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Dendrimer Nanocarriers as Versatile Vectors in Gene Delivery

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ABSTRACT

The successful delivery of nucleic acids to particular target sites is the challenge that is being addressed using a variety of viral and non-viral delivery systems, both of which have distinct advantages and disadvantages. Non-viral vectors offer the advantage of safety and flexibility over viral vectors, although they lack efficiency. Dendrimers are novel, three dimensional polymers that have the ability to interact with various forms of nucleic acids such as plasmid DNA, antisense oligonucleotides and RNA to form complexes that protect the nucleic acid from degradation. The interaction between the dendrimers and the nucleic acids is purely electrostatic where the cationic dendrimer condenses the anionic nucleic acids. Because cell membranes are negatively charged, the net positive charge of the dendrimer nucleic acid complex determines the transfection efficiency, although highly cationic systems are also cytotoxic. The nature of the dendrimer nucleic acid complex depends on various factors like stoichiometry, concentration of dendrimer amines and nucleic acid phosphates, as well as bulk solvent properties like pH, salt concentration, buffer strength, and dynamics of mixing. This article aims to review the role of dendrimers as novel gene delivery vectors both in-vitro and in-vivo. Dendrimer based transfection reagents have become routine tools for in-vitro transfection, but in-vivo delivery of therapeutic nucleic acids remains a challenge.

INTRODUCTION

Gene therapy is an approach that aims to cure inherited and acquired diseases by correcting the overexpression or under-expression of defective genes. The success of gene therapy is largely dependent upon the development of a vector that delivers and efficiently expresses a therapeutic gene in a specific cell population. Gene therapy protocols were originally designed to correct inheritable disorders, such as adenosine deaminase deficiency, cystic fibrosis, Gaucher's disease, and Duchenne muscular dystrophy. However, gene therapy has been considered more recently as a promising tool for treating acquired diseases

such as cancer and acquired immunodeficiency syndrome (AIDS) virus after elucidation of the genetic basis of such diseases came to the fore [1,2]. Of the two potential strategies for gene therapy, the *ex-vivo* and the *in- vivo*, the *ex-vivo* strategy allows a wider range of therapies, although there are limitations to its practicality in humans. In this type, cells would be extracted from patients, transfected with the therapeutic gene, grown in culture, and reimplanted in the patient. In the *in-vivo* approach, genes are to be administered to the patient to transfect the cells *in-vivo* [3]. To administer a therapeutic gene (genetic medicine) into the body of the patient, a delivery system is required. These medications include gene therapy, DNA vaccination, ribozymes, and antisense oligonucleotides. In gene therapy, successful DNA transfer results in the production of therapeutic protein that is encoded by the transgene. Most gene therapy experiments and clinical trials use viral vectors for gene delivery. The safety concerns (immunogenicity, oncogenicity, etc.) and difficult large-scale production limits the usefulness of recombinant viral vectors [4,5]. An attractive alternative would be to prepare nonviral delivery systems without immunogenicity and other problems of safety while retaining the efficiency of viruses: a feat that has yet to be universally achieved.

1.1 Gene delivery by nonviral vectors: delivery barriers:

The biodistribution or pharmacokinetics of a drug is determined by its interaction with the body, a factor that relies on both physicochemical and biological properties. Therefore, drug targeting can be achieved by altering the physicochemical properties of a drug with the aid of drug delivery systems. For low-molecular-weight drugs as well as biologically active proteins, drug targeting has been achieved by controlling the physicochemical properties of any given drug-carrier complex (or conjugate) such as the particle size, molecular weight, and surface charge, and/or by using a specific ligand such as monoclonal antibodies or carbohydrates [6,7]. Plasmid DNA can be delivered to target tissue/cells employing a similar strategy. Plasmid DNA is a large polyanion that readily forms electrostatic complexes with cationic nonviral vectors, and there are many complex factors that influence the biodistribution of the administered delivery system; this determines the ultimate fate of the DNA. Therefore, efficient and target-specific gene transfer is difficult to achieve. The route of administration determines the number of barriers that need to be overcome for successful in vivo gene transfer by a nonviral vector. In addition to these delivery barriers, an ideal nonviral vector and its therapeutic cargo should be stable in the test tube as well as in the body, being biodegradable, nontoxic, and nonimmunogenic.

1.2 Physicochemical properties:

Cationic lipids and polymers form complexes with DNA via electrostatic interaction. When formed, a DNA complex has its unique particulate characteristics depending on the properties of the vector used, the mixing ratio, and the diluents used in the mixing protocol. Of the various properties, particle size is an important factor determining the tissue distribution process, such as passage through the capillaries (≈5 μm) and through the fenestrae between discontinuous endothelial cells (30 to 500 nm). The entrance of a particle into cells via endocytosis is also a size-limiting process [1,8]. Overall electrical surface charge of the complex greatly affects the biodistribution. Cell membranes are negatively charged due to presence of glycoproteins and glycolipids, and the negatively charged membrane is a good target for cationic complexes to induce cellular uptake. However, this nonspecific interaction is also a major hurdle for cell-specific delivery of DNA after its local or systemic administration [6].

1.3 Interaction with blood components:

Plasmid DNA or the vector-gene complex administered into the blood distributes to downstream tissues, and along the way it interacts with serum proteins and/or blood cells; its biodistribu-tion will depend on newly acquired physicochemical properties that are generally difficult to control and predict. Negatively charged proteins such as albumin are present in abundance, so they will very likely bind with cationic nonviral vectors. This has been evidenced where the efficiency of cationic liposome-mediated gene expression decreases in the presence of serum [9]. Negatively charged proteins adsorb onto the surface of the complex thereby neutralizing its charge and increasing its size, which decreases the transfection

efficiency. It has been reported that, after exposure to mouse serum, cationic lipid–DNA complexes become negatively charged, significantly increasing in size, and eventually disintegrate prematurely releasing the DNA cargo, which is rapidly degraded by serum nucleases [10,11]. The rate of disintegration depends on the lipid composition of the liposome, and should the DNA-vector complex diameter exceed 5 μm, it will result in capillary embolism of downstream tissues. In some cases, the mixing of plasmid DNA and cationic carrier in vitro resulted in large aggregation with sizes close to that of capillaries [12]. Surface modification with hydrophilic compounds like polyethylene glycol (PEG) has been shown to prevent the aggregation of the delivery system, although it has the propensity to mask the positive charge of the complex thereby hindering interaction of the complex with the cell membrane. A common approach to overcome this has been to conjugate targeting ligands to the surface of the complexes.

1.4 Recognition by the immune system:

Upon administration of plasmid DNA to humans, it is recognized as foreign material by the cells of the mononuclear phagocytic system (ie, Kupffer cells in the liver and splenic macrophages). This immune reaction is part of the innate mechanism that recognizes foreign materials and removes them from the body. CpG (cytosine-phosphate-guanine) sites are regions of DNA where a cytosine nucleotide occurs alongside guanine nucleotide in a linear sequence of bases along its length. It has been reported that an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines induces immune reactions [13]. Bacterial DNA or synthetic oligonucleotides containing such a sequence can trigger B-cell proliferation and release of several proinflammatory cytokines. Thus, CpGs have been used as adjuvants in genetic vaccination. However, this property of CpGs is highly undesirable in gene delivery where such strong immune responses have resulted in fatal consequences. In the caseof cationic liposome–DNA complexes, which show minimal toxicity in animal and clinical studies after local administration, high levels of cytokines such as interferon-γ and tumor necrosis factor-α are observed after intratracheal instillation or intravenous injection. The immune systems response to plasmid DNA is amplified by the use of cationic liposomes. These cytokines not only cause significant toxicity in the treated animals but also inhibit transgene expression [14,15]. Plasmid DNA delivered by cationic liposomes has been reported to show a higher immune response, which is beneficial for genetic immunization but undesirable in gene therapy.

1.5 Vascular permeability:

Intravascular delivery of viral or nonviral DNA complexes results mostly in gene expression in vascularly accessible cells such as endothelial cells. When the target cells are readily accessible, the complex does not need to permeate across the blood vessels. Even if the target cells localize outside of the blood vessels, the transgene product secreted from the endothelial cells to the extravascular space has been shown to reach the target. However, many other cases will require extravasation of the DNA complex. The structure of capillary walls varies depending on their resident tissue and can be divided into three general types: continuous, fenestrated, and discontinuous endothelium. DNA complexes can pass through only the vascular wall composed of discontinuous endothelial cells under normal conditions. Discontinuous endothelium exists only in the liver, spleen, and bone marrow, having gaps of 30 to 500 nm between neighboring endothelial cells, and partially or completely lacks a basement membrane. Only relatively small DNA complexes are able to readily pass through the blood vessels and directly interact with parenchymal cells [1].

The transport of the molecules across the blood vessels by improving vascular permeation can be achieved by several approaches such as preadministering hypertonic solutions or by using vasodilating agents. It has been reported that viral vector–mediated gene transfer in brain tumors or skeletal muscles is increased by the administration of bradykinin or histamine, respectively [16,17].

1.6 Uptake by target cells:

Cellular uptake of cationic DNA complexes is a nonspecific process. Because of the presence of a net

positive charge, the complex binds to the negatively charged cell membrane and is subsequently internalized by endocytosis. To improve the cell specificity of gene expression by a DNA complex, incorporation of a homing device into the complex looks promising. When galactose is attached to the surface of the complex, it is internalized by hepatocytes via the asialoglycoprotein receptor at a much faster rate than that of native cationic complexes [18]. After endocytosis, the DNA-containing particles are largely retained in perinuclear endosomes/lysosomes, which limit their transport into the cytoplasm and is one of the major barriers to eventual transfection. To increase the specificity of DNA uptake by target cells, monoclonal antibodies, other macromolecules, or low-molecular-weight ligands such as galactose have been used [19]. However, a DNA-vector complex must reach the target cells intact so that the ligand can bind with its receptors on the cell surface and internalize. Electric pulses have also been employed to facilitate cellular translocation whereby permeability of cell membranes is increased, and plasmid DNA adjacent to the cells can enter the cytoplasm by passive diffusion as well as by electrophoretic and electro-osmotic transport [20].

1.7 Endosomolysis:

Plasmid DNA taken up by endosomes is largely retained within the endosomes/lysosomes and is eventually degraded. For effective transfection to occur, early escape of the DNA from the endosomes is necessary. In one approach, fusogenic lipids are employed that fuse to and rupture the endosomal membrane releasing the plasmid. In another approach, cationic polymers like polyethyleneimine (PEI) are employed. These polymers undergo protonation in the acidic pH of maturing endosomes resulting in rapid swelling of the polymer matrix and subsequent rupturing of the endosomes and thus releasing its contents.

1.8 Cytoplasmic stability:

Plasmid DNA is rapidly degraded within the cytoplasm by cytoplasmic nucleases. The half-life of plasmid DNA within the cytoplasm is typically 50 to 90 minutes. Microinjection of free DNA directly into the nucleus can of course bypass this degradative process and results in much higher levels of gene expression than that of microinjection of DNA into the cytoplasm [21,22]. On the other hand, no detectable level of expression was obtained when a cationic liposome–DNA complex was injected into the nucleus. This suggests that the lipid coating of the DNA inhibits transcription and must be shed prior to nuclear transfer [22].

1.9 Nonviral vectors for gene delivery

Cationic liposomes

Cationic liposomes are widely explored nowadays for the delivery of DNA into eukaryotes. Several kinds of cationic lipid, such as quaternary ammonium detergents, cationic derivatives of cholesterol and diacylglycerol, and lipid derivative of poly-amines, have been developed. Some cationic lipid-DNA complexes (lipoplexes) have been used in clinical trials for the treatment of cancer and cystic fibrosis [23,24]. The addition of cationic lipids to plasmid DNA decreases its negative charge and facilitates its interaction with cell membranes. Neutral lipids such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol are generally added as “helper lipids” in cationic lipid-DNA complexes to facilitate the release of plasmid DNA from the endosome after endocytic uptake of the complex. To date, cationic lipid-DNA complexes have been used successfully to deliver plasmid DNA to the lungs, brain, tumors and skin, by local administration, or to vascular endothelial cells after systemic, intravenous injection [25-27]. Incorporation of cationic polymers, such as polylysine and protamine, into cationic liposome-DNA complex leads to a tight condensation of DNA and prevents aggregation and nuclear degradation.

Cationic polymers

High-molecular-weight cationic polymers are more effective in condensing DNA than are cationic liposomes and have also been used as a delivery system for DNA. They include poly-L-lysine (PLL), poly-

L-ornithine, PEI, chitosan, and Starburst dendrimers (Dendrimer Nanotechnologies Inc., Mount Pleasant, Michigan). These polymers enhance the cellular uptake of plasmid DNA by nonspecific adsorptive endocytosis, as cationic lipids do. PEI can mediate efficient gene transfer without the use of an endosome disruption component (viruses, fusogenic lipids, or peptides) because it enhances the release of plasmid DNA entrapped in endosome/lysosome by buffering this compartment ("proton sponge" effect). Because the biodistribution of cationic polymer-DNA complexes after systemic administration is more easily controlled than that of cationic liposome-DNA complex, active targeting of the former system to a specific population of cells is attempted. Polymers such as PLL and PEI have been covalently modified with targeting ligands including asialoglycoproteins, carbohydrates, transferrin, antibodies, and lung surfactant proteins. In addition to such targeting ligands, endosome-disrupting molecules are also tethered to these cationic polymers to increase the cytoplasmic delivery of plasmid DNA after endocytic uptake of the complex.

Dendrimers

Dendrimers have generated a great deal of interest as controlled and targeted drug/gene delivery systems due to their exceptional structural properties such as monodispersity (≈1.0), high density of peripheral functional group, and well-defined globular shape and multivalency [31-33]. Dendrimers (Figure 1) are globular, nanoscaled macromolecules with a particular architecture constituted of three distinct domains: (i) a central core that is either a single atom or a group having at least two identical chemical functionalities, (ii) branches emanating from the core, composed of repeat units having at least one junction of branching, whose repetition is organized in a geometric progression that results in a series

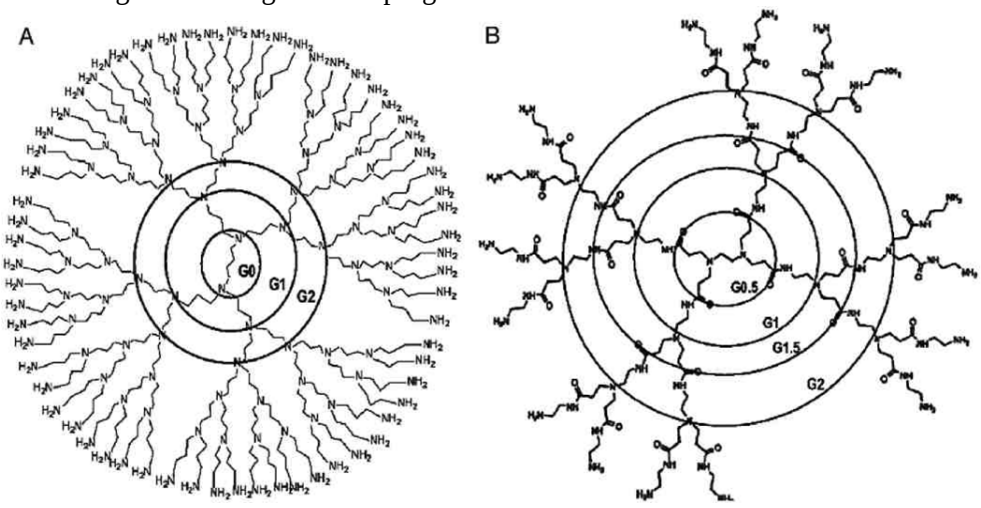

Figure 1. Schematic representation of two commercially available dendrimers: (A) PPI; (B) PAMAM. The first two generations (G0 to G2) are highlighted by concentric circles

(From Parekh HS. The advance of dendrimers: a versatile targeting platform for drug/gene delivery. Curr Pharm Des 2007;13:2837-50. Reprinted with permission from Bentham Science Publishers Ltd.).

of radially concentric layers called generations (G), and (iii) many identical terminal functional groups, generally located in the exterior of the macromolecule, which play a key role in their gene-complexing or drug-entrapping ability.

2.1 Physicochemical properties:

Because of their molecular architecture, dendrimers show some unique physical and chemical properties, which make them particularly interesting for drug and gene delivery applications. In contrast with linear polymers, the intrinsic viscosity of dendrimer solutions does not increase linearly with mass but shows a maximum at a specific generation [34,35]. This is likely to be because of the way in which dendrimer shape changes with generation (ie, lower generations adopt a more open planar-elliptical shape with transition to a more compact spherical shape for higher generations). The compact shape also reduces the likelihood of

entanglement, which affects larger classic polymers. Because of the unique structure of the dendrimers, the molecular density is theoretically highest at the periphery where the largest numbers of surface functional groups reside. However, back-folding of the terminal branches toward the core leads to a more uniform and reverse density profile [36]. The actual conformation for dendrimers, however, depends on the nature of the solvent; for example, G3 (third generation) poly(propyleneimine) (PPI) dendrimers display an extended conformation (Figure 2) in a good solvent such as water (polar) or chloroform (acidic), whereas they display a poor, inwardly crowded

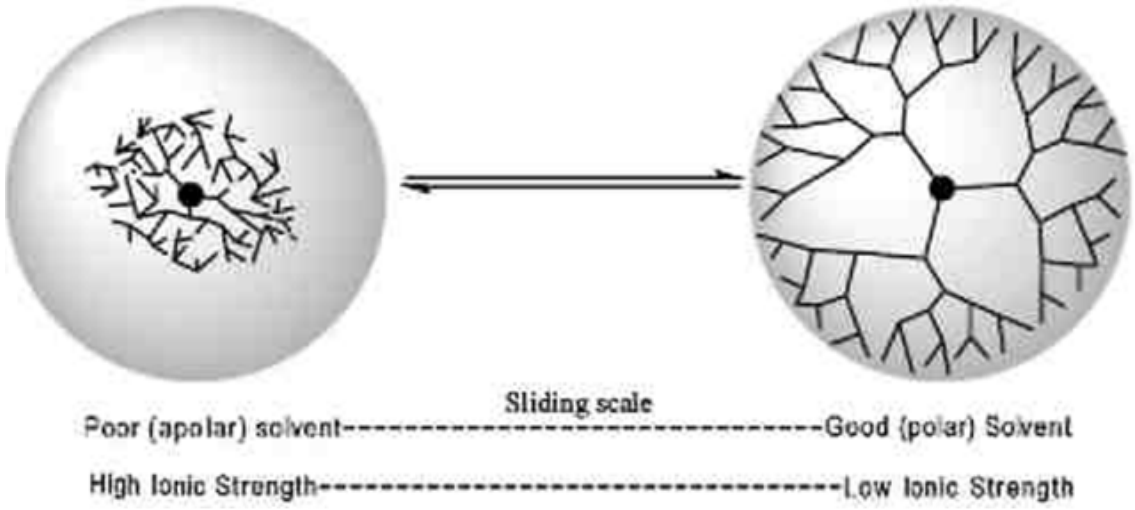

Figure 2. Conformation of dendrimers in polar and apolar solvents

conformation in a poor solvent like benzene (nonpolar) [37,38]. The presence of numerous terminal groups facilitates multiple simultaneous interactions with solvents, surfaces, or other molecules and, as a consequence, dendrimers tend to show high solubility, reactivity, and binding. This multivalency is of general importance for biological interactions but may be of particular importance for biomedical applications, as the multimeric binding through statistical and/or cooperative effects can increase affinity, avidity, and specificity of binding. Statistical effects are those involving numerous binding entities on a multimeric ligand that are also associated with a single binding site on a given receptor. These effects allow a multimeric ligand to bind a receptor based on its increased local effective concentration thereby increasing the binding affinity. The multiple interactions between surface amines and nucleic acid phosphates are also important for the formation of dendrimer-DNA complexes [39,40]. Furthermore, the multiple surface groups can be derivatized simultaneously to afford systems tethered to targeting ligands or hydrophilic copolymers (eg, PEG) for steric stabilization.

2.2 Toxicologic properties:

Dendrimers with free amine groups at the periphery are reported to show concentration-dependent and generation number–dependent toxicity; this has without doubt limited their clinical application *in-vivo* [41-43]. Many functionalization strategies have been employed to mask the terminal amino groups and thereby reduce the inherently associated positive charge and resultant cytotoxicity. Functionalization of dendri-mers has also been found to impart many other properties beneficial in gene/drug delivery, including modification of their physicochemical properties to make them more suitable for a proposed particular application, enhancing the peripheral congestion to improve container properties, and attachment of targeting groups to the periphery. Hemolytic toxicity and cytotoxicity of polyamidoamine (PAMAM) and PPI dendrimers have been widely reported. Both PAMAM and PPI dendrimers are extremely toxic to red blood cells, causing their rupture and release of hemoglobin even at a concentration as low as 10μg/mL [41,44,45]. Surface functionalization was found to reduce the hemolytic activity and cytotoxicity of PPI

dendrimers [45-49]. Cytotoxicity of dendrimers is directly attributed to the number of terminal amino groups and the positive charge density. Hence, masking of the amino groups by surface functionlization leads to a decrease in cytotoxicity. Both PAMAM and PPI dendrimers and their surface-functionalized derivatives have been reported to be nonimmunogenic [41,48]. Toxicologic investigations in vivo on surface-engineered G5 PPI dendrimers versus native PPI dendrimers showed the latter predominately accumulate in the liver and cause damage to the liver and hemopoietic system, whereas functionalized dendrimers are devoid of such toxicity [50].

2.3 Dendrimer-based gene delivery:

Complexation of dendrimer with nucleic acids

Dendrimers interact with all forms of nucleic acids such as DNA, RNA, and antisense oligonucleotides by electrostatic interaction to form complexes, which condense/compact the nucleic acid. During complexation, the extended configuration of the nucleic acid is changed and a more compact configuration results, with the cationic dendrimer amines and the anionic nucleic acid phosphates reaching the local charge neutralization resulting in the formation of dendrimer-nucleic acid complexes ("dendriplexes"). The nature of the complex is not only dependent on the stoichiometry and concentration of the DNA-phosphates and dendrimer-amines but also on the bulk solvent properties (eg, pH, salt concentration, buffer strength) and even the dynamics of mixing. High ionic strength (ie, increased amounts of NaCl) both interferes with the binding process and appears to help to establish equilibrium. The medium in which complexes are formed not only affects their morphology but also modifies other properties and even stability *in-vivo* [51-53]. With each increasing dendrimer generation, the number of surface amine groups, which are most likely to bind DNA, effectively doubles. This also affects the nature of complexes formed by the different generations; a model for the binding of PAMAM dendrimers to DNA has been put forward that explains the observation of increased binding with higher generation dendrimers (G7 vs. G2, G4). A recent study suggests that smaller dendrimers (ie, PAMAM G2), which do not induce a wrap around, may in fact bind DNA relatively better than the larger PAMAM G6, potentially because of the more fluid structure of these smaller dendrimers. Higher-generation PPI dendrimers in higher concentrations form water-soluble dendriplexes, whereas the G1 and G2 PPI dendrimers lead to the formation of electroneutral complexes, even at dendrimer: DNA charge ratios greater than 1. The higher-generation dendrimers are able to produce charged soluble complexes because of their ability to form "over-stoichiometric" (ie, >1) complexes with a net positive charge. Initial studies of DNA complexes formed by PAMAM dendrimers found that their morphology was quite similar to complexes formed with other cationic polymers such as PLL or PEI. In all cases, the formation of toroidal structures (Figure 3) of around 45 nm was observed. PLL and intact PAMAM dendrimer-based complexes, in particular higher-generation dendrimers, were found to have a tendency to form clusters rather than distinct units, in contrast with those complexes observed for the PEI and fractured PAMAM. Complex size tended to decrease with increasing polymer: DNA ratio for the fractured PAMAM dendrimer [55].

Transfection by dendriplexes

The binding of cationic DNA complexes to the cell membrane is in general based on an initial electrostatic attraction between the cationic complex and negatively charged cell surface moieties. The complexes once taken up by endocytosis depend on timely endosomal escape mechanisms to be able to reach the cytoplasm and traffic to the nucleus. Eukaryotic cell membranes contain many lipids (glycerolipids, sphingolipids, cholesterol) as well as proteins, and the physical properties of biological membranes are linked to their lipid composition. The difference between lipids such as their melting temperature and nature of their aliphatic chains leads to the formation of glycerol- or sphingo-domains in which the phospholipids have either rapid lateral or rotational diffusion or limited mobility. Cholesterol plays a role in endocytosis, tends to partition with sphingolipids to occupy the free space between acyl chains, and therefore promotes the formation of liquid ordered domains [56,57]. Microdomains of cholesterol and sphingolipids in the exoplasmic leaflets of the plasma membrane are commonly termed "membrane rafts," being characterized by their resistance to solubilization with non-ionic detergents. It has been reported that cholesterol and membrane rafts are

involved in transmembrane trafficking, signaling, protein and lipid sorting, bacterial infection, as well as binding and internalization of viruses [58-60]. It has been observed that depletion of plasma membrane cholesterol profoundly affects the gene delivery mediated by dendriplexes and that membrane rafts are involved in the cellular uptake of dendriplexes. Although cholesterol is involved in both binding of the dendrimers to a cell surface and endosomal escape, the most important effect is on dendriplex-internalization [61]. Reports also suggest that caveolae-mediated uptake is the main route of internalization of dendriplexes in cells expressing caveolae. There is now good evidence supporting the importance of dendrimer buffering capacity to act as a proton sponge and facilitate efficient endosome disruption. The high buffering capacity of polymers such as PEI and PAMAM leads to a decelerated acidification of the endosome, an increased accumulation of osmotically active Cl^-, and induces a significant (140%) increase in endosome volume [62]. The notion that the amount of dendrimer is not only important in creating the excess positive charge, which supports cellular association and uptake, but also for the intracellular trafficking process is also supported by data for cyclodextrin-dendrimer complexes.

The transfer of DNA from the cytoplasm to the nucleus is the critical final step in the process leading to transfection. Fluorescence microscopy of dendrimer-AS complexes [Oregon green conjugated $PAMAM_{G5}$ and TAMRA (tetramethyl-6-carboxyrhodamine) labeled antisense (AS) oligonucleotide] suggests that the dendrimer itself has the ability to accumulate to some extent in the nucleus; this is also the case for PEI [63]. The major difference between lipidic gene delivery systems and polymeric systems lies in their intracellular process. Whereas for lipid-based systems dissociation of the complex at the level of the endosome seems to be obligatory, this does not necessarily hold true for polymeric systems, which appear to have at least some activity even when still complexed. Specifically $PAMAM_{G5}$ dendrimer AS oligonucleotide complexes seemed to be active although a large proportion of AS in the nucleus seemed to be still complexed [64]. However, an early study suggested that PAMAM dendrimers do inhibit the initiation of transcription in vitro but did not affect the elongation of the RNA transcript [65].

The first report of the use of Starburst PAMAM dendrimers as transfection agents demonstrated that these agents could efficiently induce expression of reporter genes in adherent and suspension cell cultures with the G6 (NH_3-core) dendrimer having optimum efficiency of all those trialed [65]. Relatively small dendrimer-DNA complexes with a significant excess of positive to negative charge (6:1) were most efficient but were strongly and adversely affected by the presence of serum [65]. Interestingly, it was also demonstrated that these materials, in contrast with PLL, were not dependent on the presence of lysosomotropic agents, suggesting that they had an intrinsic ability to escape from the endosome. The authors suggested that this ability may be related to the ability of the internal dendrimer amine groups to buffer pH changes in the endosome [65-67]. This has been proposed as a general mechanism that facilitates escape from the endosome because of the accumulation of Cl^- counterions and subsequent osmotic swelling of the endosome [68]. This hypothesis has also been supported by recent experiments that studied the effect of various polyamines on endosome swelling. Although PAMAM Starburst dendrimers of generation G3 to G10 were found to form stable complexes with DNA, their ability to transfect different cell lines varied considerably. Overall, the higher-generation dendrimers (G5 to G10) were found to be of superior efficiency, showing a near exponential increase of efficiency with generation in Rat2 cells [69]. In some cell lines, the ability to create stable clones was also quantified and found to be of the order 10^{-3} to 10^{-5}. The nature of the core, ammonia (NH_3) or ethylenediamine (EDA), was found to be less significant, highlighting the greater importance of the surface functionalities in the nature of the complex. This may, however, be less clear for smaller dendrimers where access to the core groups is sterically less restricted. The major advantage of dendrimers is their sequence independence and the ease with which even large DNA constructs can be accommodated. In a comparative evaluation of various polyplexes based on linear, branched, and dendritic polymer structures, it was demonstrated that the transfection activity between these polymers varied by three orders of magnitude [70]. The same study also indicated that factors such as incubation time of the complexes with the cells or cell density affect different polymers to a varying degree.

The ability of PAMAM dendrimers to deliver oligonucleotides is well documented [71]. $PAMAM_{G3}$ was found to increase cellular uptake of phosphorthioate oligonucleotide by a factor of 50 compared with that of oligonucleotide alone [72]. It has been demonstrated that the transfection activity of dendrimers is dramatically enhanced (N50-fold) by heat treatment in a variety of solvolytic agents (eg, water or n-butanol). Such treatment induces significant degradation of the dendrimers at the amide linkage (for PAMAM), resulting in a heterodisperse population of compounds with molecular weights ranging from as low as 1 kDa up to several tens of kilodaltons. The fragments facilitating transfection in this case are thought to be the high molecular components. The reason behind the increased transfection efficiency after the heating process is principally due to the increase in flexibility, enabling fractured dendrimers to be compacted when complexed with DNA and swell when released from DNA [73}.

2.4 Gene delivery by dendrosomes:

Dendrosomes are novel, vesicular, spherical, supramolecular entities containing the entrapped dendrimer-DNA complex. They have a significant advantage over dendrimers in that they possess negligible hemolytic toxicity, higher transfection rates, and better in vivo acceptability. The transfection efficiency and toxicity of dendrosomes composed of PAMAM and PPI dendrimers have been extensively investigated previously with promising results [44,74]. Dendrosomes prepared with PAMAM displayed superior transfection efficiency in vitro when compared with a number of other nonviral gene delivery vectors44. PPI-based dendrosomes have been employed for genetic immunization against hepatitis B, and the results indicate that dendrosomes hold great potential in DNA vaccination [74].

In-vivo gene expression

The ability of nonviral systems such as dendrimers to efficiently transfect various cells *in-vitro* has made synthetic vectors a routine tool in molecular biology. However, their impact on the translation to true genetic therapies in the clinic has to date been met with much scepticism. One impediment is that it remains a significant challenge to make valid and accurate predictions on the *in-vivo* behavior of synthetic vectors. When one considers the vastly increased complexity of the biological system that it is being introduced to along with the range of possible interactions between an array of endogenous macro-molecules and cells, this comes as no surprise. The challenge can be reduced significantly by circumventing the vascular compartment, and consequently, many applications of dendrimers in vivo have focused on their use for local or *ex-vivo* administration. Despite these challenges, evidence is emerging that dendrimer-based systems have significant potential in gene therapy *in-vivo*.

Ex-vivo gene delivery

A transfection efficiency of 6% to 10% was observed on direct application of activated PAMAM (Superfect, Qiagen Inc., Valencia, California) complex (18:1 ratio) ex vivo to rabbit and human corneas [75]. Intravitreal injection of lipid lysine dendrimers with an antisense oligonucleotide was found to inhibit neovascu-larization of the choroid by downregulation of VEGF (Vascular Endothelial Growth Factor) over a period up to 2 months [76]. Intramuscular injection of 100 μg HSV-tk (Herpes Simplex Virus Thymidine Kinase) suicide vector complexed with Super-fect PAMAM dendrimers at 3:1 ratio (wt/wt) led to a pronounced growth delay. The plasmid contained Epstein-Barr virus (EBV) sequences with the ability to replicate and persist in the nucleus of the transfected cells (carrying the EBV nuclear antigen, EBNA1, and oriP). The animals received up to four weekly cycles (single injection of complex followed by 100 mg $kg^{\sim}$ day^{-} of the prodrug ganciclovir for 6 days) [76]. The measured level of 3-gal expression of the plasmid employing the EBNA1/oriP system was eight times higher than that in a conventional plasmid, and in conjugation with a vector expressing Fas ligand, the injection of 10 *fig* plasmid complexed with dendrimers (Superfect) at a ratio of 10:1 (wt/wt) also led to a pronounced tumor growth delay. A growth delay was also demonstrated after intratumoral injection of plasmids coding for the anti-angiogenic peptide angiostatin or the tissue inhibitor of metalloproteinase (TMP)-2 genes in special dendrimers/plasmid/oligonucleotide complexes. Efficient local delivery of an In-labeled oligonucleotide to tumor cells in an intraperitoneal

tumor model was demonstrated when complexes with $PAMAM_{G4}$ were injected intraperitonealy. Intratracheal instillation of complexes, with fractured PAMAM dendrimers (Superfect), at N/P (Nitrogen to Phosphate ratio) ratio of 4.7, resulted in gene expression 130-fold lower than that for the branched PEI 25 kDa formulation (N/P 10:1). Using direct injection, in a murine cardiac transplant model, $PAMAM_G5$ dendrimer complexes demonstrated more widespread and prolonged expression compared with that of the naked plasmid, and when combined with a viral interleukin, 10 genes were able to prolong graft survival. The efficiency of the procedure was improved at a higher charge ratio of 20:1 and in combination with electroporation.

Systemic administration

Systemic administration of dendriplexes results in significant deposition within the lungs as evidenced with $PAMAM_{G9}$ complexes (200 *fig* DNA complexed with 650 *fig* dendrimers) that led to expression mainly in the lung parenchyma but not in other organs. The systemic administration of dendrimers was also explored for a $PAMAM_{G3}$ and conjugates of a-cyclodextrin with the terminal amines of $PAMAM_{G3}$. After 12 hours, the spleen was the dominant organ, and the concentration became at least one order of magnitude higher than the next highest organ, the liver.

RNA interference

Posttranscriptional gene silencing by RNA interference (RNAi) appears to be a promising approach to the targeted inhibition of gene expression *in-vitro* and *in-vivo*. Similar to its predecessors, such as antisense oligonucleotides, ribozymes, and DNAzymes, RNAi is a process by which a specific messenger RNA (mRNA) is targeted for degradation as a means of inhibiting the synthesis of encoded proteins[75]. Although the commercially available dendrimers Polyfect and Superfect are designed for the delivery of plasmids, they have also been used for the delivery of short interfering RNA(siRNA), siRNA delivered to HeLa cells using Polyfect (Qiagen Inc., Valencia California) achieved silencing of the target genes by 90% as determined by the absence of the target proteins P120RasGAP and p130Cas. Superfect-mediated delivery of siRNA achieved knockdown by more than 50% of the target Erbin protein that acts in the localization and signaling of ERBB-2 receptor in epithelia in rat pheochromocy-toma–derived (PC12) cells. More recently, the polyamine siPORT (Applied Biosystems, Carlsbad, California) has been specifically designed for siRNA delivery. A knockdown of 90% of both focal adhesion kinase (FAK) mRNA and protein was achieved when siPORT was used to deliver siRNA to human pancreatic ductal adenocarcinoma cells [76].

CONCLUSION

Dendrimer-based delivery systems have shown considerable promise as tools for the development of gene therapies. Whereas most of the applications to date have focused on their use for local or *ex-vivo* administration, it has been demonstrated that dendrimers have desirable properties that also make them attractive for *in-vivo* administration. The obstacle of safe and efficient delivery of genetic medicine largely remains, and the suitability of any gene delivery system will always have to be matched with the clinical situation, the specific disease, and the chosen therapeutic strategy. Overall, the reports available to date certainly suggest that dendrimer-based delivery systems hold great promise and potential in gene delivery.

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