Dendrimers as DNA Carriers

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1 Introduction

Recently, numerous polycations have been used for formulating gene and oligonucleotides (ODN) into complexes now termed "polyplexes", which efficiently transfect cells (Wagner 2004). Polycations include histones, polylysine, polyethyleneimine (PEI), polypropyleneimine (PPI), cationic dendrimers, poly(2-(dimethyl-amino)ethyl methacrylate) and chitosan (De Smedt et al. 2000). The properties of these polymers and their uses in transfections have been reported previously (Abdallah et al. 1995; Garnett 1999; Ruponen et al. 2003).

Of the various polymers for gene transfer, dendrimers are highly branched three-dimensional macromolecules with well-defined structures constructed around a multifunctional central core (Tomalia et al. 1985). They have novel structural properties such as a single molecular weight, a large number of controllable peripheral functionalities and a tendency to adopt a globular shape (Tomalia et al. 1990). Indeed, they differ from classical monomers, oligomers and hyperbranched polymers. There are two synthetic approaches that have been used for the preparation of dendrimers: the divergent approach and the convergent approach (Grayson and Frechet 2001).

There are now more than fifty families of dendrimers, each with unique properties, since the surface, interior and core can be tailored to different sorts of applications (Klajnert and Bryszewska 2001). Table 1 summarizes the various types of cationic dendrimers and their conjugates for gene and ODN transfer. Polyamidoamine (PAMAM) starburst dendrimers (Bielinska et al. 1995; Haensler and Szoka 1993), partially hydrolyzed (degraded) PAMAM dendrimers (Tang et al. 1996), PPI dendrimers and phosphorous dendrimers (Loup et al. 1999) have been used for their delivery. The potential use of polylysine dendrimers for gene delivery has been reported as well (see the chapter by T. Niidome, this volume).

Of the various cationic dendrimers, PAMAM starburst dendrimers have steadily grown in popularity in the past decade in a variety of disciplines, ranging from materials science to biomedicine. Indeed, PAMAM starburst dendrimers are safe, nonim-

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Table 1. Various dendrimers and their conjugates for gene and oligonucleotide transfer

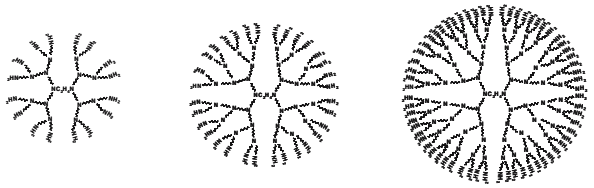
Dendrimers
Amphipathic asymmetric dendrimers
Cyclic core dendrimers
Degraded polyamidoamine (PAMAM) dendrimers (SuperFect)
Hydroxyl-terminated polyamidoamine dendrimer
Phosphorus dendrimers
PAMAM starburst dendrimers
Poly(ethylene glycol)-block-poly(L-lysine) dendrimers
Polypropyleneimine dendrimers
Polypropyleneimine (PPI)-DAB dendrimers
Dendrimer conjugates
PAMAM dendrimers conjugated with the fluorescent dye Oregon green 488.
PAMAM starburst dendrimer conjugate with cyclodextrins
Dendrimer conjugates with antibody

munogenic and can function as highly efficient cationic polymer vectors for delivering genetic material into cells (Esfand and Tomalia 2001). They have been shown to be more efficient and safer than either cationic liposomes or other cationic polymers for in vitro gene transfer (Gebhart and Kabanov 2001).

The major structural differences in PAMAM dendrimers relate to the core molecule, either ammonia (NH_3), as trivalent initiator core, or ethylenediamine (EDA), as a tetravalent initiator core, which dictates several structural characteristics of the molecule, including the overall shape, density, and surface charge (Tomalia et al. 1990). As shown in Fig. 1, the size of the PAMAM starburst dendrimer (EDA) is generally determined by the number of layers or generations present in the polymer (Klajnert and Bryszewska 2001). Each additional layer of a PAMAM dendrimer enlarges the size of the molecule by approximately 1 nm while doubling the number of surface amine groups. At the present, PAMAM dendrimers (Sigma-Aldrich (St. Louis, MO) and Dendritech Nano Technologies (Mount Pleasant, MZ)) and PPI dendrimer or Astramol (Sigma-Aldrich (St. Louis, MO) and DSM Fine Chemicals (Heerlen, Netherlands)) are commercially available (Cloninger 2002). In addition, the degraded PAMAM dendrimer Superfect can be obtained from Qiagen (Hilden, Germany).

2 Transfection Efficiency

Both the gene transfer activity and the cytotoxicity of PAMAM starburst dendrimers are significantly generation dependent. In general, the transfection activity of dendrimers with a high generation number is likely to be superior to those with a low generation number (Zhang and Smith 2000). For example, in the case of the PAMAM starburst dendrimers, optimum gene transfer is obtained with a molecular mass in excess of 20 kDa (Tang et al. 1996). In the presence of DEAE-dextran, in Rat2 cell line, a definitive relationship between the PAMAM dendrimer generation (G) and the transfection efficiency was observed, whereas an exponential increase in transfection efficiency was seen by increasing the generation number from G5 to G10, with a plateau in activity after G8 (Kukowska-Latallo et al. 1996). In addition, maximal trans-



Generation 2 **Generation 3** **Generation 4**

$\sim\sim$ $=-C_2H_4CONHC_2H_4-$

Generation	Molecular weight	Measured diameter (nm)	Surface groups
0	517	1.5	4
1	1,430	2.2	8
2	3256	2.9	16
3	6909	3.6	32
4	14215	4.5	64
5	28826	5.4	128
6	58048	6.7	256
7	116433	8.1	512
8	233383	9.7	1024
9	467162	11.4	2048
10	934720	13.5	4096

Fig. 1. Physical characteristics of polyamidoamine (PAMAM) dendrimers (ethylenediamine core)

fection efficiency was obtained using G6 (NH_3) PAMAM starburst dendrimer rather than higher-generation dendrimers (Haensler and Szoka 1993), possibly due to the rigid structure and cytotoxicity of dendrimers with a generation number >G7. Thus, the use of dendrimers as gene delivery agents has largely been focused on high-generation (>G5) PAMAM dendrimers. However, there are a few reports on the use of PPI dendrimers containing 100% protonable nitrogens for gene delivery (Zinselmeyer et al. 2002). For example, lower-generation (G2) PPI dendrimers have been shown to be effective gene-transfer agents (Zinselmeyer et al. 2002). The potential use of PPI as a carrier of ODNs has been demonstrated as well (Santhakumaran et al. 2004).

Szoka et al. developed an activated PAMAM dendrimer, Superfect (Tang et al. 1996), which represents a new class of transfection reagents based on activated-dendrimer technology, in which some of the branches have been removed. Activated dendrimers assemble DNA into compact structures. Indeed, Superfect has been shown to enhance transfection activities due to the increased flexibility of the fractured dendrimers, which enable them to be compact when forming complexes with DNA and to swell when released from DNA (Hudde et al. 1999, Tang et al. 1996).

Recently, dendrimers have been reported to be able to transfer various types of DNA, including Epstein-Barr virus (EBV)-based plasmid vectors containing the

EBNA1 gene and oriP (Maruyama-Tabata et al. 2000) and a 60-Mb mammalian chromosome into murine and hamster cells (de Jong et al. 2001).

3 Factors Affecting Transfection Activity

There are various barriers to transfection by non-viral vectors, e.g. physicochemical properties, enzymatic stability, cellular association, endosomal release, cytoplasmic translocation, nuclear uptake, localization in the nucleus, transcription activity, epigenetic events and the cytotoxicity of the polyplexes (Nishikawa and Huang 2001; Pitkanen et al. 2003). In the following sections, each factor influencing the transfection activity of the polyplexes together with dendrimers and their conjugates is described.

3.1 Physicochemical Properties

Dendrimers offer many advantages for the in vitro transfection of cells. The DNA/dendrimer complexes are very soluble in most aqueous solutions and stable for many weeks in solution, mediating high-efficiency transfection in a wide variety of cell lines, including primary cells and non-adherent cell lines (Kukowska-Latallo et al. 1996).

In general, the size and surface charge of DNA complexes with cationic polymers determine the transfection efficiency (Bielinska et al. 1996; Kukowska-Latallo et al. 1996). For example, the size of the ODNs/DNA is an important factor in optimizing the efficiency of the dendrimer, and the great majority (>90%) of transfection activity is carried out by low-density and soluble complexes, which represent approximately only 10–20% of total complexed DNA (Bielinska et al. 1999). However, large polyplex aggregates have been found to be more active in transfection compared with small particles (Ogris et al. 1998). Thus, the relationship between size and transfection activity of DNA complexes with dendrimers is far from being resolved (Gebhart and Kabanov 2001), and size dependency cannot be generalized, but may instead be specific for each transfection reagent (Kiefer et al. 2004). Using atomic force microscopy, DNA nanoparticle formation in the presence of PAMAM dendrimers with some degree of uniformity was observed (Choi et al. 2004), although lipoplexes were reported to have a heterogeneous morphology. Therefore, polyplexes seem to have properties that are advantageous for gene transfer. Further elucidation of the relationship between the size and the morphology of the DNA complex with dendrimers, and of the effect on transfection efficiency is required.

Dendrimers protect DNA degradation by DNase through complexation, indicating that the therapeutic success of gene delivery depends on the availability and retention of intact DNA within the cell for prolonged periods of time (Santhakumaran et al. 2004). Taken together, the physicochemical properties of DNA complexes with dendrimers for DNA delivery are preferable to those of lipoplexes.

3.2 Cellular Uptake Mechanism

Cell transfection is a complicated multistep process that, in many cases, appears to be mediated by endocytosis, and there is evidence that exit from the endosome is the step that controls transfection efficiency.

The uptake mechanisms of DNA complexes with dendrimers are unlikely to be simple. While previously it was thought that polyplexes enter cells through adsorptive endocytosis (Thomas and Klibanov 2003; Lechardeur and Lukacs 2002; Hollins et al. 2004; Jevprasesphant et al. 2004), recent evidence suggests that they enter cells via a raft-dependent pathway (Manunta et al. 2004). Importantly, the different membrane lipid compositions, the pH and intracellular trafficking between endosomes and caveosomes have been reported to play important roles in transfection and may explain differences in transfection efficiencies (Bathori et al. 2004; Nichols 2003; Parton and Richards 2003). Since endocytotic mechanisms are cell-dependent in most cases, further investigation regarding the endocytotic mechanism of DNA complexes with dendrimers in a variety of cell types is needed.

3.3 Endosomal Release

Endosomal escape of DNA complexes with non-viral vectors is critical for gene transfer, and various membrane-active agents have been reported (Wagner 1998). The high transfection efficiency of dendrimers may not only be due to their well-defined shape but may also be caused by the low pK of the amines (3.9 and 6.9), which permits the dendrimer to buffer the pH change in the endosomal compartment (Klajnert and Bryszewska 2001). Thus, the enhanced transfection efficiency has been attributed to the dendrimer acting as a proton sponge similar to PEI in the acidic endosomes, leading to osmotic swelling and lysis of the endosomes/lysosomes (Tang et al. 1996; Haensler and Szoka 1993). In fact, chloroquine, a lysosomotropic drug, is ineffective in improving the transfection efficiency of DNA complexes with dendrimers (Haensler and Szoka 1993) and PEI (De Smedt et al. 2000). An alternative model for endosomal release has been proposed in which electrostatic interactions between the surface of dendrimers and charged groups on the lipid bilayers cause bending of the membrane (Zhang and Smith 2000).

3.4 Cytotoxicity

A major concern with the use of dendrimers as vectors for DNA delivery is their cytotoxicity, which may be due to the interaction between the positively charged dendrimer and the negatively charged cellular structure, especially glycosaminoglycans (heparan sulfate, hyaluronic acid and chondroitin sulfate). The toxicity of the dendrimers increases with increasing molecular size (Morgan et al. 1989), suggesting that the availability of multiple contact points between the dendrimer molecules and glycosaminoglycan is implicated in the toxicity of these molecules.

PPI dendrimers are likely to show molecular-weight-dependent cytotoxicity. For example, the cytotoxicity for B16F10 cells increased in the order of G2 < G3 = G4 dendrimers (Malik et al. 2000). The toxicity of PPI dendrimers against a human epidermoid carcinoma cell line has also been shown to be molecular weight-dependent (Zinselmeyer et al. 2002), but is lower than that of PEI in Vero cells (Lim et al. 2002a). Interestingly, SuperFect may be much better tolerated at longer exposure times of cells to the DNA complexes at any confluency (Gebhart and Kabanov 2001). It is important to note that the cytotoxicity of polyplexes appears to be lower than that of lipoplexes.

The mechanism of cytotoxicity resulting from polycations is not yet fully understood, although cationic lipids induce apoptosis (Aramaki et al. 1999). The cell death

induced by PEI and its DNA complexes shows features of necrosis, as evidenced by an early membrane leakage without changes in nuclear morphology (Fischer et al. 2003).

3.5 Ex Vivo and In Vivo Applications

There are a few reports regarding the ex vivo and/or in vivo applications of DNA complexes with cationic polymers. DNA/dendrimer complexes can mediate gene transfer into murine cardiac transplants ex vivo (Qin et al. 1998). Billinska et al. (2000) demonstrated local application of the surface coating or incorporation of dendrimer/DNA complexes into poly(DL-lactide-co-glycolide) or the use of collagen-based biocompatible membranes as a possible means to facilitate transfection of dermal cells. Likewise, not only efficient adventitial gene delivery to rabbit carotid artery with plasmid complexes containing fractured PAMAM dendrimer (G6) (Turunen et al. 1999) but also intravascular and endobronchial DNA delivery to murine lung tissue using PAMAM starburst dendrimer (G9) were reported (Kukowska-Latallo et al. 2000). Moreover, applications of DNA complexes with dendrimers in tumor therapy have also been reported. The efficacy of dendrimer-mediated angiostatin and TIMP-2 gene delivery on the inhibition of tumor growth and angiogenesis after intratumor injection of these DNA complexes with dendrimers was demonstrated (Vincent et al. 2003). Intraperitoneal tumor targeting and imaging of intraperitoneal tumors by use of antisense oligo-DNA complexed with dendrimers and/or avidin in mice were reported (Sato et al. 2001).

From the in vivo safety point of view, attention should be paid to the occurrence of side effects. PAMAM dendrimers are not immunogenic or carcinogenic, enhancing their potential as vectors for an in vivo gene transfer system (Roberts et al. 1996), but dendrimers and PEI appear to activate the complement system after in vivo administration (Plank et al. 1996).

4 Combining Dendrimers with Other Strategies

Several strategies to enhance the gene expression of non-viral vectors are currently under development, e.g. the application of helper and pH-sensitive lipids, endosome-disruptive peptides, nuclear proteins, and nuclear localization signals (Davis 2002, De Smedt et al. 2000). For example, electroporation (Wang et al. 2001) and cyclodextrins (CDs) (Roessler et al. 2001) have been combined with DNA-dendrimer systems. Electroporation caused significant increases in the gene expression ability of a DNA: dendrimer-containing solution compared with direct injection plus electroporation, suggesting that the former allows plasmid vectors to directly enter a multitude of cell types (Wang et al. 2001). The addition of amphoteric or sulfonated β-CDs to PAMAM dendrimer (G5 EDA) caused the formation of smaller and more monodisperse particles, leading to an increase in the transfection efficiency of the dendrimer. By contrast, the potential use of a ternary complex of PPI-(1,4-diaminobutane) dendrimer, DNA, and cucurbituril as an example of a spontaneously assembled supermacromolecular gene delivery carrier was reported (Lim et al. 2002b). Other reports have shown that the combination of DNA/dendrimer complexes and surfactants improves gene transfer activity, e.g. the synthetic lung surfactant Exosurf and its component, tyloxapol, constitute a powerful enhancer for dendrimer-mediated gene transfer in vitro owing to

improved complex internalization and intracellular release from endosomes rather than an increase in membrane permeability (Kukowska-Latallo et al. 1999).

The translocation of polyplexes into the nucleus is a critical step in gene expression, but gene delivery by the polyplex is limited by inefficient transfer of DNA from the cytoplasm to the nucleus. In order to improve transfer, Ritter et al. investigated the effect of combining a tetramer of the nuclear localization signal of the SV40 large-T-antigen peptide with DNA:dendrimer complexes. The combination resulted in a strong increase in transfection efficiency, indicating that the peptide mediates nuclear accumulation of transfected plasmid DNA (Ritter et al. 2003).

The potential uses of the combination (hybrid vectors) of viral vectors and dendrimers have been discussed (Schmidt-Wolf and Schmidt-Wolf 2003), e.g., reciprocal enhancement of gene transfers by combinatorial adenovirus transduction and plasmid DNA transfection in vitro and in vivo (Bonsted et al. 2004). Another example is efficient large-scale production and concentration of HIV-1-based lentiviral vectors for use in vivo (Coleman et al. 2003). Thus, the combination of dendrimers and other DNA transfer methods may be effective for the delivery of genes and ODNs.

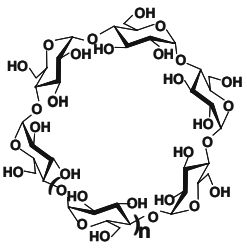
5 Dendrimer Conjugates

Significant advances have been made in the synthesis and study of dendrimers with sugars (Cloninger 2002) and peptide (Sadler and Tam 2002) have recently been made. Table 1 summarizes the various types of dendrimer conjugates. Conjugating the fluorophore Oregon green 488 with a G5 dendrimer (G5) yielded a much better delivery agent for antisense compounds than unmodified dendrimer, possibly due to an increase in endosomal escape (Yoo and Juliano 2000). The use of CD-conjugated PAMAM dendrimers has also been described (Fig. 2). Arima et al. (2001) prepared PAMAM starburst dendrimers (G2, G3 and G4) conjugates (CDE conjugates) with CDs. As measured by luciferase gene expression using CDE conjugates with α-, β- or γ-CD, the transfection efficiency of PAMAM dendrimers (G2) functionalized with α-CD (α-CDE conjugate) was about 100 times higher than that of unfunctionalized PAMAM or non-covalent mixtures of PAMAM and α-CD (Arima et al. 2001). Of various α-CDE conjugates, an α-CDE conjugate (G3) with a degree of substitution of 2.4 had the best transfection efficiency as well as low cytotoxicity (Kihara et al. 2002, 2003). Moreover, the α-CDE conjugate was induced gene expression in the spleen after intravenous injection of the DNA-complex-containing suspension (Kihara et al. 2003). In addition, polyethylene glycol functionalization of PAMAM dendrimers (G5) produced a 20-fold increase in transfection efficiency using plasmid DNA relative to partially degraded PAMAM dendrimers (Luo et al. 2002). Dendrimer conjugates with antibody for cell-specific DNA delivery have also been demonstrated (Li et al. 2000). Thus, the conjugation of dendrimers with hydrophobic and hydrophilic compounds or antibodies is of interest.

6 Conclusion

This review has focused on the potential use of dendrimers, the combination of dendrimers with other transfection techniques, and the use of dendrimer conjugates for DNA and ODN delivery in vitro and in vivo.

(A)



CyDs	Molecular weight	Cavity diameter (Å)	Inclusion affinity with membrane component
n = 1, α-CyD	972	4.7 ~ 5.3	Phospholipids
n = 2, β-CyD	1135	6.0 ~ 6.5	Cholesterol
n = 3, γ-CyD	1297	7.5 ~ 8.3	

(B)

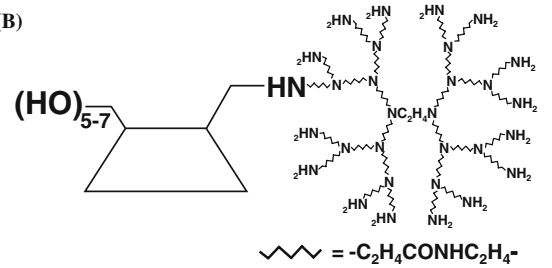


FIG. 2. Chemical structures **A** of α-, β- and γ-cyclodextrins and **B** PAMAM starburst dendrimer (G2) conjugate with α-, β- and γ-cyclodextrins

Recently, other biologically functional small nucleic acids, such as small interfering RNA and micro RNA, have been developed for clinical applications. Dendrimers may be useful as carriers for these small RNAs. In fact, we have shown the use of α-CDE conjugates as a carrier for siRNA in vitro.

Currently, there are no clinical trials using either dendrimers or other cationic polymers, despite the large number of in vitro and in vivo studies showing the potential applications of DNA complexes with dendrimers. The most important reason is the low efficiency of gene transfection, in vitro and in vivo, obtained with polyplexes. Thus, a more rational and intelligent molecular design of dendrimers and their conjugates is needed in order to improve transfection efficiency and to minimize cytotoxicity and side effects. In addition, the pharmaceutical technological aspects should be emphasized so that the carriers are safe for clinical use.

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