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Perfectly shaped siRNA delivery

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“Dendrimers are now emerging as nonviral vectors for siRNA, the wide range of chemical variation introducing the possibility of improved, (perfectly shaped) siRNA delivery complexes in the near future.”

Dendrimers are hyperbranched macromolecules, ideally monodisperse, with a perfectly regular and globular structure in which every repeated sequence represents a higher generation molecule [1,2]. Their precise core-shell nanostructures enable various methods of designing drug formulations, which involve interior encapsulation, surface adsorption or chemical conjugation of the drug by the dendrimer [3]. Dendrimers have been employed as delivery vehicles for small-molecule drugs [4] and nucleic acids [5] in the past. Recently, the number of publications reporting siRNA delivery by dendrimer-based nonviral vectors has strongly increased. In contrast with plasmid DNA (pDNA), which needs to be transported to the nucleus for efficient transgene expression, RNA interference (RNAi) requires only that the siRNA be trafficked into the cytosol of the cell for incorporation into the RNA-induced silencing complex (RISC) [6]. However, siRNA appears to be less easily formulated than pDNA [7], and due to its susceptibility to hydrolysis by serum nucleases, the difficulty related to complexation with the carrier is a major obstacle for efficient RNAi. Serum stability of siRNA has been favorably increased by modifications at the 2' position on the sugar-phosphate backbone [8]; however, encapsulation by a carrier, which shields the

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negative charge of siRNA and renders the particles nanosized, provides better protection, uptake and biological activity.

Based on previous approaches of attempting to use vehicles for siRNA delivery that had actually been optimized for pDNA, researchers have learned that although both pDNA and siRNA are double-stranded nucleic acids, there are fundamental structural differences that primarily affect their ability to be complexed. As recently described, dsRNA is less flexible than pDNA [9,10], which can lead to incomplete encapsulation or the formation of undesirably large complexes [11]. Since the use of low-generation dendrimers (e.g., 1–3) has not consistently led to the formation of uniformly small complexes [12–14], recent studies of dendrimer-mediated siRNA delivery have typically focused on the use of high generations, such as G6 or G7 [12,14–19]. However, Morilla *et al.* showed that the dendriplex preparation in low ionic strength media could yield small dendriplexes using lower generation polyamidoamine (PAMAM) (i.e., G4–7) that was efficiently taken up by cells [16].

Chemistry

Polyamidoamine and its derivatives are certainly the best-studied dendrimers with respect to siRNA delivery, as is currently reflected by approximately 20 *in vitro* and two *in vivo* reports. While ethylenediamine cores are the most widely used [12,16,20–24], a cystamine core structure has been employed for the synthesis of so-called dendriworms by polyvalent conjugation of G4 dendrimers onto an elongated magnetic nanoparticle host [25]. In addition, Peng *et al.* synthesized PAMAM dendrimers (G4–7) with a triethanolamine core to increase flexibility [17]. Since cationic PAMAM dendrimers with amino-terminated surface groups were found to be more cytotoxic in Caco-2 cells than negatively charged PAMAMs with carboxylate-terminated surface groups [26], it was concluded that the biocompatibility of dendrimers is related to their surface charge, as well as their structure, molecular weight and generation number [27]. To reduce the cytotoxicity of positive surface charges, a certain fraction of the primary amines of PAMAM dendrimers have been acetylated [28]. Although these acetylated derivatives afforded reduced transfection efficiency, their activity could be improved by quaternization of the internal amines, which increases cationic charge at physiological pH [29]. In other approaches aimed at reducing the cationic surface charge, Minko *et al.* internally synthesized cationic and hydroxyl-terminated PAMAM G4 dendrimers [30], while Parekh *et al.* formulated dendrosomes by encapsulating PAMAM dendriplexes within a lipophilic shell that could shield the positive charges [31]. For transfection of neurons, which are especially prone to polycation-mediated toxicity, biodegradable arginine esters of PAMAM were synthesized by conjugating arginine to PAMAM-OH functionalized dendrimers by using ester linkage. The resulting e-PAM-R was found to be readily degradable under physiological conditions [22]. Juliano *et al.* conjugated the arginine-rich HIV-derived transactivator of transcription (TAT) peptide to G5 PAMAM dendrimers to increase delivery of siRNA into multi-drug resistant mouse embryonic fibroblast cell line NIH3T3 MDR cells [32]. Other PAMAM conjugates that have been employed for siRNA delivery contained α -cyclodextrin (α -CD), which was shown to have a stabilizing effect on the enzymatic degradation of siRNA [20,21,23]. For active targeting, the cyclic arginine–glycine–aspartic acid (cRGD) motif, which has a high affinity for integrin receptors, was coupled to the peripheral primary amines of PAMAM with a sulfo-LCSPDP linker [24], EGF was coupled via a triglycine spacer [18], and luteinizing hormone-releasing hormone (LHRH)-hemisuccinate was attached by reacting it directly with the peripheral hydroxyl groups of G4 PAMAM [30]. Leroux *et al.* formed PAMAM-core polyion complex micelles (PICMs) by coupling Fab-fragments to the distal end of PEG-b-P(PrMA-co-MAA) copolymers that formed the shell [33].

“The latest class of dendrimers exploited for siRNA delivery are triazine dendrimers.”

Another highly branched dendrimer terminated with high-density amino groups that is commonly used for gene delivery is polypropylenimine (PPI). PPI was first described for siRNA delivery by Minko *et al.* [34]. Minko and co-workers used conjugates of G5 PPI with a synthetic analog of LHRH for targeted siRNA delivery that was layer-by-layer caged with a dithiol containing cross linker and coated with PEG [34], as well as G5 PPI surface-modified superparamagnetic iron oxide nanoparticles (SPIONs) that were additionally conjugated with PEG and receptor-binding ligands for target-specific, noninvasive MRI monitoring of siRNA delivery and therapy outcome [35]. In collaboration with He's group, Minko *et al.* designed gold-containing inorganic nanoparticles for nucleic acid packaging and delivery. These particles could be internalized by cancer cells, and the cell mRNA silenced after the gold core was eliminated from the resulting nanoparticles [36].

Dendritic poly-L-lysine (PLL) has been studied as a gene-delivery vector for many years [37]. While unmodified sixth-generation PLL (KG6) was used successfully by Niidome *et al.* [19], a number of structural modifications have also been investigated. Okitsu *et al.* mixed dendriplexes of unmodified K6G with the weak base amphiphilic peptide Endo-Porter [14]. PLL dendrimers with a silsesquioxane cubic core that were coupled to the cRGD using a PEG spacer might even be considered a new class of dendrimers [38]. In another report, a G3 tetraoleoyl lysine (TOL) dendrimer was bound to the hydrophobic surface of single-walled carbon nanotubes (SWNT) and complexed 2'-O-F and thiol-modified siRNA [39].

Another silicon-containing and rather novel class of dendrimers are the carbosilane dendrimers, which were only recently adopted for siRNA delivery. In the first report dated 2007, Muñoz-Fernandez performed alcoholysis of Si-Cl bonds with amino alcohols and subsequent quarternization with methyl iodide [40]. A year later, the 2G-NN8 and 2G-NN16 derivatives were used for siRNA delivery [41], and additional reports of siRNA delivery using 2G-NN16 followed soon afterwards [42,43].

The latest class of dendrimers exploited for siRNA delivery are triazine dendrimers [9,10], which were synthesized from cyanuric chloride in a divergent strategy to yield G2 and G3 'rigid', 'bow-tie' and 'flexible' backbones [44], with a number of different peripheries that varied in the number of amines, guanidines, hydroxyls and aliphatic groups [45].

***In vitro* efficacy**

Owing to the described formulation problems, dendrimer-mediated siRNA delivery has not always been successful, particularly because siRNA transfection using reagents that have been optimized for DNA delivery leads to nucleolus localization of the load, as shown with PAMAM [29]. In addition, as described above, limited biological activity of both PAMAM and TAT-conjugated PAMAM has been attributed to incomplete endosomal release of the siRNA [32]. Conversely, the dendriworm constructs efficiently induced a high proton sponge effect, which enhanced endosomal escape of siRNAs [25]. To overcome the obstacle of endosomal trapping, other approaches have focused on reducing the cationic charge on the surface of PAMAM dendrimers to improve both the cytotoxicity and intracellular release of siRNA. However, as shown for PAMAMs, partial acetylation reduced cytotoxicity, but often also diminishes the ability to mediate gene knockdown [28]. Quaternization of the internal amines to increase cationic charge at physiological pH was intended to address the problem of reduced cellular uptake [29]. Internally cationic and hydroxyl-terminated PAMAM G4 dendrimers afforded only slight reduction (~10%) in BCL2 protein expression [30]. However, gene knockdown could be increased to approximately 60% when the

dendrimers were conjugated with LHRH. In addition to peripheral group manipulations, the core and generation number of PAMAM dendrimers have been modified to affect siRNA transfection efficiency. Flexible triethanolamine core PAMAM dendriplexes of higher generation mediated Hsp27 gene knockdown in human PC-3 prostate cancer cells, with the G7 PAMAM dendrimer showing almost no cytotoxicity in (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays [17]. This indicates that the flexible core may reduce the expected toxicity for the higher generation PAMAM structures. Cytotoxicity was also reduced by conjugating α -CD onto PAMAM, while enhanced transfection efficiency without off-target effects was maintained [20]. Later, the beneficial effect of preventing enzymatic degradation of siRNA and improving the distribution of siRNA within the cytoplasm using α -CD was observed. Using this construct in transient and stable luciferase-expressing NIH3T3 cells, efficient gene knockdown was observed [23]. The shielding of positive charges by creating dendrosomes of PAMAM dendriplexes within a lipophilic shell also led to reduced cytotoxicity and 80% green fluorescent protein (GFP) knockdown *in vitro* [31]. Delivery of anti-high-mobility group protein B1 (HMGB1) siRNA with biodegradable e-PAM-R reduced both basal, H₂O₂-, and *N*-methyl-D-aspartic acid (NMDA)-induced HMGB1 levels in neurons in which cell death was significantly suppressed [22]. With the RGD conjugate of PAMAM and anti-enhanced GFP (EGFP) siRNA, only modest silencing of the EGFP expression was achieved in stably-transfected U87 cells, although the uptake of RGD-PAMAM/siRNA complexes was increased compared with the nontargeted dendriplexes. Interestingly, the RGD motif prevented adhesion of U87 cells to fibrinogen-coated plates via modulation of cell-ECM interactions [24]. The EGF-targeted PAMAM conjugate G4.0-GGG-EGF was taken up in an EGF-dependent manner, as shown by microscopy of quantum-dot-labeled dendriplexes, and led to specific knockdown of yellow fluorescent protein (YFP) only when EGF was coupled to the G4 dendrimer. The knockdown of 70% was quantified by western blotting [18]. pH-responsive PICMs with a PAMAM core and methacrylic acid (MAA) corona were designed to lose their corona upon endocytosis and protonation of the MAA in the endosome. Targeting the G5-PICMs to PC-3 cells with an anti-transferrin Fab caused greater BCL2 knockdown than nontargeted PICMs or commercial PAMAM dendrimers while maintaining decreased nonspecific cytotoxicity as compared with PAMAM [33].

“...the dilemma of endosomal trapping was solved by mixing the amphiphilic peptide Endo-Porter with preformed poly-L-lysine dendriplexes.”

Layer-by-layer complexes of siRNA with conjugates of G5 PPI coupled to a synthetic analog of LHRH of 100–150 nm in size showed specific intracellular delivery to LHRH receptor-positive cancer cells, release of siRNA in the cytoplasm, and efficient gene silencing of BCL2 [34]. G5 PPI surface-modified SPIONs for MR imaging packaged large numbers of siRNAs, protected them during the delivery process, and mediated siRNA escape from the endosomes [35]. Inorganic engineered nanostructures of gold nanoparticle-anchored PPI were efficiently internalized by cancer cells. Particles fabricated by Au-G3 PPI were even more efficient in silencing BCL2 mRNA expression than those fabricated by G5 PPI. The 72% knockdown mediated by siRNA complexed with Au-G3 PPI was explained by the formation of spherical particles compared with randomly aggregated nanofibers made of low-generation dendrimer alone [36].

Since it is known that PLL does not exhibit the ‘proton-sponge effect’ as efficiently as other carriers such as PEI, the dilemma of endosomal trapping was solved by mixing the amphiphilic peptide Endo-Porter with preformed PLL dendriplexes [14]. The dendrimeric nanoglobules with 3D compact globular morphology formed using PLL with a silsesquioxane cubic core mediated 50% sequence-specific luciferase knockdown in U87 cells when PEG-RGD was coupled. The efficiency could be increased to 75% knockdown after co-delivery of doxorubicin, although this approach also increased the cytotoxicity of

the nontargeted complexes [38]. Sequence-specific knockdown of 80% of ApoB in FL83B mouse hepatocytes was achieved with lipid- and natural amino acid-modified nanotubes, which delivered siRNA as efficiently as lipofectamine at even lower cytotoxicity [39].

In the first report of carbosilane dendrimer-mediated siRNA delivery, silencing of GAPDH expression and reduced HIV replication in SupT1 and PBMC were described [41]. Later, selective blockage of HIF1- α synthesis in neurons [43] and inhibition of cyclooxygenase-2 gene expression in HIV-infected nervous system cells [42] were reported. However, it was also found that the dendrimer alone caused repression of IL-17F, IL-23R and IL-23A in macrophages [46].

“Complexes of triazine dendrimers with siRNA were found to be stable in circulation.”

While computer simulations have shown that the backbone structures of triazine dendrimers designed to be ‘flexible’ actually exhibited a collapsed topology [10], the ‘rigid’ backbone eventually mediated significant and sequence-specific luciferase knockdown [9]. Alkylated triazine dendrimers were hypothesized to be taken up by different mechanisms compared with dendrimers with amine and hydroxylperipheries, and consequently exhibited improved endosomal escape [9].

***In vivo* evaluation**

To date, only two reports of PAMAM-mediated *in vivo* siRNA delivery can be found in the literature, which might reflect the decreased efficiency of some PAMAM dendrimers that have been structurally altered to optimize biocompatibility. One of the success stories, however, is the local, intracranial injection of biodegradable e-PAM-R that notably reduced the infarct volume in the postischemic rat brain by silencing HMGB1 [22]. The other PAMAM derivative that specifically and significantly suppressed gene expression *in vivo* were the dendriworms. These nanoparticles were able to penetrate throughout the brain parenchyma after intracranial infusion and suppressed EGFR levels *in vivo* in a transgenic mouse model of glioblastoma [25].

Concerning PPI/siRNA complexes, only body distribution has been investigated after *in vivo* administration to date. While only trace amounts of dendrimer and siRNA were found in an A459 tumor, LHRH receptor-targeted dendrimer and delivered siRNA were found predominately in the tumors [34].

Dendritic PLL mediated knockdown of ApoB in healthy C57BL 6, without causing hepatotoxicity, and maintain a significant reduction of LDL-cholesterol in serum in apolipoprotein E-deficient mice after intravenous injection [19]. The same gene was silenced in the liver when siRNA was complexed with TOL7 dendrimers that were attached to SWCNTs. It was found that the nanotubes inhibited the biodegradation of siRNA and the drug clearance *in vivo* [39].

Complexes of triazine dendrimers with siRNA were found to be stable in circulation, while passive targeting to the lung and comparably low liver uptake were observed [9].

Imaging

Important parameters for efficient *in vivo* delivery systems are their circulation times and their bio-distribution, since nanocarriers are easily captured by the reticulo-endothelial system. Systemic bioavailability and pharmacokinetics of triazine dendrimer-complexed siRNA were recently investigated. After radiolabeling siRNA [9] the distribution of dendriplexes within the whole body was followed by single-photon emission computed

tomography imaging of radiolabeled siRNA [9], as well as by tracking of fluorescently labeled siRNA [34]. In the case of LHRH-PPI, tumor accumulation was confirmed by fluorescence imaging and colocalization of bioluminescence of luciferase-transfected A549 cells inoculated for the tumor model. In addition, organ distribution of fluorescence carrier [25,34] and load [9,34] were determined on the microscopic level by confocal laser scanning microscopy.

Conclusion

Dendrimers have been extensively investigated for therapeutic purposes, including pDNA and antisense oligonucleotide delivery, but these structures are only just emerging as nonviral vectors for siRNA and shRNA-expressing plasmids. Owing to the wide range of chemical variation possibilities for both backbones and peripheral groups, we can be sure that improved, perfectly shaped siRNA delivery complexes will be designed in the near future.

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Biographies

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