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Review

Dedicated to Professor Bogdan C. Simionescu on the occasion of his 70th anniversary

DENDRITIC ARCHITECTURES AS NON-VIRAL GENE DELIVERY VECTORS: CHALLENGES AND PERSPECTIVES

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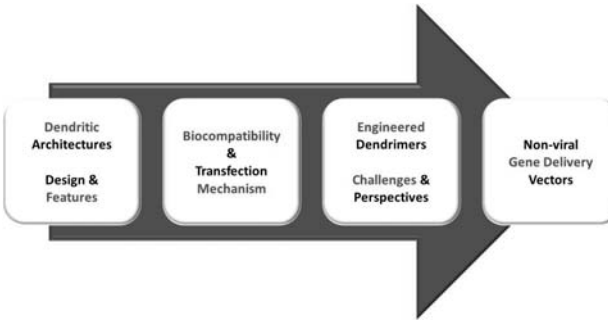
Dendritic structures represent one of the most captivating macromolecular systems which have evolved in the past three decades and created the means for the birth of a new family of materials with applications ranging from microelectronics to nanomedicine.
These remarkable engineered architectures are able to unlock a plethora of relevant traits for the development of synthetic gene delivery vectors: well-defined, monodisperse systems, highly ordered and densely packed structures, tailorable sizes, abundant surface groups, plenty of possibilities for processing and surface functionalization, and increasing commercial availability.
This review focuses on the current challenges and perspectives which arise in the use of dendritic architectures as non-viral vectors in gene therapy, dealing with some of the most used species in the field and key aspects for the transfection output like molecular design, physical and chemical features, dendrimeric core and generation, surface functionalities and modifications, and others.



INTRODUCTION

Gene therapy came forth in the last two decades as a pioneering tool to address several inherited and acquired diseases, [1-4] or as a viable substitute for conventional chemo- or radiotherapy.[5-7] Its real potential is underlined by the great number of research articles (almost 200 per year, according to Web of Science) dealing with gene delivery vectors and clinical trials (almost 2000 until now,[8] more than half dealing with cancer) being carried out worldwide. The holy grail of a safe and efficient gene vector implies a handful of challenging features: high transfection performance, suitable stability, proper cell or tissue specificity, good biodegradability and biocompatibility, and ease of synthesis and manufacture.[9-11]

Initial efforts in the field were directed towards viral vectors (e.g. adeno-, lenti- and retro-viruses)

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which are able to provide very high transfection outputs both *in vitro* and *in vivo*, but also display disturbing safety issues and production-related drawbacks.[12-14] As a consequence, most of research switched towards non-viral DNA delivery systems (e.g. cationic lipids, polymers, dendrimers) which ensure an improved safety, higher synthetic versatility and tunability and provide the conditions for large scale production.[15] However, their current performances are inferior to the viral counterparts in terms of efficiency and the quest for successful non-viral options is far from finished.[12,15]

Dendrimers and macromolecular structures based on them distinguish among these vectors due to a set of exclusive structural features and the ability to complex DNA in compact nanostructures which can be further functionalized in order to obtain the desired specificity.[8,13] These remarkable engineered architectures unlock a collection of relevant traits for the development of synthetic gene delivery vectors: well-defined, monodisperse systems, highly ordered and densely packed structures, tailorable sizes, abundant surface groups, plenty of possibilities for processing and surface functionalization, and increasing commercial availability.[1,5,12,16] Nevertheless, dendrimers are less than ideal non-viral vectors, suffering from the same imperfect efficiency as the other non-viral gene delivery tools.[17]

There are many substantial reviews describing the state-of-the-art in the field of dendrimers for biomedical applications,[1,5,12,16,18] and therefore the present one focuses on the current challenges and perspectives which arise in the use of dendritic architectures as non-viral vectors in gene therapy. The review is also dealing with some of the most used species in the field and key aspects for the transfection output like molecular design, physical and chemical features, dendrimeric core and generation, surface functionalities and modifications, and others.

SHORT NOTES ON DENDRIMER SYNTHESIS

Although both are macromolecular architectures, dendrimers severely differentiate from traditional polymers (another type of well-performing gene delivery vehicles) in terms of synthesis and physicochemical features.[19] Dendrimers are built in a well-controlled stepwise manner which leads to three-dimensional hyperbranched systems of high symmetry and precise molar mass. These attributes ensure a quite precise management of the entire preparation process and provides a certain reproducibility of the pharmacokinetic conduct of the obtained compound.[20] The latter is a major advantage as compared to classical polymers, which often circumvent clinical trials because their polydisperse character leads to irreproducible pharmacokinetic conduct. Moreover, the high degree of control over the final properties of the dendrimeric architecture is simply out of the reach of conventional polymer synthesis.

There are two major synthetic pathways to generate dendrimeric structures – the so-called divergent and convergent procedures. Both of them imply an iterative sequence of synthetic steps (each step presuming the growth of an additional layer of 'branches' or generation) and exhibit particular benefits and drawbacks. Dendrimers were first prepared in the early 1980's by Denkewalter, Tomalia and Newkome through a divergent procedure.[12] This method is building dendrimers beginning from a branched core towards the periphery by adding one dendron (generation) after the other, as it can be observed in Figure 1.[19]

By comparison, the convergent procedure, introduced in the early 1990's by Hawker and Fréchet,[21] implies the step-by-step growth of dendrimers from the outer shell towards the branched core, as depicted in Figure 2.[19] The seminal work of these groups was soon upscaled by Meijer and his group,[22] who developed the kilogram-scale preparation of polymers and made their entry on the market.

The divergent method excels in providing high generation structures and in the possibility to modify the functional groups from the periphery, with major implications in final properties and applications. Its major drawbacks come from the incomplete or side reactions of the exponentially growing end groups, which make it difficult to obtain flawless high generation dendrimers and turn purification into a nightmare.[23-25] On the other side, the convergent strategy implies fewer and more distinct reactive sites, which translate into lower reaction times and easier purification. However, this method is able to provide only lower generation structures, due to the steric difficulties of connecting the large-sized dendron to the smaller dendrimer core.[26,27] Despite these issues, both methods deliver large, commercial quantities of well-defined, monodisperse (almost monodisperse: at large scale, Mw/Mn gets close to 1.0005) products by employing rather common reagents like acrylates and diaminoethane.[28,29] The two synthetic pathways have provided more than 100 distinct dendritic families and tenfold modified architectures, which create unlimited traits and potential applications.[26]

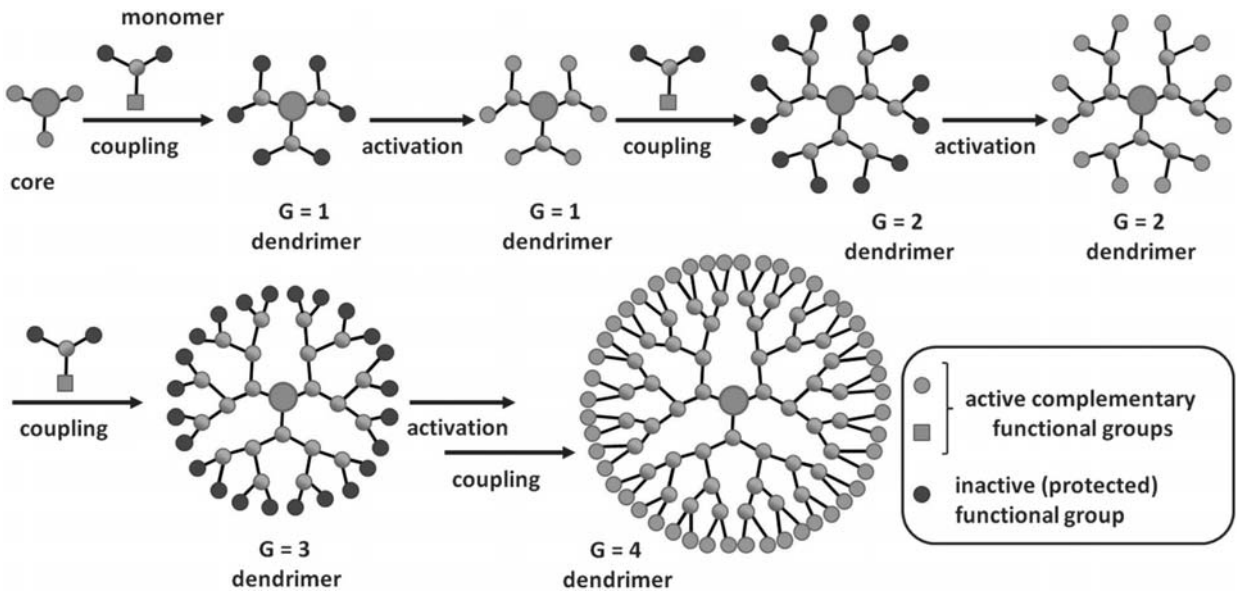

Fig. 1 – Dendrimer synthesis by the divergent method. Reproduced from reference 19 with permission from The Royal Society of Chemistry (RSC) on behalf of the European Society for Photobiology, the European Photochemistry Association and the RSC.

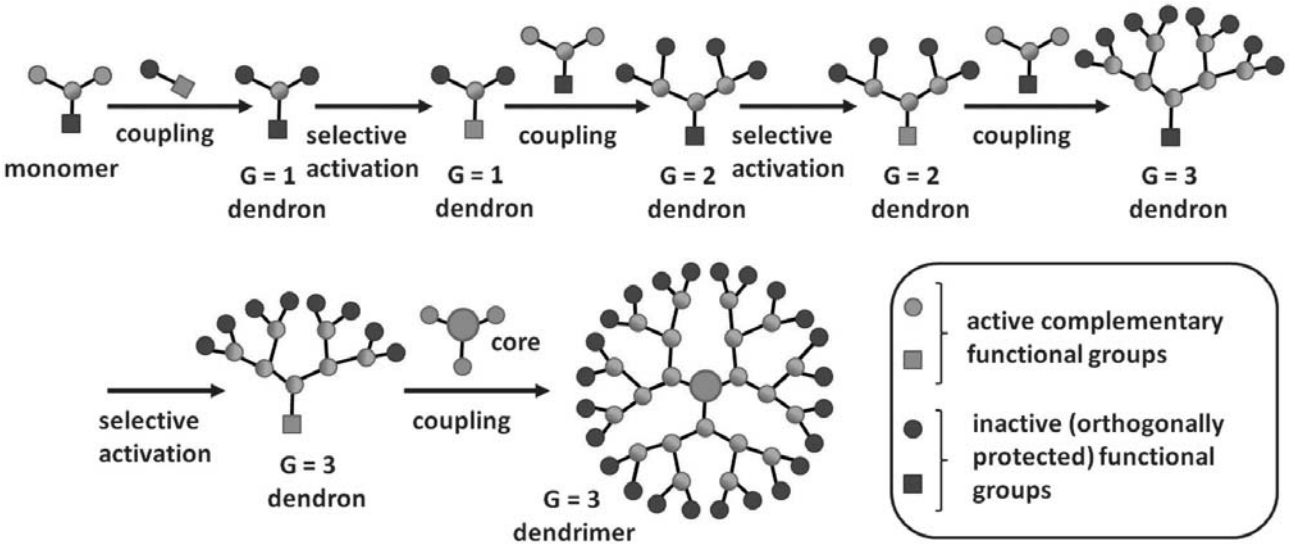

Fig. 2 – Dendrimer synthesis by the convergent method. Reproduced from reference 19 with permission from The Royal Society of Chemistry (RSC) on behalf of the European Society for Photobiology, the European Photochemistry Association and the RSC.

STRUCTURE AND PROPERTIES OF DENDRIMERS

The hyperbranched, fractal framework of dendrimers and alike is a very common one in the biological world, covering the entire scale length, from meters (trees) and centimeters (lungs) to micrometers (neurons) and nanometers (glycogen).[26] Although (still) not as complex (in structure and function) as the above-mentioned examples, these types of synthetic hyperbranched structures are able to provide a collection of exclusive characteristics, especially in terms of interfacial conduct and functional behavior.

The clear-cut architecture of dendrimers is built on three different elements, as represented in Figure 3:[30] (1) a branched central core with several reactive sites which represent the starting points of the symmetrical growth of the dendrimer; (2) dendrimer generations, namely identical, individual layers of branches; (3) closely packed surface groups which multiply in an exponential manner with each generation. The dendrimeric core is usually a small-sized molecule (as compared to the rest of the structural elements) and is responsible for the shape and multiplicity of branching. The generations determine the size and the number of the functionalities (end groups) of the dendritic

construct. The outer shell formed by the closely packed, surface end groups (neutral or ionic) is a platform for the attachment of various motifs and for a broad spectrum of distinct biological and physicochemical features.

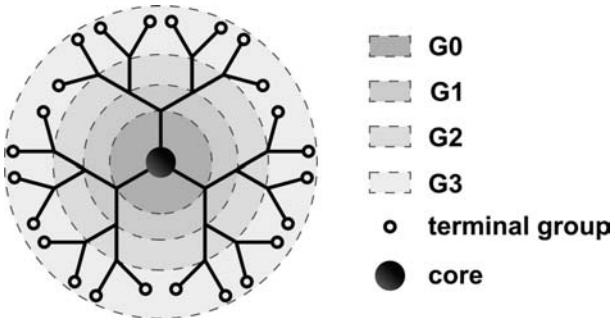

Fig. 3 – Structural components of a dendrimer. Reproduced from reference 38 under Creative Commons Attribution 3.0 Unported License.

Last but not least, this type of framework implies the existence of several microenvironments or internal cavities (their number and degree of congestion are determined by the core and their size by the steric nature of the branching and the generation number) which can act as a host or pocket and accommodate different types of guest molecules by non-covalent encapsulation.[31]

This type of a complex architecture requires a well-defined set of parameters to insure proper understanding, controlled synthesis and modifications, and tailorable characteristics. Therefore, a collection of critical nanoscale design parameters was developed:[32-34] elemental composition, size, shape, surface chemistry, flexibility, and overall architecture. By the adequate management of any of these critical design parameters, well-structured, nanosized dendritic constructs with distinct features and commercial relevance can be obtained. This turns out to be a crucial argument for the exploration of dendritic structures as non-viral gene delivery vectors, as it will be described later.

Dendrimers exhibit a predictable and controllable three-dimensional hyperbranched framework of a monodisperse nature. Outer functionalities and internal microenvironments provide a multitude of substitution patterns and geometries and can be employed or altered by turn or at once to generate multifunctional products for specific applications.[35,36] In addition, the functionalization can be performed in a site-selective manner, since dendrimers are prepared in a stepwise, iterative style.

The multivalent peripheral domain of the dendrimer drives most of its physical properties, from solubility to hydrophobicity. On the other side, the core is almost completely isolated at high generations by the densely packed surface motifs and provides specific site-isolation properties of certain interest for the medical field. Moreover, the core itself can act as a functional moiety or is able to provide different patterns and geometries.

Although puzzling in nature, the dendritic framework is quite small in size, ranging for usual dendrimers from a couple to tens of nanometers in diameter, which can arise as an important advantage in terms of biodegradability.[37]

Dendritic structures are usually represented or described as being spherically in shape, which is just half of the picture. In a similar fashion to proteins, this type of products is able to embrace extreme conformations, from tight 'native' to loose 'denatured', in deep correlation with the surrounding pH, ionic strength or polarity (as confirmed by molecular dynamic studies).[16]

The successful combination of peculiar features and versatile adjustment possibilities results in (almost) endless structural possibilities and multiple applicative perspectives, especially in the biomedical area: drugs, drug encapsulation and delivery, antimicrobial agents, artificial receptors, tissue engineering, medical diagnostics, biosensors, some of them successfully making it into the market.[38,39]

BIOCOMPATIBILITY OF DENDRIMERS

Gene therapy is basically about effectively delivering a certain gene to a specific site with the aid of a stable, biodegradable, and/or biocompatible vehicle. Besides the crucial DNA-binding skills, this process imposes a handful of quite demanding, bio-related challenges:[12,16] non-toxicity, close-to-zero immunogenicity, suitable lifetime in blood (as to generate a proper clinical effect), capacity to cross a couple of physiological barriers (e.g. cell membranes), and specificity (the ability to target specific sites). The complexity of this puzzle is responsible for the lack of an ideal, both efficient and non-toxic, dendrimer-based non-viral gene delivery vector.

A critical and comprehensive monitoring of some of the most solid reviews in the field comes to the conclusion that all these conditions are directly or indirectly related to the above-mentioned critical nanoscale design parameters of dendrimers.

First of all, a small discussion regarding dendron – nucleic acid interactions is needed. Dendrimers are able to complex nucleic acids and gene plasmids in an efficient manner, electrostatic interactions representing the predominant interplay between the two elements.[32,40,41] The best players in the field are the cationic dendritic frameworks, which display a well-controlled quantity of amine groups on their outer (and many times inner) layers. Additionally, they are able to provide nucleic acids the proper protection from enzymatic degradation.[42,43] On the other side, polycationic structures in general, dendrimers included, display a hemolytic effect and trigger a destabilization of the cellular membrane and generate cell lysis,[44,45] following a mechanism which is not completely understood. Therefore, the number of cationic functionalities or of the corresponding amine groups (directly related to the generation number or the molecular weight) is an additional key parameter influencing the viability of a dendritic structure as a non-viral vector. As a consequence, the toxicity of these hyperbranched structures is generation-dependent, with low or middle generation dendrimers (usually up to generation six) being suitable for gene delivery. This issue proves to be a practical advantage, since low dendrimers require less preparation time, reagents and purification, can be prepared by both synthetic methods, and display a higher structural perfection as compared to their superior homologues. Moreover, once the free cationic dendrimer is complexed with the corresponding nucleic acid, the cytotoxicity and membrane damage are decreasing.[16] However, some of the non-toxic, low generation dendrimers are not able to provide very stable complexes with nucleic acids and lose some of their efficacy.[12]

The replacement of the cationic, amino surface functionalities with anionic, carboxylic ones would be an interesting alternative to the troublesome toxicity of cationic dendrimers. Recent studies have shown that carboxylate-decorated polyamidoamine (PAMAM) dendritic frameworks exhibit low to none (descending in dendrimer generation) cytotoxicity and hematotoxicity as compared to their cationic counterparts.[46,47]

Nevertheless, there is more to the biocompatibility of dendrimers than their outer layer. Same studies proved that the introduction of several aromatic groups in the internal layers of a polyether-like, dendritic framework generates serious hemolysis, with the high number of aromatic motifs bearing the blame for this issue.

There are only a couple of serious studies assessing the *in vivo* toxicity of dendrimers,[48,49] most of them dealing with the favored PAMAM skeleton. The overall conclusion is that PAMAM dendrimers up to the fifth generation are not toxic to mice (at a concentration of 10 mg/kg), regardless of the outer shell amine functionalities.

The same PAMAM dendrimers show low immunogenicity up to the seventh generation.[48,50] This can be further decreased by employing hydrophilic motifs like polyethylene glycol oligomers in the dendritic construct, a strategy which also proves efficient in increasing the lifetime in blood.[51]

Other studies showed that PAMAM dendrimers are eliminated by a generation- and charge-dependent mechanism.[50,52] Their size is small enough up to the fifth generation to be cleared *via* the glomerular filtration from the renal excretion. When the dimensions of the dendrimers are near in size to the filtration threshold, the charge comes into play, and anionic dendrimers for example prove quite hard to be eliminated (the filtration membrane is also anionic).

THE TRANSFECTION MECHANISM OF DENDRIMERS

The gene transfection pattern of dendrimers (and macromolecular delivery agents in general) is like a puzzle with a couple of missing pieces – a general proposed picture is largely accepted, even if not all of its components are fully known (Figure 4).[23] Dendrimers successfully condense nucleic acids into positively charged nanoparticles called dendriplexes.[53] The dendriplexes bind to the negatively charged cellular membrane through electrostatic interactions and are internalized in the cellular cytoplasm through endosomal uptake.[54] The surface and internal layers of most of the dendrimeric families studied as gene vectors own a high density of tertiary amine units which can be easily protonated in endolysosomal-specific pH conditions (pH 5.0 – 7.4). This generates a pH buffering ability and promotes an efficient endosomal escape of the dendriplexes *via* a so-called 'proton-sponge' mechanism.[55,56] The subsequent dissociation of the nucleic acid from this complex (most likely due to the intervention of cationic lipids) and its entry into the cellular nucleus represent cardinal pieces of the transfection mechanism which are not yet fully comprehended.[15]

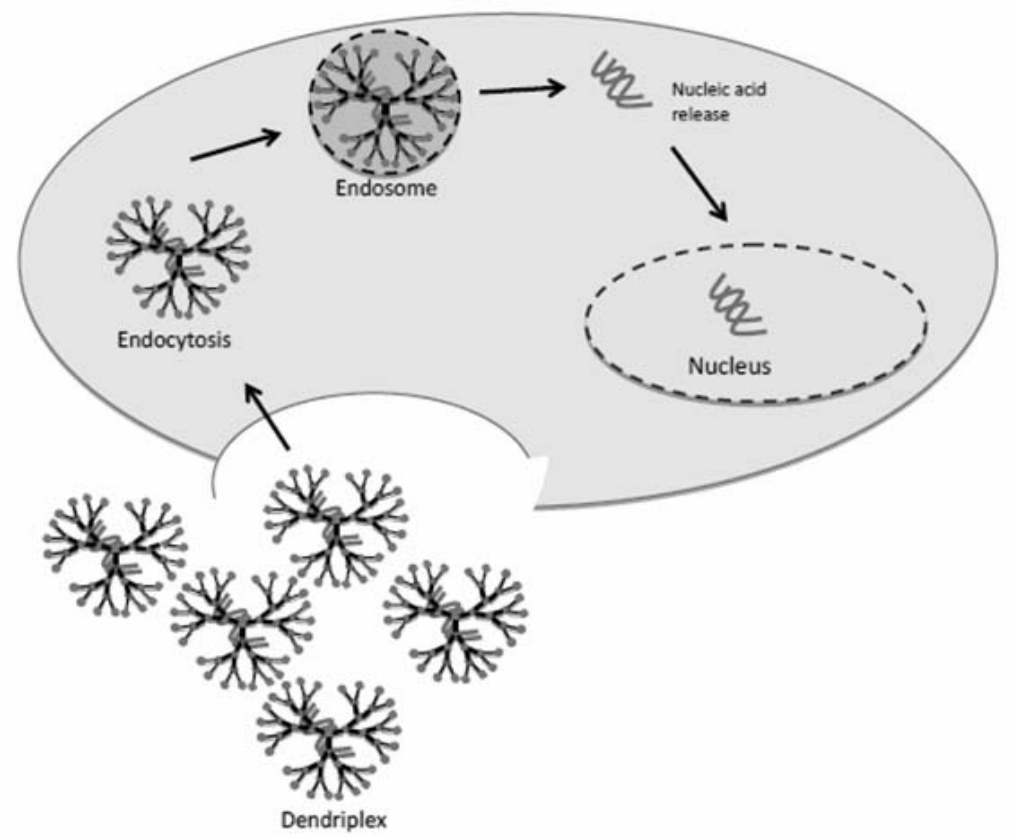

Fig. 4 – Schematic representation of the gene delivery mechanism of dendrimers. Reproduced from reference 23 under Creative Commons Attribution License and Scholars Open Access publishing policies.

DENDRIMERS IN GENE DELIVERY

Design and features

As mentioned earlier, one of the objectives of this review is to underline essential aspects belonging to the best players with dendritic constructs in the field of gene delivery and to focus on the most important challenges and prospects which they are facing.

Positively charged, water-soluble, polyethylenimine (PEI) linear and dendritic frameworks were considered for quite some time the gold standard in plasmid DNA delivery, both *in vitro* and *in vivo*.[57-59] This opinion changed however in the last years due to many troublesome issues regarding high cytotoxicity, deficient transfection, and poor stability of the complexes (polyplexes or dendriplexes) formed between DNA and PEI derivatives.[60] Various modified PEI architectures with improved transfection and diminished cytotoxicity are still being developed,[61-63] but they are still far away from any relevant clinical applications.

The search for new, more effective, more stable and less toxic gene delivery vehicles revealed two viable dendritic alternatives (Figure 5): polyamidoamine (PAMAM) and polypropyleneimines (PPI),[8,12] along other promising dendrimeric frameworks, like poly-*L*-lysine,[20,64] triazine,[65] carbosilane,[66] and viologen dendrimers.[67] The following pages are dealing with the dominant species in the field, PAMAM and PPI dendrimers, and condense some key issues regarding the transfection output like molecular design, physical and chemical features, dendrimeric core and generation, surface engineering, and others.

PAMAM dendrimers

The PAMAM framework is the most common and important dendrimer species investigated and used a gene carrier.[8,9] Firstly synthesized in the early 1980's, this type of dendrimers entered the market in 1990 and were advanced as potential gene vectors three years later.[12,26] They are nanospherical, flexible, monodisperse, cascade macromolecules with a high solubility in aqueous media and an undemanding preparation procedure. PAMAMs are obtained through a two-stage iterative, divergent method (Figure 6) which consists in the Michael addition of a small nucleophilic core (usually ammonia or ethyl-

enediamine) to methyl acrylate and the subsequent amidation reaction of the prepared ester with the same ethylenediamine or similar diamines. The result is a well-defined core-shell structure with a high density of amine units (which increase exponentially with every layer of branches) and diameters between 22 Å for the first generation (8 amine moieties, 1430 Da molecular weight) and 135 Å for the tenth one (4096 amine moieties, 934720 Da molecular weight for the perfectly structured dendrimer).[23]

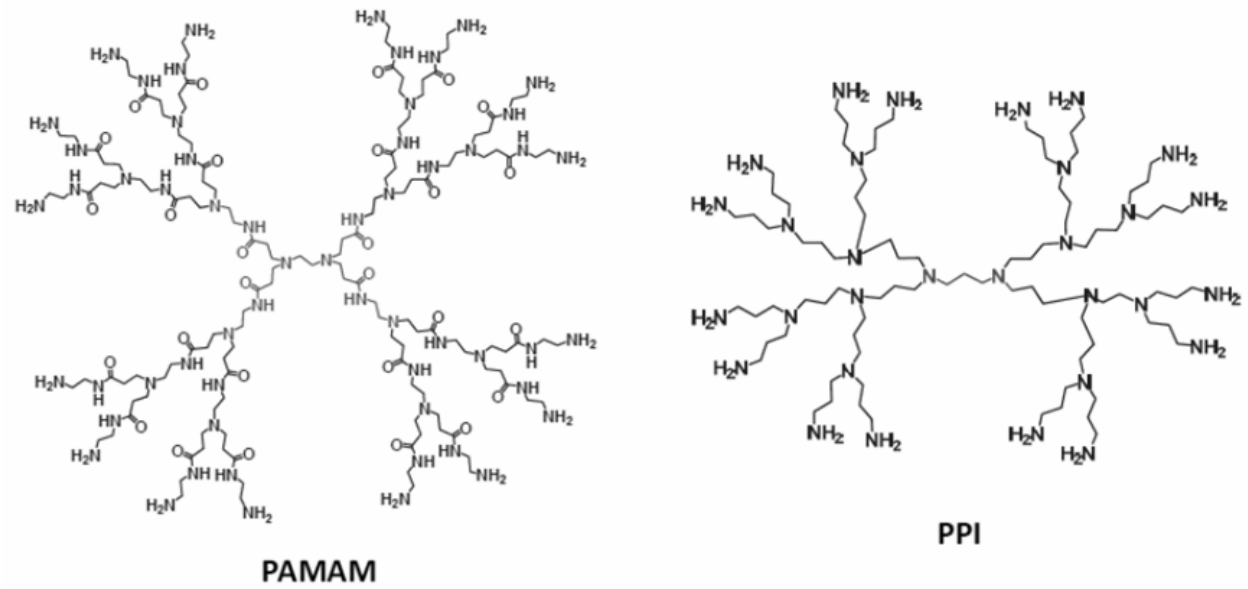

Fig. 5 – Structure of PAMAM and PPI dendrimers. Adapted from reference 20 under Creative Commons Attribution License.

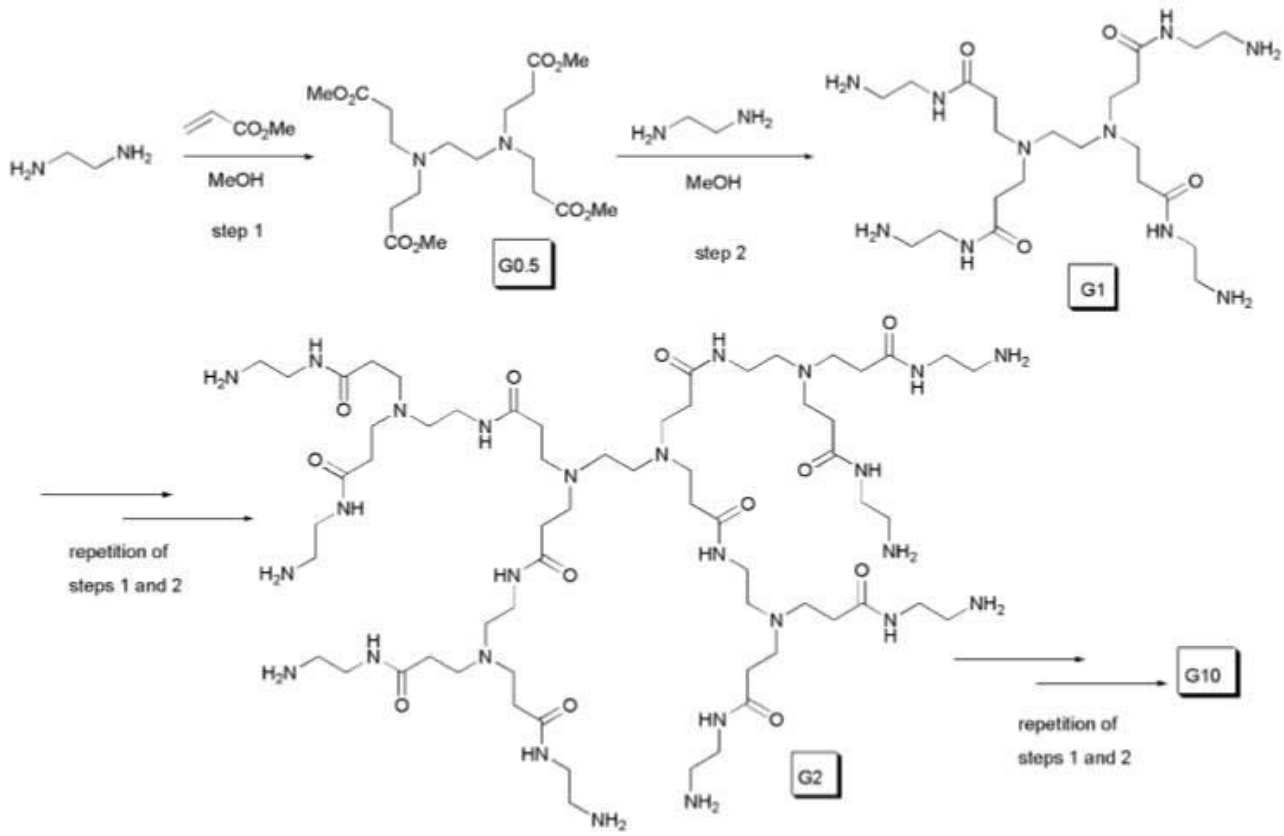

Fig. 6 – PAMAM dendrimers synthesis. Reproduced from reference 19 with permission from The Royal Society of Chemistry (RSC) on behalf of the European Society for Photobiology, the European Photochemistry Association and the RSC.

The attractiveness of these dendrimers lies on their high efficiency in interacting with and condensing (negatively charged) nucleic acids with the help of electrostatic interactions of the outer shell, positively charged amine functionalities. Moreover, they are able to provide a safe passage through the cellular membrane, a protective action against nuclease degradation and a safe release of the active species at the desired site, mainly due to the earlier described 'sponge effect' (which also involves the inner shell amine motifs).[9]

The transfection ability of PAMAM dendrimers is conditioned by the generation number and the corresponding concentration of functional groups, dimension and conformation.[5,68] *In vitro* studies showed that the dentritic frameworks with generations four to six display the highest transfection efficiency (between generations one to eight), even if their cytotoxicity is above their lower generation counterparts.[60] While still acceptable, their cytotoxicity and following biocompatibility is a first-hand limiting issue of their clinical use.

PAMAMs also exhibit a very interesting feature: partially degraded dendrimers obtained by thermal degradation in proper solvents are 50 times more efficient in terms of transfection output as compared with the initial, structurally perfect dendrimers.[12] One reason for this behavior is the superior flexibility of the fractured dendrimer, which generates more compact dendriplexes with the nucleic acid.[26] Moreover, an excess of the cationic amines from the dendrimer as reported to the negatively charged phosphates from DNA determines maximum transfection output.

The high number of amine groups placed on the external layer of PAMAM structures generates relatively facile adjustment possibilities, like the simple modification of cationic groups or the more elaborate connection of various motifs (described later) in order to provide superior transfection output and optimal cytotoxicity. All this tuning can be performed on commercially available high generation PAMAM dendrimers like SuperFect™ (fractured sixth generation PAMAM) and PolyFect ™ (intact sixth generation PAMAM), two of the dominant gene transfer kits used in DNA and RNA delivery research.[9] Even if their laborious and time-consuming preparation makes these products quite expensive,[59] they can be successfully used as building blocks in the construction of efficient gene carriers which could find a solution for some *in vivo* issues in the field like storage and colloidal stability.[5]

PPI dendrimers

Firstly proposed as a gene delivery vehicle in 1999,[12] PPI dendritic constructs are the second family of dendrimer-based transfection agents which dominate the gene delivery area.[70] Similarly to PAMAM, PPI dendrimers are nanospherical, flexible, monodisperse, cascade polymers with a clear-cut architecture.[12] The most relevant differences as compared to PAMAM are the smaller dimensions (smaller branches), the more hydrophobic character and the 100% protonable nitrogen content.[71] They are prepared by a two-step divergent strategy which implies the Michael-type addition of acrylonitrile to a small-sized core (usually butylenediamine or ethylenediamine) and the subsequent hydrogenation of the nitrile groups.[70]

As PAMAM frameworks, PPI dendrimers display a high density of amine units at the surface (the same number of outer amine units per generation as PAMAM) and therefore have similar DNA-binding skills, but also kindred cytotoxicities and haemolytic effect.[71]

Their transfection ability is also fixed by the generation number, with the second and third generation dendrimers being the most efficient while maintaining a reasonable toxicity.[72,73] Opposite to PAMAM carriers, PPI dendrimers do not exhibit a generation-dependent haemolytic effect.[70] A comparison between the transfection efficiencies of commercially available generation four PAMAM and PPI dendrimers concluded that the latter is the least efficient vehicle.[74] On the other side, *in vivo* tests showed that PPI dendrimers are capable of a preferential expression of genes in the liver, a very useful trait in the targeted gene therapy of cancer.[75]

The commercial availability of the PPI dendrimers (Astramol ™, PPI generations from one to five) and the multitude of functionalization or conjugation possibilities add to the high potential of these dendrimers to deliver optimum non-viral gene delivery vectors.

Engineering dendrimers for improved transfection

The field of non-viral, dendrimer-based gene carriers faces a constant conflict between the favourable transfection efficiency, DNA-binding and protection abilities and the unpleasant toxicity, stability or specificity-related problems. As a consequence, the quest for new, versatile gene vectors created an impressive collection of

dendritic structures with engineered cores, generations, conformations, surface functionalities and physicochemical traits.[1,5,12,13] The majority of these creative efforts are based on the earlier mentioned dendritic families, especially on PAMAM and PPI constructs.

However, the vectors obtained till now are far from perfect and the holy grail of the ideal gene carrier is still untouchable. Transfection performances, structure-function relationships and a better understanding of the mechanistic insights seem to be the last hurdles to overcome. Even if a small variation in the dendrimeric structural organization strongly affects the transfection conduct, a single dendritic mutation is not able to tackle the entire complexity and multiple obstacles of gene delivery.

A deep literature survey led to the conclusion that there are two major pathways to optimize the molecular structure of the dendritic carriers (from the initial synthesis or through surface modification) and to enhance their biological output: (i) the mutation of the dendrimeric structure and corresponding properties by employing new core molecules, and (ii) engineering the surface of the dendritic framework as to adjust the physicochemical and biological traits. The latter strategy has three dimensions: (1) conjugation of several segments to the amine-decorated surface of the dendrimer in order to improve all stages of the gene delivery mechanism, (2) the use of various targeting ligands as to increase the specificity of the carrier, (3) the connection of hydrophilic macromolecular backbones to the dendritic construct towards superior efficiencies and stabilities and diminished toxicities.

It is evident that the refinement of any nanoscale design parameter has a major cascade effect upon the final behaviour of the dendrimeric carrier. For example, even an apparently minor modification of the dendritic core greatly influences its transfection performance,[76,77] since this focal point is responsible for the dimension, functionality density, conformation and overall architecture of the final dendrimer.[78]

This leads to severe modifications of the DNA-binding behaviour, hydrophilic hydrophobic balance, and dendriplex delivery.[79] For instance, the use of triethanolamine instead of the widely employed ammonia core in PAMAM dendrimers increases the distance between the first generation branches, enhances the flexibility of the dendritic construct and improves its DNA-complexation ability.[80] The use of the larger, tetrafunctional jeffamine molecule as a core further rises the number of and distances between the dendrons, which results in a superior transfection output.[81] Moreover, if the classical core of PAMAM is replaced with lipids,[1] the resulting dendrimer will exhibit a completely different hydrophobic behaviour and will prefer to cooperate with phospholipid-based cellular membranes.

As described earlier, the success of cationic dendrimer-based vectors in gene therapy is based on the high density of protonable, primary amine groups on their outer (and many times inner) layers. The downside of this feature is the cytotoxicity generated by the resulting polycationic structures. Therefore, the choice of the amine feature has a cardinal role in the delivery behaviour, with all its components: complexation of the nucleic acid, stability of the resulting dendriplex, and release pathway.[12,13] Several efforts were dedicated to conceal, adjust or shield the strength of the positively charged amines, and quaternary ammonium and oligoamine conjugation seem to be the most feasible options in this regard.[12,26] The toxicity of the highly charged cationic dendrimers is reduced by employing the quaternary ammonium route, while the DNA-complexation skills were enhanced by applying various oligoamines. Moreover, an 'odd-even' effect was observed for the latter: an even number of aminoethylenes conjugating the surface of the dendrimers generates superior transfection performances as compared to their odd numbered counterparts.[26,82]

Accordingly, the generation number, being directly responsible for the density of the cationic motifs is of high importance to transfection efficiency. Higher generations usually imply better gene interaction and transfection but also poor DNA release and higher toxicities.[8,13] The most viable options seem to be generation six for PAMAM and generation three for PPI (as detailed earlier), even if this rule does not seem to apply to all types of cells.[15]

The external layers of the dendritic architectures were customized with various functional or targeting motifs, like bio-inspired lipids, saccharides, proteins, and amino acids, or synthetic fluorocarbons, cationic segments, and low polymers, with impressive effects on the transfection output, biocompatibility, and hydrophilic behavior. Several excellent reviews in the field provide a detailed description of these functionalization procedures and the ups and downs of the resulting engineered dendrimers.[5,8,9,12,13,32] Some general conclusions can be extracted from all the research endeavors of customizing the dendrimer surface: (i) incorporation of lipids has a hydrophobic

effect and usually improves the cellular assimilation; (ii) functionalization with saccharides, proteins and peptides has a positive influence on the delivery performance and also renders specificity to the resulting systems; (iii) each amino-acid (arginine, histidine, phenylalanine) employed has an individual impact on different stages of the transfection pathway (binding capacity, endosomal escape, cellular uptake, respectively) and improves the overall biocompatibility; (iv) perfluoroalkyls drastically enhance the transfection efficiency (especially for PAMAM) and also help in reducing toxicity; (v) connection of various macromolecular segments (especially polyethylene glycols) leads to a serious upgrade in delivery performance, biocompatibility, and blood circulation time; (vi) self-assembled, well-balanced (between the hydrophilic and the hydrophobic motifs), amphiphilic dendrimers display interesting features and certain fundamental and applicative potential in the gene therapy area.

Finally, it must be underlined that each element of the dendritic construct plays a complex role in the final gene delivery performance. To puzzle the matter even more, a dendrimeric gene carrier can be the most efficient only for a certain type of cell, so the specific cellular structure becomes an additional limiting factor of the transfection performance.

CONCLUSIONS

The concept of employing macromolecular dendritic tools in the field of gene delivery represents an encouraging pathway to generate highly ordered, monodisperse, non-viral vehicles with tunable size, shape and surface functionalities. The quest for efficient and nontoxic gene delivery vehicles created an impressive collection of dendritic structures with engineered cores, generations, conformations, surface functionalities and physicochemical traits. These hyperbranched architectures were customized with various motifs like lipids, saccharides, proteins, amino acids, fluorocarbons, cationic segments, and low polymers in order to provide superior transfection output, biocompatibility, and hydrophilic behavior.

However, much progress is still needed to reach the right features of (almost) perfect vectors, suitable for successful clinical applications. Transfection performances, structure-function relationships and a better understanding of the mechanistic insights seem to be the last hurdles to overcome. Even if a small variation in the dendrimeric organization strongly affects the transfection conduct, a single dendritic mutation is not able to tackle the entire complexity and multiple obstacles of gene delivery.

Therefore, the evolution of 'smarter', more versatile dendrimers with programed and improved functions, superior delivery efficacy, and cell/tissue specificity is expected. This implies the development of novel strategies and pathways to synthesize, functionalize and adjust dendritic architectures and to unlock the close-to-ideal non-viral gene delivery vector.

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