the suitability of any gene delivery systems will always have to be matched with the clinical situation, the specific disease and the chosen therapeutic strategy.

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