Knocking Down Gene Expression with Dendritic Vectors

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Abstract: The aim of this review is to provide the reader with an overview on the potential of dendritic polymers in the antisense delivery technology. Special emphasis has been placed on the different types of dendritic structures that have been reported and the modifications performed to increase their efficacy and safety. Therefore the advances made in their chemistry and how it has been adapted to meet the specific requirements of the antisense delivery technology are reviewed and discussed.

Key Words: Dendrimers, dendriplexes, gene silencing, antisense therapy, antisense delivery, siRNA delivery, gene therapy, knocking down.

1. INTRODUCTION

The discovery in the late 1970s that the expression of a specific gene product could be inhibited using a short DNA sequence, represents the first step towards the development of the antisense approach [1]. The rationale behind this antisense technology is that specific DNA or RNA molecules are able to down-regulate the expression of disease-causing proteins by inhibiting gene expression at the level of mRNA [2]. One of the main advantages of the antisense strategy over conventional pharmacological therapies relies on its specificity, as these DNA or RNA molecules are designed with a sequence complementary to that of the target mRNA. In fact, the antisense strategy allows the rational design of sequence-specific nucleic acid that can target and even destroy a given mRNA. By contrast, conventional therapies utilize compounds which act upon the protein itself and often require the non-rational approach of screening thousands of compounds to find an active molecule. Moreover, selection of drugs on the basis of DNA sequence has a reduced potential for toxicity, should result in fewer side effects, and therefore should eventually yield safer drugs than generally less specific conventional therapies.

There are several molecules that can down-regulate the protein expression at the mRNA level and are currently under investigation. These include: antisense oligonucleotides (AS-ODN), small interfering RNA (siRNA), ribozymes and DNA enzymes (DNAzymes). Progress in understanding the genetic bases of the pathogenesis of diseases has prompted the rapid evolution of these gene-silencing strategies. The clinical status of the antisense strategy has been recently reviewed [3, 4]. A number of these approaches are currently under clinical evaluation and are expected to offer considerable improvements to our current therapeutic armamentarium, where an unmet clinical need exists. At present numerous clinical studies have been completed or are underway for the evaluation of the efficacy of antisense strategies in different diseases, including cancer, cardiovascular diseases, inflammations and infections [5]. Moreover, an antisense oligonucleotide has already been approved by the FDA as a therapeutic agent: Vitravene® (Fomivirsen) [6], which is used for the treatment of ocular cytomegalovirus retinitis by local injection.

However, although much work has been accomplished, the development of an efficient delivery system still remain a major challenge for the wide clinical application of these antisense technologies. In the following sections - after a brief introduction of the different antisense strategies - the potential and contributions of dendrimers in the development of effective non-viral delivery systems for the antisense therapy will be discussed.

2. ANTISENSE STRATEGIES

2.1 Antisense Oligonucleotides

Antisense oligonucleotides (AS-ODNs) are synthetic single-stranded DNA fragments that bind to complementary intracellular mRNA strands forming a short double helix. They consist of short sequences, composed of about 13 to 25 nucleotides. Although AS-ODNs have been recognized as a naturally occurring gene regulation approach, the precise mechanism of action for these antisense molecules still remains unclear. Two major mechanisms have been proposed and are widely accepted, namely translational arrest and RNase H activation [7, 8]. In the translational arrest, an AS-ODN binds to the complementary single mRNA strand by Watson-Crick base pairing, thus forming a double-helix hybrid that sterically blocks the translation of this transcript into a protein [7]. These double-helix segments prevent the binding of factors necessary for the initiation and/or modulation of the translation process. In addition, the hybrid formation may block the movement of ribosome along the mRNA [9]. On the other hand, according to the second

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hypothesized mechanism a RNase-H mediated cleavage of the target mRNA takes place. RNase-H is a ribonuclease that recognizes RNA–DNA duplexes and selectively cleaves the RNA strand. This is a catalytic mechanism: once a RNA molecule is cleaved, the AS-ODN dissociates from the duplex and becomes available to bind a second target mRNA molecule [8]. Due to their great instability against degradation, one of the major challenges in the development of antisense strategies include the investigation on antisense chemistry to improve their stability. The most notable discovery was the replacement of the non-bridging oxygen of the phosphodiester backbone by sulphur addition, resulting in the synthesis of phosphorothioate AS-ODNs and leading to a significant increase in their stability, without major changes in their ability to hybridize with their target mRNA [10].. Other chemical modifications include methylphosphonate AS-ODN, boranophosphate AS-ODNs, morpholino ODN (non-ionic DNA analogues), locked nucleic acids, or peptide nucleic acids. However, no further details on chemically modified AS-ODNs will be given in this review because they have not been used in dendrimer mediated delivery. The reader can refer to comprehensive reviews published on this topic [11, 12].

2.2 RNA Interference

RNA interference (RNAi) is the induction of sequence-specific gene silencing via the introduction of double-stranded RNA [12, 13]. Like the antisense strategies, RNAi relies on complementarities between the RNA and its target mRNA. In this process, long dsRNAs are recognized by a dsRNA-specific endonuclease called Dicer, which cleaves long dsRNAs into small interfering RNAs of about 21–23 nucleotides [14]. Then, this siRNA is incorporated into a nuclease complex called RISC (RNA-induced silencing complex), where the duplex is unwound by a helicase, resulting in two single strands, one of which (the antisense strand) remains bound to the RISC complex [15]. This single strand RNA/RISC complex locates mRNA sequences within the cytoplasm with homologous nucleotide sequences and induces cleavage of mRNA, thereby preventing its translation into protein [16].

Unmodified RNAs duplexes are more stable than unmodified single-strand RNA. For this reason, although chemical modifications have been proposed in the design of synthetic siRNA [17, 18], they are much less necessary for siRNA than for antisense oligonucleotides.

2.3 Ribozymes and DNAzymes

Ribozymes (from *ribo*nucleic acid *enzy*me, also called RNA enzyme or catalytic RNA) are catalytic RNA molecules that inhibit gene expression by the direct hydrolysis of the target mRNA [19, 20]. More recently DNA enzymes (DNAzymes) that are essentially the DNA analogues of ribozymes, have been added to the antisense technology [21, 22]. The major advantage here is that the biologically unstable ribonucleotide motifs essential for the catalytic activity of ribozymes are now replaced with the more stable DNA chemistry. The versatility of DNA chemistry also allows for more synthetic options in producing modified DNAzymes with enhanced biological stability.

Despite the apparently simple idea to down-regulate the expression of disease-causing proteins by sequence-specific short molecules complementary to a mRNA, several problems have to be overcome for successful clinical application. In fact, AS-ODNs, siRNA, ribozymes and DNAzymes face several pharmaceutical problems that have limited their clinical use. They are generally all polyanionic macromolecules which do not readily cross biological barriers [2, 23]. Moreover, they show a poor stability towards nuclease activity, low intracellular penetration and low bioavailability. Fig. (**1**) shows a schematic representation of the extra- and intracellular barriers that hamper efficient gene silencing [24, 25]. Although chemical modifications were brought to the basic AS-ODNs and siRNAs, their sensitivity to degradation and poor intracellular penetration is still hampering their widespread clinical applications. In fact, the major bottleneck in the development of these antisense therapies is the delivery of these macromolecules to the target cells, tissues or organs. Therefore the development of more efficient delivery systems for these antisense macromolecules is regarded as one of the most promising strategies to solve these pharmaceutical hurdles. For that reason, improvements on effective delivery of antisense macromolecules have progressed rapidly. Currently, several clinical trials using specific delivery systems for different antisense macromolecules are underway (www.clinicaltrials.gov).

Among the different approaches under study, dendrimers are attracting a great interest for their well defined structure and great versatility in their chemistry that offer a unique platform for the rational design of efficient antisense delivery systems.

3. DENDRIMERS: GENERAL ASPECTS

Since their conception in the late 1970s and early 1980s, the unique properties of these highly branched three dimensional macromolecules have attracted the interest of many investigators making them the focus of much research in different fields, including the pharmaceutical one. They consist of a central core which acts as the root from which a number of highly branched, tree-like arms grow in an ordered and symmetric fashion. This molecular architecture confers them a number of unique properties which differentiate dendrimers from other polymers; in fact the gradual stepwise synthesis generate macromolecules with a well defined size and a low polydispersity index [26]. Furthermore, dendrimers offer a great versatility in their chemistry, with almost limitless possibilities of modifications, enabling them to be engineered to meet specific biopharmaceutical needs. Moreover, the surface of dendrimers provides an excellent platform for the attachment of functional groups to improve particular characteristics or add new functionalities, such as cell-specific ligands, solubility modifiers, transfection enhancing groups or imaging tags. Recent successes in synthetic procedures, such as the introduction of the "lego" and "click" approaches, have greatly simplified the synthesis of dendrimers providing a vastly expanded variety of dendritic compounds while reducing the cost of their production. Thanks to recent advances in synthetic chemistry and characterization techniques, a rapid development of this kind of polymers has been made and a variety of dendritic

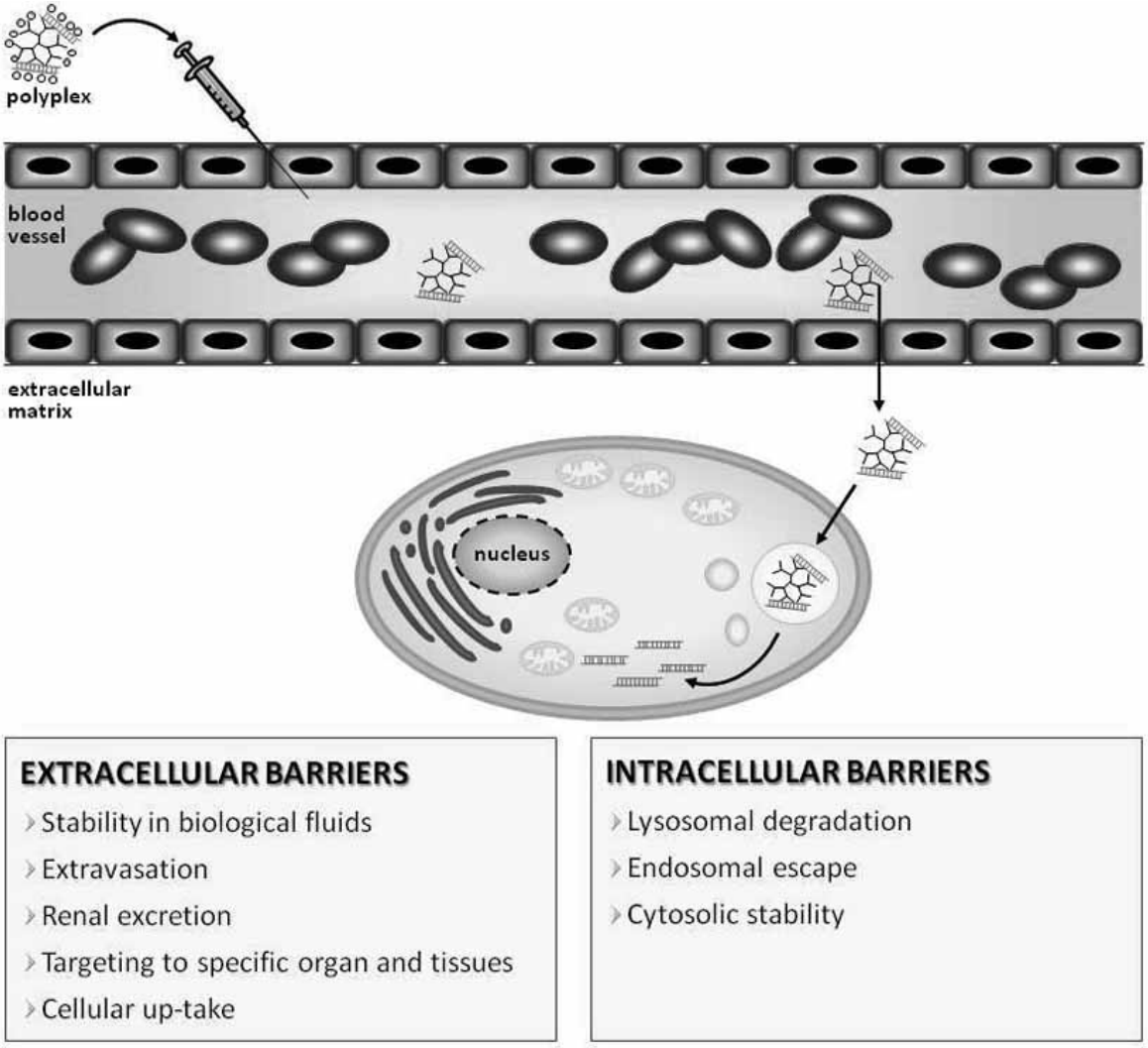

Fig. (1). Barriers to nucleic acids delivery.

scaffolds has become accessible with defined nanoscopic dimensions and discrete numbers of functional end groups [27]. The control over size, shape, and surface functionality makes dendrimers one of the "smartest" and customizable nanotechnologies currently available. For these reasons, they have spawned a whole range of new research areas ranging from drug and gene delivery applications to processing, diagnostics and nanoengineering [28]. Although nice reviews on dendritic gene delivery have been recently published [29, 30], our aim was to focus the attention on dendrimers for specific gene knockdown applications. For that purpose, in this review, the authors will provide the readers with an overview on the advances of the specific use of dendrimers in the antisense delivery strategy and how their chemistry has been adapted to meet its specific requirements. Highlighted features are included in Table **1**.

Table 1. Highlighted Effects of the Structural Modification of Dendrimers on their Gene-Knockdown Activity

Dendrimer	Surface modification	Internal modification	Nucleic acid	Structure-activity relationship	Refs.
PAMAM	Oregon 488 (hydrophobic fluorescent dye)	------	AS-ODN	- Improved *in vitro* delivery of the AS-ODN. - Suggested enhanced ability of disrupting endosomal membranes.	[43]
PAMAM	cholesterol	------	AS-ODN	- Improved *in vitro* delivery of the AS-ODN. - Suggested enhanced ability of disrupting endosomal membranes.	[50]
PAMAM	TAT peptide	------	AS-ODN and siRNA	- Increased cellular uptake. - No enhanced silencing efficiency. probable incomplete intracellular release of AS-ODN and siRNA.	[32]

(Table 1) Contd…..

Dendrimer	Surface modification	Internal modification	Nucleic acid	Structure-activity relationship	Refs.
PAMAM	acetylation	quaternization with MeI	siRNA	- Reduction of the cytotoxicity improved intracellular delivery of the siRNA. - No gene silencing efficiencies reported.	[60]
PAMAM	α-cyclodextrin	------	siRNA	Improved delivery of anti-luciferase siRNA in NHI cell line.	[53]
PAMAM-OH	arginine	------	DNAzymes and AS-ODN	- Easy release of AS-ODN through fast hydrolysis of the dendrimer. - Reduction of the cytotoxicity.	[51]
PLL	lipidic groups	------	AS-ODN	Better ability of crossing biological membranes.	[81]
PPI	quaternization with MeI	quaternization with MeI	DNA	Limited toxicity only for the lower generation dendrimers.	[71]
PPI	acetyl groups or triglycol gallate (PEG-like) groups	quaternization with MeI and MeCl	DNAzyme	Transfection ability as high as that obtained with DOTAP, with lower cellular toxicity.	[72]

3.1 Polyamidoamine (PAMAM) Dendrimers

Among all dendritic vectors applied so far for knocking down gene expression, polyamidoamine (PAMAM) dendrimers and their derivatives currently dominate this area. This predominance can be linked to the fact that PAMAM dendrimers were one of the earliest dendritic systems synthesized at high generation (G) numbers and that they are commercially available. Moreover, the promising efficiencies achieved with PAMAM dendrimers early prompted researches to investigate their ability to efficiently deliver molecules that could inhibit the expression of disease related proteins.

The PAMAM dendrimers are normally based on ammonia as a trivalent initiator core or ethylenediamine as a tetravalent initiator core [31]. This starts the stepwise polymerization process and dictates the number of branches of the molecule. Using a divergent approach the molecule is constructed in a sequential fashion by exhaustive Michael addition of methylacrylate followed by amidation of the resulting ester with ethylenediamine [31]. Each complete reaction sequence results in a new "full" dendrimer generation (i.e. G3, G4) with terminal amine functionality, whereas the interruption of the sequence at the first step leads to a "half" generations (i.e. G2.5, G3.5) with ester terminal groups [31]. A schematic representation of a PAMAM dendrimer is illustrated in Fig. (**2**).

Amine-terminated PAMAM dendrimers develop high positive charge densities at their surfaces when they are at physiological pH or when they are dissolved in water. This enables them to electrostatically interact with the negatively charged phosphate groups of nucleic acids, including AS-ODNs, ribozymes and siRNA [32-34]. As a result of this electrostatic interaction, dendrimer-nucleic acid complexes are formed. Several factors have been reported to influence this complex formation, ionic strength and pH above all. In fact, the interaction between AS-ODN and PAMAM dendrimers was reported to be unstable at high ionic strength [33]. Moreover, it was also found that greater AS-ODN/dendrimer binding occurred at lower pH [33]. As a result of this dendrimer/AS-ODN interaction, it was found that PAMAM dendrimers could efficiently protect AS-ODN from degradation and facilitate its cellular uptake [33, 35].

In order to down-regulate gene expression, antisense molecules must penetrate into the targeted cells and reach the cytoplasm. Although the exact mechanism of PAMAM mediated oligonucleotide internalization is unclear at this point, several studies conducted with PAMAM dendrimers have suggested different possible mechanisms of cellular internalization. Indded, it has been reported that positively charged amine-terminated PAMAM dendrimers induce the formation of nanoscale holes upon interaction with artificial lipid bilayers, facilitating therefore dendrimer uptake [36]. This effect has been atributed to high generation dendrimers (G5-G7) and possible mechanisms of hole formation include dendrimer-mediated removal of lipids from the membrane [37] or direct insertion of the amine-terminated dendrimers into the membrane [38]. Concerning the cellular internalization of dendrimer/nucleic acid complexes it has been reported that cholesterol pays an important role in this process, as depletion of cholesterol from the plasma membrane profoundly affects the gene delivery mediated by dendrimers [39]. However other mechanism have also been described, such as the caveolae-mediated cellular uptake, suggesting that dendrimer/nucleic acid complexes may use different internalisation pathways in different cell lines [40]. Once internalized by the cells one of the mayor limiting steps leading to low gene-transfer efficiency is their accumulation in the endosomes. In the particular case of PAMAM dendrimers, `the proton sponge´ hypothesis has been described as a possible mechanism of endosomal encape leading to efficient transfection [41]. According to this hypothesis, the buffering capacity presented by several polymers (such as polyethilenimine) leads to osmotic swelling and rupture of endosomes, resulting in the release of the vector into the cytoplasm.

Regarding its silencing efficiency, the first evidence of the efficacy of PAMAM dendrimers as transfection agents for the delivery of AS-ODN was reported by Bielinska and coworkers in 1996 [42]. Generation 7 PAMAM dendrimers with an ethylenediamine core were complexed with an AS-ODN and transfections were performed into clones generated from D5 mouse melanoma and Rat2 embryonal fibroblast cell lines expressing luciferase cDNA. These AS-ODN/G7 dendrimer complexes were able to inhibit about 30 % of the luciferase expression [42]. Later on studies performed by Joo

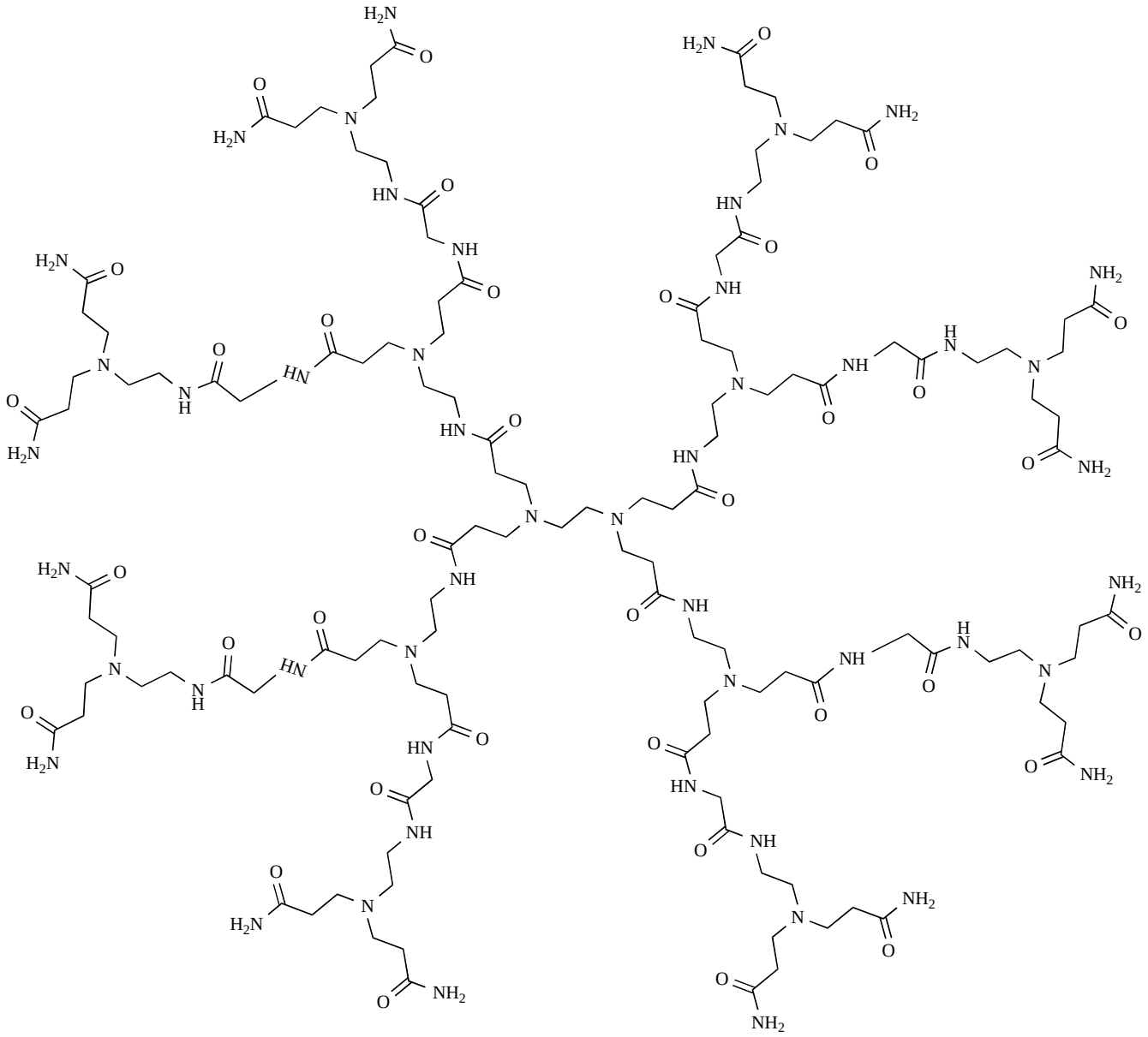

Fig. (2). Generation 2 PAMAM dendrimer.

et al. demonstrated that G5 PAMAM dendrimers had the ability to efficiently deliver phosphorotioate oligonucleotides in the presence of serum proteins, thus pointing out the possibility for an *in vivo* application of these dendrimers [43]. The bioavailability of siRNA and AS-ODN is generally decreased by interactions with serum proteins, as it was observed that AS-ODN bound to serum albumins (or other proteins) do not enter the extra-vascular space. Therefore in the presence of serum proteins higher doses of AS-ODNs are generally required to achieve the desired therapeutic effect [44-46]. Compared to Lipofectamine, PAMAM dendrimers were more effective in delivering oligonucleotides in the presence of serum proteins [43]. The oligonucleotide dose, the charge ratio of oligonucleotide to dendrimer and the generation of the dendrimers were found to be all critical variables for the antisense effect. Although no size measurements were performed, the best performance of the dendrimer was attributed to the molecular rather than particulate nature of the dendrimer/AS-ODN complex [43].

On the other hand, partially degraded PAMAM dendrimers exhibited a greater capacity in pDNA delivery than the perfect dendritic PAMAM structure [47]. This behavior has been attributed to the more flexible structures of partially degraded PAMAM dendrimers that entails a more efficient interaction with DNA and improve-ment in the buffer capacity of the internal amino groups within the endosome [47]. To the best of our knowledge, no studies concerning the direct comparison of conventional and partially degraded PAMAM dendrimers for the delivery of short oligonucleotides have been performed. However, Wu and co-workers have reported the design and synthesis of PAMAM dendrimers with a more flexible structure than the commercial ones. To this end they have synthesized a series of PAMAM dendrimers with an increased distance between the core and the first branching unit via the insertion of an ethylene glycol unit [34, 48]. These dendrimers are expected to have less densely packed branching units at end groups than the commercially available PAMAM dendrimers, where the

branching starts immediately at the central amine core. First studies were conducted to evaluate the ability of this dendrimers (generation 4) to efficiently bind and subsequently inhibit ribozyme RNA activity [34]. Subsequent cell culture studies also evidenced the potential of these dendrimers to efficiently delivery siRNA to cells [48]. In this work, dendrimers up to generation 7 were synthesized and gene silencing was found to be more effective when generation number of the dendrimer was increased from 1 to 7 [48]. However, whether this enhancement of the dendrimer flexibility results in an enhanced siRNA or rybozime delivery remains unclear, since no comparisons with conventional PAMAM dendrimers were performed.

Several researchers have taken advantage of the multiple possibilities of closely controlling the size, shape and chemistry of dendrimers to develop structures that could enhance their ability to efficiently deliver antisense molecules, such as those aiming at improving their transfection efficiency or reducing their toxicity. In general, due to their easy synthetic production, low cost and reduced cytotoxicity they mainly focused on low generation (from G2 to G5) PAMAM dendrimers.

One of the first modifications reported was the coupling of relatively hydrophobic small molecules to the PAMAM structure. In order to investigate the fate of AS-ODN/dendrimer complex after cellular internalization, generation 5 PAMAM dendrimers labeled with a small hydrophobic fluorescent dye (Oregon 488) were synthesized and complexed with TAMRA (Tetramethyl-6-Carboxyrhodamine) labeled AS-ODN [49]. Surprisingly, all labeled conjugates were more active in delivering the AS-ODN *in vitro* than the original dendritic vector. These results suggested that the introduction of relatively hydrophobic small molecules in PAMAM dendrimers could enhance their efficacy as delivery agents for nucleic acids. Although the bases for this effect were not clear at that time, a proposed explanation was that relatively hydrophobic fluorescent moieties enhanced the ability of the dendrimer to disrupt endosomal membranes and thus traffic to the cytosol and nucleus [49]. To pursue that issue, a series of cholesterol (Fig. (**3**)) conjugated generation 5 PAMAM dendrimer at increasing derivatization degree in cholesterol units were prepared. The obtained results confirmed that an appropriate increase in the hydrophobicity of the dendrimer enhance the delivery efficiency of AS-ODN into cells [50].

As one of the main problems for the non-viral gene delivery systems is their lower transfection efficiency compared to viral vectors, many methods attempting to mimic viruses have been used to overcome such an obstacle. In a first study, a generation 5 PAMAM dendrimer labeled with Bodipi (boron-dipyrromethene fluorescent dye) was conjugated to a Tat peptide, a cell penetrating peptide derived from HIV-1 virus that is expected to increase cellular uptake. These dendrimers resulted to be highly effective in delivering both AS-ODNs and siRNA designed to inhibit MDR1 gene expression (codes for P-glycoprotein, a transmembrane protein that is a drug transporter) in NIH/3T3 cell line [32]. However, beside the increased cellular uptake, the addition of the Tat peptide to PAMAM dendrimers failed to enhance their silencing efficiency. Although the reason for these results was not clear, the limited biological activity was attributed to the incomplete release of AS-ODN and siRNA inside the cells. In fact, cell penetrating peptides, such as Tat, are highly cationic and these positive charges contribute to their delivery ability. As the PAMAM dendrimers are also highly positively charged at physiological pH, it was suggested that the presence of Tat residues was essentially redundant. Whether or not this is the reason of the low efficiency, it has not been clarified until the moment. However, in a ingenious approach, biodegradable PAMAM dendrimers with arginine (Fig. (**3**)) modified surface groups have been recently reported for the effective delivery of DNAzymes and AS-ODNs [51]. Indeed, arginine is one of the most representative basic aminoacids in the protein transduction domains family of the cell penetrating peptides.

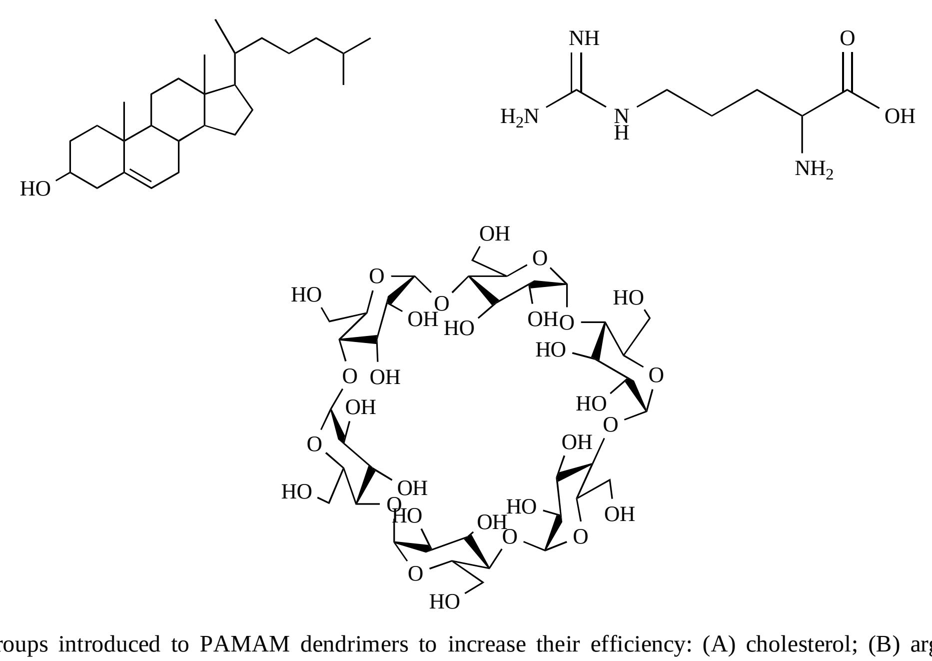

Fig. (3). Chemical groups introduced to PAMAM dendrimers to increase their efficiency: (A) cholesterol; (B) arginine residue; (C) α-cyclodextrin.

Arginine residues were coupled to PAMAM-OH dendrimer (from G2 to G4) through a biodegradable ester bond. On one hand, the introduction of the arginine residues on the dendritic structure was expected to enhance the transfection efficiency. On the other hand, the biodegradation into PAMAM-OH was expected to facilitate the release of the associated molecule and improve the toxicity profile of the system. In fact, cationic dendrimers have shown to exhibit a greater toxicity than anionic or neutral PAMAM dendrimers [52]. Therefore, the PAMAM-OH dendrimer, with neutral hydroxyl groups on its surface was expected to improve the biocompatibility of the system. Comparison with a conventional PAMAM dendrimer modified with arginine groups attached through an amide bond indicated that the introduction of biodegradability was significantly important for DNAzyme delivery [51]. This improvement was attributed to the easy release of oligonucleotides through fast hydrolysis of PAMAM-OH-arginine dendrimer. Moreover, toxicity studies revealed the positive effect of these modifications on cell viability.

Other PAMAM conjugates that have shown to efficiently delivery antisense molecules, specifically siRNA, include covalent conjugates of α-cyclodextrin (Fig. (**3**)) to low generation (G3) PAMAM dendrimers [53]. The synergic effect between the nucleic acid complexing ability of the PAMAM dendrimer and the interaction of the cyclodextrin with cellular membranes components, such as phospholipids and cholesterol, was expected to result in a delivery vector with enhanced transfection ability [54]. Silencing studies performed with α-cyclodextrin-PAMAM dendrimers complexing a siRNA directed against the model protein luciferase revealed an efficient delivery of the siRNA in NHI cells [53].

On the other hand, and considering that one of the major concerns associated with the development of new materials is the issue of toxicity, several modifications have been performed to develop PAMAM dendrimers with a low toxicity. In fact, PAMAM dendrimers posses concentration- and generation- dependent toxicities, confirming that a high density of cationic amine groups is damaging to cellular membranes [55-57]. Several studies have been conducted and recently reviewed illustrating that both negatively charged and neutral dendrimers were nontoxic, clearly demonstrating the structure/ toxicity relationship that is governed primarily by the functional groups on the dendrimer surface [58,59]. For that reason, a novel internally quaternized and surface-acetylated poly(amidoamine) generation 4 dendrimer for siRNA delivery has been recently developed [60]. The modification of surface amino groups by acetylation followed by internal quaternization was reported to reduce the cytotoxicity of the dendrimers. Moreover, the presence of internal charges preserved the ability of the dendrimers to form well defined polyplexes with siRNA and therefore facilitate the intracellular delivery of siRNA. However, whether they could efficiently mediate a gene knock-down ability remains unclear, as no gene silencing efficiencies were reported.

An alternative approach to reduce the cytotoxicity of PAMAM dendrimers consists in the partial surface derivatization of amino groups with poly (ethylene glycol) (PEG) or fatty acids. This observation can be explained by the reduced overall positive charge of these surface modified dendrimers. Partial derivatization with as few as six lipid chains or four PEG chains on G4 PAMAM dendrimers, respectively, was sufficient to substantially lower their cytotoxicity in Caco-2 cell line [52]. Same results were obtained by Kim *et al*. who performed a systematic investigation on a series of PAMAM-PEG conjugates, prepared varying the degree of substitution and PEG chain length [61]. They found that less than 25% of surface-modification by shorter PEG chains (PEG550/PEG750) may significantly reduce the cytotoxicity of amine-terminated PAMAM dendrimers, while maintaining good water-solubility [61]. More insights into the effects of PEGylation on the decrease of cytotoxicity at the molecular level have been given by Wang *et al*. [62]. These authors observed that conjugation with PEG could effectively reduce the PAMAM-induced cell apoptosis by attenuating the reactive oxygen species production and inhibiting PAMAM-induced mitochondrial membrane potential collapse [62].

Regarding the ability of these modified dendrimers to efficiently deliver nucleic acids it was recently observed that PEG conjugation to generations 5 and 6 (G5 and G6) PAMAM dendrimers greatly improve their characteristics as gene delivery carriers [63]. Compared with unconjugated PAMAM dendrimers, PEG conjugation significantly decreased the *in vitro* and *in vivo* cytotoxicities and hemolysis of G5 and G6 dendrimers, especially at higher PEG molar ratios: dendrimers conjugated with more PEG of higher molecular weight were much less cytotoxic. Meanwhile, the transfection ability of these PEG conjugated PAMAM dendrimers was unaltered as indicated by the efficient muscular gene expression observed when plasmid-DNA/dendrimer polyplexes were injected intramuscularly to the quadriceps of neonatal mice [63]. However, at the high PEG conjugation the transfection efficacy was markedly reduced probably due to a decreased interaction with the cell membrane caused by the greater shielding effect of PEG chains to the surface amine groups of the PAMAM dendrimers. In addition, authors suggest a wrapping of the PEG chains to the DNA molecules, thereby blocking the intracellular release of DNA [63]. On the contrary, from the best of our knowledge no data is available concerning the ability of PEGylated PAMAM derivatives for the delivery of short polynucleotides. For that reason, althougth the benefits provided by the PEG groups in terms of citotoxicity have been evidenced, more studies should be performed to determine appropiate degree of substitution or PEG length for the effective delivery of these specific antisense molecules.

3.2 Polypropylenimine (PPI) Dendrimers

Another class of dendritic macromolecules that have emerged as attractive cationic vectors for the delivery of nucleic acids are polypropylenimine dendrimers (PPI) [64,65]. They have been developed in 1993 at DSM Research (The Netherlands) on the base of the pioneer work of Vogtle and are now commercially available.

PPI dendrimers are synthesized by a divergent method (from the core to the periphery) starting from 1,4-diaminobutane used as the core molecule, Fig.(**4**). They are grown by a reiterative sequence consisting of a Michael addition of acrylonitrile to a primary amine group followed

by hydrogenation under pressure of nitrile groups to primary amine groups in the presence of Raney cobalt [66]. Due to their structural characteristics rich in amine groups, they were investigated as delivery vehicles for nucleic acids. High generation PPI dendrimers were first evaluated for nucleic acid delivery, however, disappointing toxicity levels of these high-generation (G8) PPI dendrimers precluded for a long time the use of the whole family of PPI dendrimers for gene delivery. Nevertheless, in 2002 Uchegbu and co-workers focused their work on low generation PPI dendrimers and demonstrated that the lower-generation dendrimers (G2 and G3) were effective gene transfer agents with a good biocompatibility profile in a human epidermoid cell line [65]. Recently, the lower-generation of PPI have also been reported to be effective gene transfer agents for antisense oligonucleotides in human epithelial cells [64]. The uptake of AS-ODN targeted to the epidermal growth factor receptor (EGFR; a receptor tyrosine kinase proto-oncogene that plays a central role in the initiation and development of several human malignancies, notably breast, brain, and lung tumors) was markedly enhanced (approximately 10-fold) when delivered as either G2 or G3-PPI complex, as compared to naked AS-ODN. The internalization mechanism appeared to be energy dependent and resulted in cytosolic localization of the AS-ODN as determined by a combination of FACS and fluorescent microscopy studies. G2 and G3 PPI dendrimers associating AS-ODN resulted in a marked knockdown of EGFR mRNA and protein expression in A431 cancer cells, as determined by RT-PCR and Western blotting respectively. They were able to inhibit target gene expression at levels comparable to those observed with Oligofectamine-mediated delivery — a commercially available cationic lipid-based transfection agent.

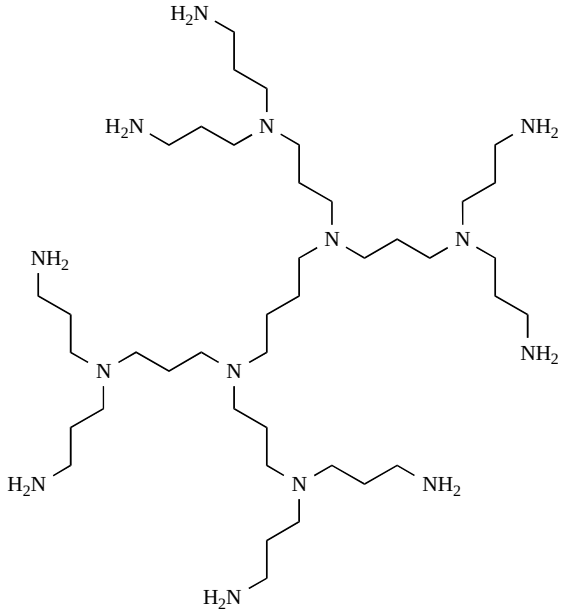

Fig. (4). Generation 2 poly-propylenimine dendrimer.

As alreday described for PAMAM dendrimers one of the mayor concerns related with amine functionalized PPI-dendrimers is the issue of toxicity, generally considered to be too high for their direct use in delivery systems. Moreover, it was observed that PPI-dendrimers can intrinsically alter the expression of many endogenous genes involved in apoptosis and cytokine signalling at doses previously suggested as non citotoxic. The extent of changes in gene expression depends on the dendrimer generation and cell type [67]. Data from literature strongly suggest that one of the main factors determining the citotoxicity of the total dendritic structure is the nature of the terminal groups, being surface amine groups considered to be ones of the more toxic [56]. For that reason, as in the case of PAMAM dendrimers, PPI-dendrimers have been chemically modified to create delivery systems with an improved toxicity profile. Two strategies have been undertaken to improve such toxicity profiles. The first one was the modification of the surface and/or the inner core by quaternization of the superficial primary amine and the internal tertiary amine respectively, which leads to multiple cationic ammonium sites [68-70]. The addition of permanent positive charges allows more nucleic acid sites to be bound by each dendrimer molecule at a minimum nitrogen/phosphate (N/P) ratio. Thus recently, PPI dendrimers quaternized with methyl iodide (MeI) have been presented as gene delivery agents [71]. The quaternization of the G2 PPI dendrimer led to an improved DNA binding and complex stability. This was accompanied by a dramatic improvement of the in vivo safety. In fact, a pDNA formulated with a generation 2 PPI dendrimer was found to be lethally toxic on intravenous injection in Balb/C mice and caused embolism-like deaths. On the contrary, pDNA complexed with generation 2 PPI quaternized dendrimers, was well tolerated on intravenous injection. The improved DNA binding exhibited by quaternized PPI dendrimers and hence the improved colloidal stability, was indicated as the main reason of this improved tolerability seen on quaternization of the dendrimer. Possibly the counter anions in the quaternized dendrimers determine to some extent the toxicities of the species and it seems favourable to use chloride counter anions instead of iodides [72].

Another strategy undertaken in an attempt to reduce the toxicity of PPI dendrimers was to modify the dendrimer structure at both the exterior with acetyl groups or triglycol gallate (PEG-like) groups and the interior by quaternization with methyl iodide (MeI) or methyl chloride (MeCl) [73]. The rationale behind this strategy is the preservation of the water solubility while generating no toxic species as the amine end groups were blocked. Moreover, the presence of the multiple cationic sites in the interior of the dendrimer is expected to maintain the ability of complex formation with nucleic acids. These dendrimers were able to efficiently complex and deliver a DNAzyme into ovarian carcinoma cells showing high transfection efficiencies usually exceeding 80%, with the acetylated quaternized dendrimers G4(MeI) and G4(MeCl) displaying the best results. In addition they were able to transfect cells at a level similar to that obtained with DOTAP, a cationic liposomal transfection agent, while inducing only a low cellular toxicity. This was the first work where it was observed that PPI-dendrimers of the higher generations (G4 and G5) displayed low cytotoxicity at concentrations used for *in vitro* transfection experiments, as measured by MTT-assay in four different cell lines (MCF7, Malme-3M, HT29 and K562-C1000).

A layer-by-layer surface modification approach was very recently adopted by Taratula et al. to improve the efficacy of siRNA complexes with PPI dendrimers, while reducing their toxocity [74]. siRNA/PPI complexes were first caged with a

cleavable dithiol containing crosslinker, such as DTBP (Dimethyl-3-3′-dithiobispropionimidate-HCl) and further modified with NHS {O-[2-(N-succinimidyloxycarbonyl)-ethyl]-O´ methylpolyethylene}from a difunctionalized PEG used for steric stabilization (MAL–PEG–NHS, α-maleimide-ω-N-hydroxysuccinimide ester poly(ethylene glycol)). Finally, a synthetic analog LHRH (Luteinizing Hormone-Releasing Hormone) peptide was bound on the nanoparticles surface as a targeting moiety to tumors overexpressing LHRH receptors. The combination of caging, PEGylation, and targeting by LHRH peptide revealed to be an effective strategy in terms of increased serum resistance, improved stability in biological liquids, tumor-specific targeting, effective penetration into cancer cells and preferential accumulation of delivered siRNA in the cytoplasm. In fact data obtained in the present study showed that the use of the LHRH peptide prevented an accumulation of siRNA in healthy organs, and enhanced both drug accumulation in tumors and its internalization by cancer cells. More important, the protective effects of cross-linking resulted to be reversible. Indeed, upon internalization into the targeted cancer cells, siRNA was able to escape from endosomes to cytoplasm, without penetrating into the nucleus, and efficiently silence the targeted mRNA.

3.3 Polylysine (PLL) Dendrimers

The idea of synthesizing PLL dendrimers came from the observation of Ryser's group at the Harvard Medical School that the uptake of radiolabelled serum albumin by sarcoma-180 cell cultures was significantly enhanced by the presence of proteins rich in lysine or of synthetic peptides obtained from lysine (L, D or LD), L-ornithine, or L-histidine [75]. Subsequent investigations indicated that individual amino acids or diamines did not have promoting effects, and that the effect of polypeptides was related to their molecular weight and to the distance between amino groups in their molecule, from which it was concluded that their attachment to the cellular membrane must take place through multiple centres [76, 77].

In the early 1980s Denkewalter patented the synthesis of L-lysine-based-dendrimers (PLL) and now a large number of synthetic PLL products (3–60 kDa) are commercially available [78]. Following a divergent route, conventional peptide chemistry was applied to the Boc-L-Lys(Boc)-OH monomer thus obtaining the lysine-based dendrimers reported in Fig. (**5**). The use of asymmetric L-lysine residues as branching units clearly distinguish the dendritic poly(L-lysine) from the classical, highly symmetric "starburst" dendrimers. Controlled synthesis yields the required monodispersity, defined topology and tailored versatility, all the properties that define a dendrimer. It was observed that the architecture of the polymer deeply affected the efficiency of gene expression mediated by PLL polymers [79]. Dendritic PLL resulted to be advantageous for endosomal escape and transcription of pDNA, even though the total amount of pDNA binding and uptake into cells were lower than those observed with linear PLL. The authors suggested that differences among linear and dendritic PLL were due to differences in the pKa values of terminal amines, that resulted to be lower for the dendritic polymer. This lowered pKa could result in a proton sponge effect in the endosomal compartment and a consequent osmotic endosomal disruption. Therefore, highly efficient endosomal escape mediated by dendritic PLL was possible, leading to a higher gene expression level than that obtained with linear PLL, even if the total pDNA uptake was lower. Another explanation provided by the same authors is related to the degree of pDNA compaction produced by dendritic and linear PLL, that is weaker for the former. Therefore RNA polymerase could easily access the weakly compacted pDNA compared to strongly compacted pDNA. If pDNA translocates into the cell nucleus as pDNA complexed with PLL, weakly compacted and easily chain-exchangeable complexes are advantageous for gene expression. These studies suggested

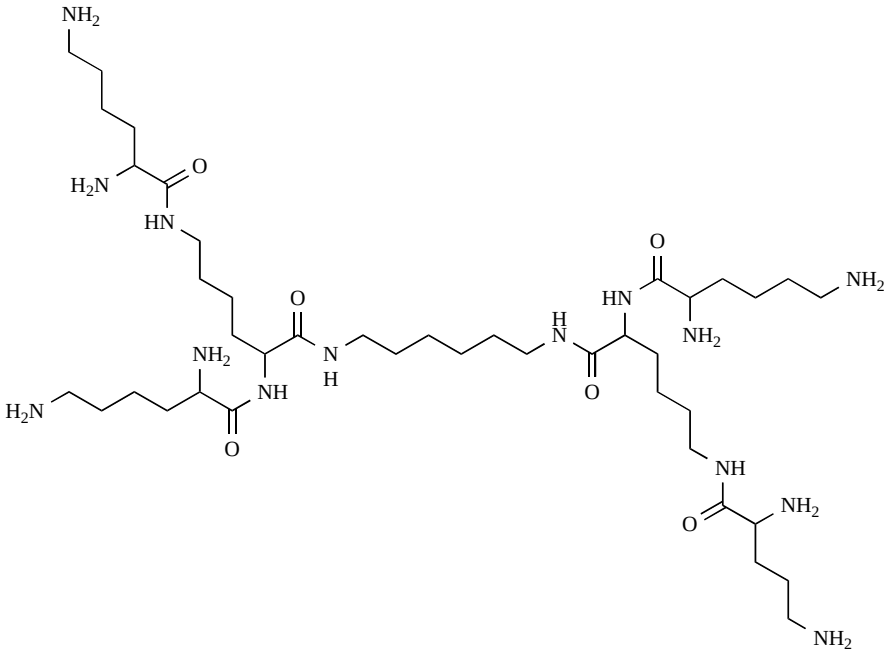

Fig. (5). Generation 2 poly-L-lysine dendrimer.

that the branch structure of dendritic PLL may play an important role for the effective delivery of pDNA. Similar results were obtained with antisense oligonucleotides, that were efficiently complexed and delivered by high-generation dendritic PPL [79]. As for the previously described dendrimers, also for PLL dendrimers the transfection efficiency increases with the number of positive charges and so with the generation number. However, when comparing with other dendrimers, a generation 4 dendritic PLL was not so effective as a generation 5 PAMAM dendrimer with similar number of surface amines [80]. In order to improve the delivery and cell uptake of nucleic acid complexed with dendritic PLL, these dendrimers were modified with lipidic tails using standard solid-phase synthetic methods [81]. In this way, by varying the length and number of the lipid residues and the number of free amino groups on the polylysine, a library of amphiphilic dendrimers with better ability of crossing the biological membranes were obtained. These dendrimers were able to produce stable complex with an AS-ODN, inhibiting the expression of the human vascular endothelial growth factor (hVEGF). These complexes were highly efficient in transfecting human retinal pigment endothelial cells *in vitro* and reducing the hVEGF concentration at higher levels than those obtained with cytofectin. Moreover these complexes were also very effective in delivering the AS-ODN to retinal cells when injected in rat eyes and inhibit the choroidal neovascularisation (CNV) induced by krypton laser photocoagulation. Fluorescein angiography revealed a reduction in the severity of CNV two months after the injection, thus suggesting the potential of the dendrimer/AS-ODN complexes for long-term CNV inhibition. Furthermore, continual ophthalmological examinations of injected rat eyes revealed no observable sign of a toxicological effects caused by the dendrimers or their complexes [82].

3.4 Carbosilane Dendrimers

Carbosilane (CBS) dendrimers have been recently proposed as new delivery vehicles of AS-ODN and siRNA. This group of water soluble dendritic macromolecules has gained special interest because they are based on a chemically inert and lipophilic skeleton, that may help to increase the biopermeability of these systems. Amine and ammonium-terminated CBS dendrimers were prepared by alcoholysis of the chlorosilane-terminated dendrimers nG-(SiCly)x, with N,N-dimethylethanolamine and subsequent quaternization with MeI to afford dendrimers of type nG-[Si($OCH_2CH_2NMe_2$)y]x and nG-[Si($OCH_2CH_2NMe_3^+I^-$)y]x, [83]. Ammonium and amine terminated generation 2 CBS dendrimers have shown a great complexation ability, electrostatically interacting with siRNA and AS-ODNs, and forming dendriplexes with at a nitrogen/phosphate (N/P) charge ratio of 2/1.

These systems are able to efficiently protect siRNA and AS-ODNs from nucleases degradation and from albumin binding [84]. These authors reported that when bound to CBS dendrimers, the nucleic acid resulted to be protected from serum albumin binding thus allowing higher effective concentration of siRNA or AS-ODNs to be maintained in the blood stream.

Generation 2 ammonium terminated CBS dendrimer possessing 16 positive charges (G2-NN16, illustrated in Fig (**6**)), resulted to be effective vehicle of different siRNAs able to inhibit HIV replication. They were able to achieve inhibition in both HIV infected PBMC (peripheral blood mononuclear cells) and SupT1 cells (human leukemia T lymphocytes), even if at a lower degree than lipofectin and electroporation [85]. However these results are very promising, considering that a substantial problem in investigating gene therapy or RNAi in the fight against HIV

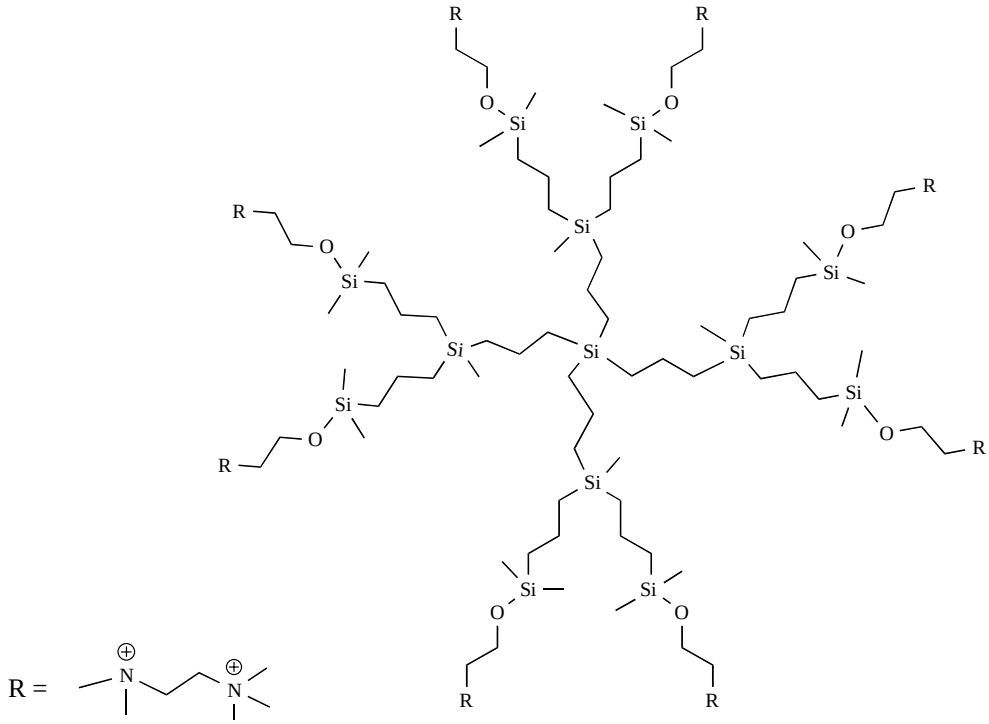

Fig. (6). Generation 2 carbosilane dendrimer.

lies in the fact that practically all HIV-susceptible cells are very difficult to transfect. The highest transfection efficiency was interestingly detected for dendriplexes formulated at N/P charge ratio of 1/1 or 2/1. For higher charge ratios not only a lower transfection efficiency was measured, but also diminished quantity of viable cells were seen due to toxic effects, as evaluated in SupT1 cells and HIV infected PBMC cells. Results from the cytotoxicity assays with these dendriplexes demonstrate their low toxicity at concentrations necessary for therapeutic treatments. However, even if they showed a low toxicity in an array of assays aimed at measuring cell viability (determined by flow cytometry), membrane rupture (LDH release), metabolic activity (MTT) and cell proliferation on T lymphocytes, the same authors have later observed multiple changes in gene expression profiles of primary cultures of human macrophages exposed to G2-NN16 at no cytotoxic doses [86]. The number of over- or under-expressed genes principally affected proliferation and transcription regulation pathways and the immune system with alterations in the immune responses.

4. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Antisense therapy has emerged as a very powerful strategy to down-regulate the expression of disease-causing proteins. However until now it was not possible to completely exploit the potential of this approach mainly due to safety and effective delivery issues. The use of targeted delivery strategies that permits systemic delivery will be a big step towards fulfilling this challenge. Moreover, decades of work on antisense oligonucleotides has provided critical technological advances that will clearly benefit the newly developed antisense strategies. From our perspective the essential requirement to antisense therapy becoming a reality, is the rational design of the systems used for the delivery of nucleic acids. We strongly believe that only with a rational approach the great number of biological barriers that hampers the clinical use of nucleic acids will be overcome. Within this context, dendritic nanotechnology may enable the construction of well-defined and versatile three-dimensional structures that may address many of these issues and therefore deliver a nucleic acid in an efficient and safe way. Indeed, different families of dendrimers have proven to efficiently deliver antisense molecules and it is expected that new dendrimer structures will continue to be developed. Furthermore, they offer a unique platform for the incorporation of specific chemical groups able to improve their gene delivery abilities, such as the inclusion of hidrophobic groups to improve the membrane permeability or the incorporation of targeting moieties to achieve a cell specific targeting. Moreover dendrimers, with their well defined structure and great versatility in their chemistry, are very promising candidates to settle and understand the basic aspects of the structure–activity relationships that will surely contribute to the rational design of more efficient delivery systems. In our opinion, the introduction of such platform is certainly altering the landscape of antisense therapy and contributing towards the development of more efficient therapeutic antisense delivery systems and helping in the future design of more sophisticated nanocarriers. In this context the great efforts performed in the design, synthesis, formulation and *in vitro* evaluation of dendrimers as antisense delivery vehicles have evidenced their great potential. However, *in vivo* studies still remain to be performed in order to provide the final evidence of the real efficacy of these delivery systems. On the other hand, more extensive and exclusive research efforts will be needed to establish the pharmacokinetic, pharmacodynamic and toxicity data of dendrimers before achieving clinical success. For instance, safety and/or toxicity represents one of the major concerns in the design of novel delivery systems. Although toxicity problems may exist, modification of the structure should resolve these issues. In this sense, the nature of the chemical groups on the periphery of dendrimers that have contact with the surrounding media is the primary factor that controls the surface-related physico-chemical characteristics of these macromolecules. Therefore, the versatility which offer dendrimers for the transformation/ tailoring of their peripheral functionalities is an easy way to change the overall behaviour of a particular dendrimer class or to impart new properties.

Finally, as any clinical application will have to be adapted with the clinical situation, the specific disease and the chosen therapeutic strategy, ideal gene medicines are far on the horizon. However, in the near future therapeutic approaches based on the exploitation and intellingent modification of current dendrimer structures can emerge. In this sense, the advances and potentialities described for dendrimers in this review suggest that we are just scratching the surface of the potential offered by dendrimers.

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ABREVIATIONS

AS-ODN	=	Antisense oligonucleotides
siRNA	=	Small interfering RNA
RNAi	=	RNA interference
RISC	=	RNA induced silencing complex
PAMAM	=	Polyamidoamine
G	=	Generation
pDNA	=	Plasmid DNA
TAMRA	=	Tetramethyl-6-Carboxyrhodamine
BODIPI	=	Boron-dipyrromethene fluorescent dye
PPI	=	Polypropylenimine dendrimers
EGFR	=	Epidermal growth factor receptor
MeI	=	Methyl iodide
MeCl	=	Methyl chloride
DTBP HCl	=	Dimethyl-3-3′-dithiobispropionimidate-

NHS	=	O-[2-(N-succinimidyloxycarbonyl)-ethyl]-O′-methylpolyethylene
MAL–PEG –NHS	=	α-maleimide-ω-N-hydroxysuccinimide ester poly(ethylene glycol)
LHRH	=	Luteinizing Hormone-Releasing Hormone
PLL	=	polylysine
hVEGF	=	human vascular endothelial growth factor
CNV	=	choroidal neovascularisation
CBS	=	carbosilane

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