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Effects of PEGylation and Acetylation of PAMAM Dendrimers on DNA Binding, Cytotoxicity and *in Vitro* Transfection Efficiency

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Abstract: Poly(amidoamine) (PAMAM) dendrimers are promising multipotent gene delivery vectors, providing favorable DNA condensation properties also in combination with the possibility of conjugation of different targeting ligands to their surface. They have been used for transfection both *in vitro* and *in vivo*, but their application is currently somewhat limited due to inherent cytotoxicity. In this work we investigate how two types of surface modification, acetylation and PEGylation, affect the DNA binding characteristics, the cytotoxicity and the *in vitro* transfection efficiency of generation 4 and 5 PAMAM dendrimers. Particularly, we address how the morphology of DNA−dendrimer complexes, formed under low salt conditions, changes upon dilution in cell growth medium, an event that inevitably occurs before the complexes reach the cell surface in any transfection experiment. We find that acetylation and PEGylation essentially eliminates the inherent dendrimer cytotoxicity. However, the transfection efficiency of the modified dendrimers is lower than that of the corresponding unmodified dendrimers, which can be rationally understood by our observations that DNA is less condensed when complexed with these modified dendrimers. Although small DNA−dendrimer particles are formed, the availability for ethidium intercalation and nuclease degradation is significantly higher in the modified DNA−dendrimer complexes than in unmodified ones. Dilution in cell growth medium has a drastic effect on these electrostatically assembled complexes, resulting in increase in size and DNA availability. Our results strongly add to the notion that it is of importance to perform a biophysical characterization under conditions as close to the transfection situation as possible, to enable conclusions regarding structure−activity relations of gene delivery vectors.

Keywords: Dendrimer; polyplex; biocompatibility; gene delivery; DNA; characterization

Introduction

Gene delivery as a concept promises to revolutionize medicine and biotechnology as well as basic research. However, despite decades of research efforts its therapeutic use is still limited. This is mainly due to the difficulties in developing safe but sufficiently efficient delivery vectors. Cationic polymers have been extensively investigated to this end because of their versatility, tolerability, and relative ease of manufacturing.[1,2] Although they still cannot compete with viral vectors in terms of efficiency, increased understanding of the barriers to gene delivery augment the possibilities for the design of improved polymeric vectors.

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Dendritic polymers were first synthesized inspired from structures found in nature.[3] The tree-like arrangement allows for synthesis of polymers with high monodispersity and well-defined sizes despite relatively high molecular weights, and dendrimers of different types have been extensively investigated in medicine and pharmacology for their abilities as imaging agents and drug carriers.[4−9] Drugs can either be bound in the internal cavities of the dendrimer, thereby promoting delivery also of drugs with low solubility, or be conjugated or adsorbed onto the dendrimer surface. Polyamidoamine (PAMAM) dendrimers are amine-terminated structures that are positively charged at physiological pH, and they are therefore able to form complexes with DNA. We and others have studied biophysical properties of such "dendriplexes", and the nature of the interaction.[10−19] The binding mode is, as expected, primarily electrostatic, which is shown by the observation that increased ionic strength decreases the affinity.[10,15] Upon binding, DNA is condensed to varying degrees depending on dendrimer generation and dendrimer-to-DNA charge ratio *r* (commonly defined as the ratio of total concentration of positive ammonium groups on the dendrimers over the concentration of negative phosphate groups on the DNA, [N]/[P]). PAMAM dendrimers have been used successfully for delivery of plasmid DNA as well as oligonucleotides and siRNA to a variety of cell types with high efficiency *in vitro*.[20−26] The most efficient transfection is seen if DNA is complexed with intermediate to higher generation dendrimers and when the dendriplexes are prepared at charge ratios of 5−10. Electron microscopy imaging of these preparations reveals the formation of heterogeneous complexes, consisting of compact spherical or toroidal structures as well as very large aggregates.[22] The structure of the complexes can be modified by the use of partly fractured dendrimers[11] or by inclusion of small anionic

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molecules such as DNA oligomers or dextran sulfate.[21,27] Both strategies result in more homogeneous populations of smaller particles, and the procedures have also resulted in increased transfection efficiency, suggesting a link between these properties. However, currently the practical use of unmodified PAMAM dendrimers has been somewhat questioned by reports that they are relatively cytotoxic,[28−32] with a suggested mechanism being the promotion of pores in cellular membranes as a result of their high surface charge density.[33,34] Attempts to increase the biocompatibility of PAMAM dendrimers involving partial acetylation or PEGylation of the surface have been promising in this respect.[30,35−37] Such modifications reduce the surface charge density of the dendrimers, which may reduce cytotoxicity. In addition, PEGylation is generally considered to increase the bioavailability of polymer−DNA complexes by enhancing solubility and increasing the circulation time in the bloodstream.[38] Only a few reports have addressed how changing the surface properties of PAMAM dendrimers affects their ability to efficiently condense DNA and mediate transfection. Shakbazau et al. reported that acetylation of G4 (i.e., fourth generation) dendrimers decreases their transfection efficiency, and proposed that this is due to an increase in hydrophobicity.[39] Kono et al., on the other hand, described how the attachment of phenylalanine to the surface of PAMAM G4 dendrimers also increases their hydrophobicity and that this results in increased transfection efficiency,[40] indicating that the understanding of how particular physicochemical properties of dendrimers and their corresponding dendriplexes affect transfection is currently somewhat limited. Further, Luo et al. reported the synthesis of a PEGylated PAMAM G5 dendrimer that has a 20-fold higher transfection efficiency than the commercially available partially fractured PAMAM dendrimer SuperFect, while the parent G5 dendrimer was found to be completely inactive.[36] They suggested that the increased transfection efficiency of their PEGylated dendrimer is partly the result of the PEG chains providing steric stabilization of the complexes, but they also speculate that the DNA dissociation inside the cell would be facilitated because of the PEG chains causing charge separation between the amine groups and the phosphates on DNA. Moreover, they suggested that, because the PEG chains have a greatly increased flexibility compared with the unmodified G5, this would also facilitate complex dissociation by giving an entropic driving force for DNA release as previously proposed for partially fractured dendrimers such as the commercially available transfection reagent SuperFect.[22,41,42] Similarly, Männistö et al. reported that PEGylation may also increase the tranfection efficiency of poly-L-lysine (PLL) dendrimers.[43] PEGylated PAMAM dendrimers have recently been used for successful transfection *in vivo* targeted to murine brain tissue,[44] showing that these polymers hold promise to become functional delivery vectors for medical applications.

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Table 1. Properties of the PAMAM Dendrimers Used in the Current Study

dendrimer	surface	charge/dendrimer[a]	MW (Da)
G4	primary amine	64	14 000
G4-ac	partial acetylation	45	14 800
G4-PEG	partial PEGylation	42	27 200
G5	primary amine	128	28 000
G5-PEG	partial PEGylation	103	43 000
SuperFect	primary amine	60[b]	35 000[b]

[a] The number of primary amines. [b] Average value, obtained from the manufacturer.

To systematically study the effects of size, surface charge and chemistry on dendriplex morphology, we have synthesized a library of dendrimers where these properties are varied one at a time (Table 1). The library contains dendrimers of two different sizes with or without surface modifications through acetylation or PEG conjugation. As reference we compare our dendrimers with the commercially available transfection agent SuperFect, which consists of fractured PAMAM G6 dendrimers and which, to date, has the highest reported transfection efficiency among commercially available PAMAM dendrimers.[22,41] In addition to the obvious exploration of modified versus unmodified dendrimers, as well as of dendrimers of different sizes, we wish to compare the effects of PEGylation versus acetylation since both modifications decrease the surface charge but PEGylation also makes the dendrimer surface more inaccessible to DNA.

A further aspect of crucial importance for understanding relations between dendriplex morphology and transfection efficiency that has to date been poorly investigated is how the dendriplexes, commonly formed under low salt conditions to obtain sufficiently stable particles, are affected by changes in the surrounding environment. Transfection is a complex process that subjects the dendriplex to many events involving changing solvent conditions in terms of pH and ionic strength, as well as presentation to potential binding partners such as proteoglycans on the surface of cells in culture or serum proteins in the bloodstream and other molecules in the extracellular matrix in tissues. As a first step toward rationalizing the effect of these properties on dendriplex stability and hence further transfection efficiency, we have here addressed this issue by monitoring how the dendriplex size and availability of DNA to ethidium is affected upon dilution of preformed complexes in cell growth medium. Our results indicate that this exposure, which inevitably must occur before the dendriplexes reach the cell surface, can significantly alter the morphology and hence properties of the dendriplexes, thereby complicating the conclusions drawn in structure−activity relationship studies where dendriplexes have been examined in simple buffers or water solutions. Further, for efficient transfection to occur the dendriplexes have to be unpacked inside the cell to allow transcription of the DNA. For this reason, we have also investigated whether a good transfection agent would easily allow transcription *in vitro*.

Materials

Generation 4 and 5 polyamidoamine (PAMAM) dendrimers were synthesized as previously described.[45] Twenty-five % acetylated generation 4 PAMAM dendrimers and 25% PEG-modified generation 4 and 5 PAMAM dendrimers were prepared as described below. All dendrimers were characterized by ^{1}H and ^{13}C NMR, and the percentage of PEG surface coverage was determined by elemental analysis. All dendrimers were stored as stock solutions at a high concentration (>10 mg/mL) in methanol and were diluted in buffer prior to use. The partially fractured dendrimer SuperFect was purchased from Qiagen (Hilden, Germany). T7 RNA polymerase, nucleotides and additional reagents and buffers were obtained from NE Biolabs (Ipswich, MA). Cell culture reagents were from PAA (Pasching, Austria). Chinese hamster ovary cells (CHO-K1) were a kind gift from Prof. Ülo Langel, Stockholm University, Sweden. All other chemicals and reagents were acquired from Sigma (St. Louis, MO).

Methods

Dendrimer Synthesis. All PAMAM dendrimer preparations were based on the work of Tomalia et al. and found to give material consistent with published data.[45−47] Acetylated generation 4 PAMAM dendrimers were prepared using a similar method as described by Baker et al.[48] Acetic anhydride was added to a solution of dendrimer in dry methanol and stirred for 72 h. The resulting product was then purified by dialysis against deionized water before being characterized by ^{1}H and ^{13}C NMR. PEG chains were connected onto the surfaces of G4 and G5 PAMAM dendrimers by the use of a thiourea linker group. This was prepared by first reacting monomethyl PEG (550) with phosphorus tribromide to give brominated PEG chains. The

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Gabriel synthesis was then used to convert the brominated PEG chains into amine-functionalized PEG chains by refluxing the bromo product in potassium phthalimide followed by removal of the phthaloyl protecting group using hydrazine. The resulting amine-terminated PEG chain was then added slowly to a solution of thiophosgene in chloroform in the presence of sodium carbonate to give the corresponding isothiocyanate-funtionalized PEG chain (ITC-PEG). A solution of the ITC-PEG in 1,4-dioxane was then added slowly to solutions of PAMAM dendrimer in deionized water, and the mixture was stirred for 18 h and then purified by dialysis against deionized water to give products that had their surface coverages determined by elemental analysis.

Cell Culture. Chinese hamster ovarian cells (CHO-K1) were cultured in HAM's F12 medium supplemented with bovine calf serum (10%) and L-glutamine (2 mM) at 5% CO_2. The cells were trypsinized and split every 3−4 days and seeded at a density of ~7,500 cells/cm^2. For transfection and cytotoxicity experiments, the cell growth medium was supplemented with antibiotics (1% penicillin/streptomycin).

Plasmid DNA Preparation. The pEGFP-C1 plasmid and the Luciferase T7 Control DNA plasmid were amplified in *Escherichia coli* strains *XL1 Blue* and *DH5a*, respectively, according to standard procedures and were purified using a QIAfilter Plasmid Giga Kit (Qiagen) according to the manufacturer's instructions. The intactness and identities of the plasmids were confirmed by agarose gel electrophoresis. For linearization, the Luciferase T7 Control Plasmid was digested with Pdm1 (Fermentas). The efficiency of the reaction was 100% as confirmed by agarose gel electrophoresis. To remove the restriction buffer, the linearized DNA was precipitated in isopropanol, centrifuged, washed once with 70% ethanol, and resuspended in a 10 mM Hepes buffer with 10 mM NaCl (pH 7.4). The plasmid stock solutions were stored at −20 °C. For each new experiment, an aliquot of DNA was thawed and diluted in 10 mM Hepes with 10 mM NaCl (pH 7.4). DNA concentrations were determined by absorbance using a Varian Cary 4000 UV−vis spectrophotometer (Varian Inc.) and an extinction coefficient of $\varepsilon_{260} = 6600\ M^{-1}\ cm^{-1}$ per base.[49]

Complex Preparation. Dendriplexes were formed by adding a small volume (2−10 μL) from a dendrimer stock solution into a 30 μM DNA solution, immediately followed by vortexing for 30 s. For *in vitro* transcription experiments, a 300 μM DNA solution was used. The dendriplexes were incubated for 10 min before use. Complexes with N/P ratios ranging from 2 to 100 were investigated. The dendriplexes were either used in transfection experiments or analyzed by biophysical techniques as described below. Samples for biophysical characterization were diluted in buffer or in serum-free cell growth medium to a final DNA concentration of 10 μM. Samples for the *in vitro* transcription experiments were prepared separately with special precautions to avoid RNase contamination (vide infra) and employing the Luciferase T7 Control Plasmid instead of the pEGFP-C1.

Transfection. Transfection was assessed by evaluating the expression of enhanced GFP, encoded in the pEGFP-C1 plasmid, using flow cytometry. The cells were seeded in 24-well plates at a density of 30,000 cells/well in complete medium 24 h prior to transfection. Dendriplexes were prepared as described above using a total amount of 1 μg of pEGFP-C1/well, diluted in 350 μL of complete medium and immediately transferred to the cells. The cells were incubated with the dendriplexes for 4 h, rinsed twice with complete medium and cultured for another 24 h. At the end of the culture period the cells were trypsinized, washed, stained with 7-AAD at 4 °C for 15 min at a final concentration of 1 μg/mL to discriminate between live and dead cells, washed again and kept on ice until flow cytometric evaluation (FACScan, BD, Franklin Lakes, NJ). All samples were prepared in triplicate, and data represent the mean of three independent experiments.

Cytotoxicity by LDH Membrane Permeability Assay. Cytotoxicity was assessed by evaluating the membrane permeability via leakage of lactate dehydrogenase from the cytoplasm of CHO-K1 cells upon exposure to dendrimers or dendriplexes using a cytotoxicity detection kit (LDH) from Roche Applied Science (Mannheim, Germany). Cells were seeded in 96-well plates at a density of 7,500 cells/well in complete medium 24 h prior to experiments (boundary wells were not used). Throughout the experiment heat-inactivated serum was used to avoid background signals due to LDH activity in the medium. Dendrimers or dendriplexes prepared as described above were diluted to specified concentrations in 50 μL of 10 mM Hepes. The cells were washed once with serum-free growth medium to remove any LDH released during the overnight culture. The dendrimers or dendriplexes were mixed with 150 μL of complete medium and thereafter immediately added to the cells. After 4 h incubation at 37 °C and 5% CO_2, 100 μL of the supernatant was removed for the LDH assay. In the assay, the activity of LDH is coupled to a catalytic reaction where a tetrazolium salt (INT) is reduced to formazan, which absorbs strongly at 490 nm and thus can be detected spectrophotometrically. The absorption measurements were done in a SpectraMax Plus^{384} plate reader (Molecular Devices, Sunnyvale, CA). All samples were prepared in triplicate, and the presented data represents the mean of three independent experiments.

Dynamic Light Scattering. Dynamic light scattering (DLS) measurements were performed using a Malvern Zeta sizer Nano ZS (Malvern, Germany) at 22 °C. The wavelength of the incident light was 633 nm, and the scattering angle was 173°. Data was collected for a period of 150 s. The decay time distribution was obtained from the autocorrelation function, and the average hydrodynamic radii were calculated using CONTIN analysis. The peak 1 value was derived based on the intensity distribution of the scattered light.

Binding Isotherms for Ethidium Bromide. The binding efficiency of the monovalent cation ethidium to compacted DNA was determined by analyzing emission spectra of

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ethidium upon titration of DNA−dendrimer samples of known concentration with small volumes of EtBr, as previously described.[10] Typically, 10 μM DNA or dendriplexes in 1 mL of buffer was titrated with aliquots of 2.5−10 μL of 150 μM EtBr. Emission spectra were recorded on a Varian Eclipse spectrofluorimeter (Varian Inc.), with excitation and emission bandpass set to 10 nm. The amount of bound and free ethidium in each titration point was determined by least-squares projection of the measured spectra onto reference spectra corresponding to completely bound and completely free ethidium. Reference spectra were recorded in separate experiments, and that for completely bound ethidium was measured on a sample where a 60-fold excess of DNA was added to a 5 μM EtBr solution. The titration data, in the form of Scatchard plots, were fitted to the McGhee and von Hippel conditional probability model of excluded site binding according to eq 1,[50]

$$\frac{\theta_{\mathrm{EtBr}}}{c_{\mathrm{f}}} = K_{\mathrm{EtBr}}(1 - s\theta_{\mathrm{EtBr}})\left[\frac{(1 - s\theta_{\mathrm{EtBr}})}{(1 - (s-1)\theta_{\mathrm{EtBr}})}\right]^{(s-1)} \quad (1)$$

with K_{EtBr} the apparent binding constant of ethidium to DNA and s the apparent binding site size. The parameter θ_{EtBr}, the ratio of bound ethidium to the number of DNA base pairs, was modified according to eq 2 by introducing a parameter x representing the apparent accessible fraction of binding sites on DNA remaining after dendrimer binding.

$$\theta_{\mathrm{EtBr}} = \frac{c_{\mathrm{b}}}{x c_{\mathrm{DNA}}} \quad (2)$$

The size of the binding site for ethidium on DNA (s) was calculated to be 2.5 base pairs using titration data for uncondensed, linear DNA. It was assumed that s does not change significantly upon dendrimer binding; hence this value was kept constant for the remaining titration data where only values of K_{EtBr} and x were calculated.

Serum Stability of Dendriplexes. The intactness of plasmid DNA protected by complexation with dendrimers was evaluated by gel electrophoresis. Dendriplexes were prepared as described above in a total volume of 16 μL. Care was taken to use the same amount of DNA in all samples. After the dendriplexes were formed, 20% (4 μL) fetal bovine serum was added to each sample. The samples were incubated with serum for 3 h at 37 °C. The serum nucleases were thereafter heat inactivated at 70 °C for 10 min before the dendriplexes were dissociated with 0.23 mg/mL heparin and 1% SDS for 45 min. The DNA was purified with isopropanol/EtOH according to standard procedures, resuspended in buffer and subjected to gel electrophoresis at 5 V/cm for 2.5 h at 4 °C. The gel was stained with 1.3 μM EtBr for 45 min and imaged using a Typhoon 9410 gel scanner (GE Healthcare, Uppsala, Sweden) with excitation at 532 nm and emission band-pass filter at 610 nm.

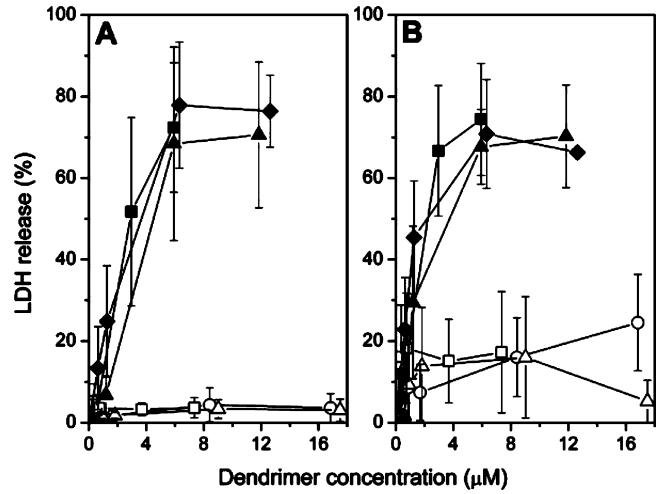

Figure 1. Cytotoxicity of dendrimers alone (A) and of dendriplexes prepared in 10 mM Hepes buffer with 10 mM NaCl (pH 7.4) (B) to CHO-K1 cells after 4 h exposure, assessed by LDH release from the cytoplasm. Data represents dendrimer G4 (▲), G4-ac (○), G4-PEG (Δ), G5 (■), G5-PEG (□), and SuperFect (◆). The dendrimer concentrations were chosen to represent charge ratios r = 5, 10, 50, and 100, when complexed with 0.7 μg of DNA. The presented data is the mean ± SD of three independent experiments. Negative and positive controls were untreated and lysed cells, respectively.

***In Vitro* Transcription Using T7 Polymerase.** The effect on transcription of compacting DNA with dendrimers was evaluated by assessing the *in vitro* production of mRNA from the DNA template. The RNA was quantified by UV−vis spectroscopy using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and an extinction coefficient of ε_{260} = 8900 $\mathrm{M^{-1}\ cm^{-1}}$ per base.[51]

Results

Cytotoxicity of Dendrimers and Dendriplexes. Figure 1 shows the cytotoxicity of dendrimers and of dendriplexes formed at different charge ratios to CHO-K1 cells assessed by monitoring the degree of LDH leakage from the cytoplasm. Both PEGylation and acetylation has a clear beneficial effect on the biocompatibility of dendrimers, rendering them completely nontoxic for all concentrations evaluated (up to 20 μM). Importantly, complexes of DNA and dendrimers appear slightly more cytotoxic than dendrimers alone. At charge ratios and concentrations commonly used for transfection, this is most notable for the partially fractured SuperFect and for PAMAM G4. Both of these dendrimers are rather well tolerated when applied alone at these concentrations, but give rise to a relatively high cytotoxicity

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(51) Cavaluzzi, M. J.; Borer, P. N. Revised UV extinction coefficients for nucleoside-5′-monophosphates and unpaired DNA and RNA. *Nucleic Acids Res.* **2004**, *32* (1), e13.

Table 2. Transfection Efficiencies of PAMAM Dendrimers at Indicated Charge Ratios *r* in CHO-K1 Cells, Determined as the Percentage of GFP-Expressing Cells[a]

	% GFP-expressing cells		
dendrimer	$r = 5$	$r = 10$	$r = 50$
G4	0.91 ± 0.39	1.17 ± 0.28	1.14 ± 1.14
G4-ac	0.24 ± 0.29	0.13 ± 0.06	0.14 ± 0.12
G4-PEG	0.06 ± 0.01	0.11 ± 0.03	0.12 ± 0.03
G5	0.73 ± 0.53	0.55 ± 0.44	1.47 ± 1.11
G5-PEG	0.05 ± 0.05	0.06 ± 0.05	0.07 ± 0.07
SuperFect	3.59 ± 1.83	6.66 ± 5.11	7.12 ± 3.88

[a] Plasmid pEGFP-C1 was condensed with dendrimers at charge ratios 5, 10, and 50 and the number of GFP-expressing cells was assessed by flow cytometry after 24 h incubation. Dead cells were excluded from the analysis via 7-AAD staining. Data is the mean ± standard deviation of three independent experiments, each performed in triplicate.

when complexed to DNA. This was also confirmed by confocal imaging of transfected cultures where cells showed compromised morphology and in some cases had started to round up and detach from the surface (data not shown).

***In Vitro* Transfection.** The *in vitro* transfection efficiency of the different dendrimers was determined by transfecting CHO-K1 cells with pEGFP-C1, encoding enhanced green fluorescent protein (eGFP), followed by flow cytometric analysis of the cells 24 h later. The data is presented in Table 2. In contrast to previous studies of the transfection efficiency of PEGylated dendrimers,[36] our G4 and G5 PEGylated dendrimers showed very modest transfection ability, with less than 1% of the cells expressing eGFP. The acetylated dendrimers showed equally poor results whereas the unmodified dendrimers G4 and G5 were moderately efficient, with G4 being slightly better than G5. None of the dendrimers in our library had comparable transfection efficiency to SuperFect. We obtained overall relatively low transfection efficiencies in the CHO-K1 cell line over the range of conditions used. It should be noted that we chose to evaluate the transfection efficiency by quantifying the percentage of transfected cells rather than using the more commonly applied method of determining the total production of the foreign protein from all cells. We noted throughout the experiments that SuperFect-transfected cells appear brighter than cells transfected with other dendrimers, and this was also observed using confocal microscopy (data not shown). Thus, interestingly, it appears that not only do cells treated with SuperFect dendriplexes show a higher transfection efficiency in terms of cell number but also each transfected cell expresses more of the foreign protein. This would cause SuperFect to appear even more efficient if a total protein quantification approach is used. The physical basis behind this particular behavior of SuperFect could be either that SuperFect mediates a higher degree of uptake of DNA into the nucleus or that SuperFect allows transcription of its cargo to a higher degree than other dendrimers.

In order to understand the links between complex morphology and transfection efficiency and thus provide a physical explanation as to why the surface-modified dendrimers in this study were not as efficient as some surface-modified dendrimers described by others, we endeavored to study the biophysical properties of our dendrimers complexed to DNA.

Biophysical Characterization of Dendriplexes. Figure 2 shows the hydrodynamic diameter of dendriplexes of varying charge ratio ([N]/[P]), as determined by dynamic light scattering. The dendriplexes were formed at low ionic strength at $t = 0$ min, monitored for 20 min and then diluted in cell growth medium at $t = 21$ min (indicated by the dotted line). For charge ratio $r = 2$ the dendriplexes are unstable in water and were observed to aggregate over time. In addition, they displayed a high degree of polydispersity. Reduction of the surface charge density, by either acetylation or PEGylation, does not improve the stability, however, partial degradation of the dendrimer has a dramatic effect since SuperFect forms small, stable particles with DNA already at $r = 2$. For $r = 5-50$, stable particles are obtained for all dendrimers as long as they are maintained in the preparation buffer (to the left of the dotted line). For $r = 50$ there is a large excess of dendrimers, which leads to a very broad size distribution with a bias toward smaller sizes. However, the smaller sizes may originate from uncomplexed dendrimers and not from dendriplexes and therefore it is difficult to estimate the "true" size of the dendriplexes from dynamic light scattering experiments. The only safe characteristic that can be settled is the presence or absence of aggregates. At even higher charge ratios data were inconclusive due to severe polydispersity (data not shown).

In the lower regime of charge ratios, surface modification of the dendrimers leads to the formation of slightly larger complexes than for the corresponding unmodified dendrimers. However, all dendriplexes are of sizes that are considered as appropriate for internalization via endocytosis.[52] The data points to the right of the dotted line, after $t = 20$ min, shows the change in hydrodynamic diameter when the dendriplexes are exposed to cell growth medium, mimicking the transfection situation. This exposure leads to immediate dendriplex aggregation, manifested by increased apparent particle size and also notably increased polydispersity. Since scattering from large particles in polydisperse solutions will be dominating over the scattering from smaller particles, it is heavily influencing the shape of the DLS autocorrelation function on which the size distribution analysis is based. Therefore, if large aggregates are present it is not possible to discern the presence of smaller dendriplexes which may still exist alongside these aggregates. We find it likely that it is these small dendriplexes that are the most transcription-competent and that aggregation upon exposure to growth medium primarily decreases the available concentration of transfection-competent complexes. It appears that the surface-modified dendrimers of both generation 4 and 5 are somewhat less prone to cause dendriplex aggregation in cell growth medium than the unmodified dendrimers and Super-

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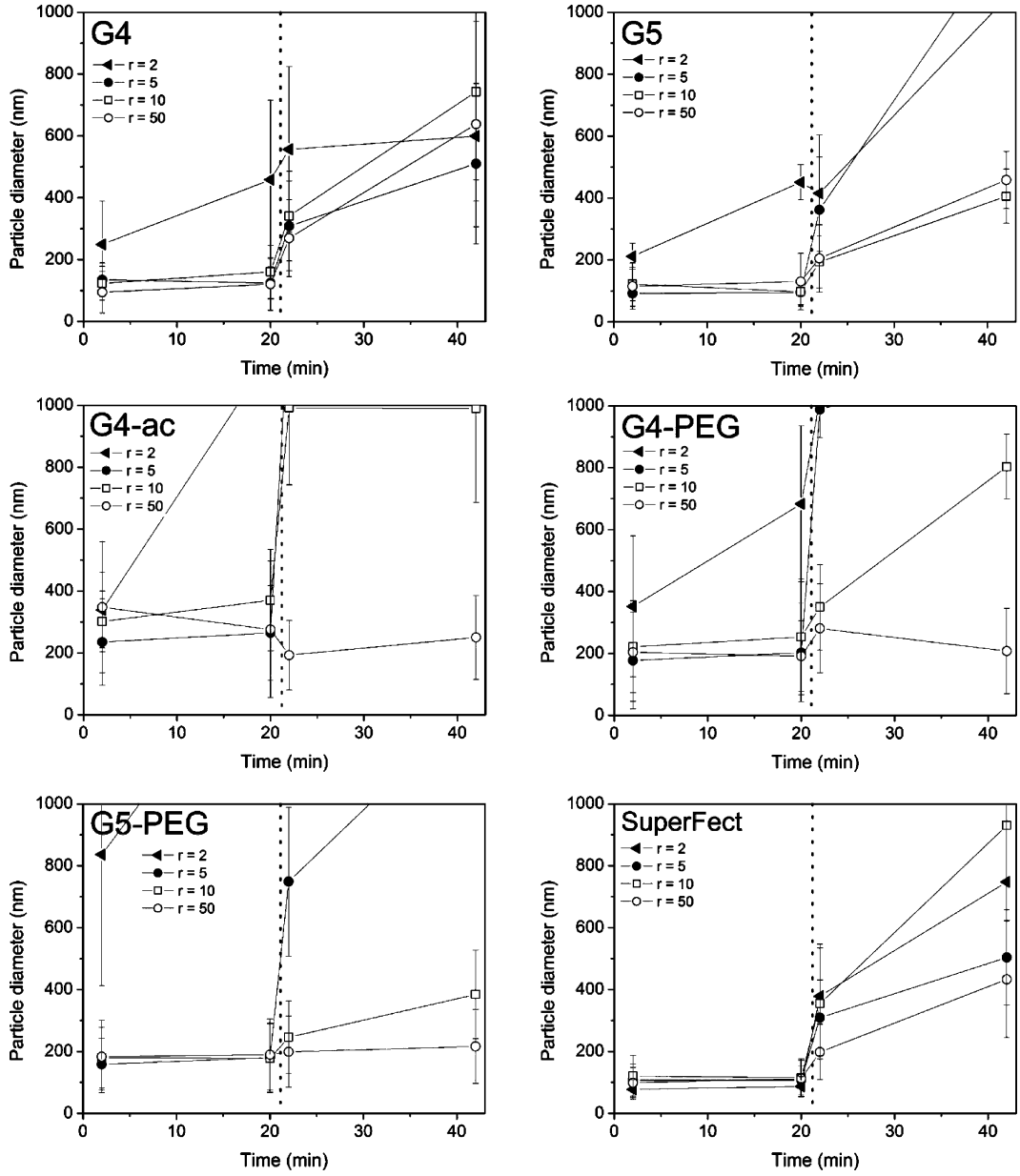
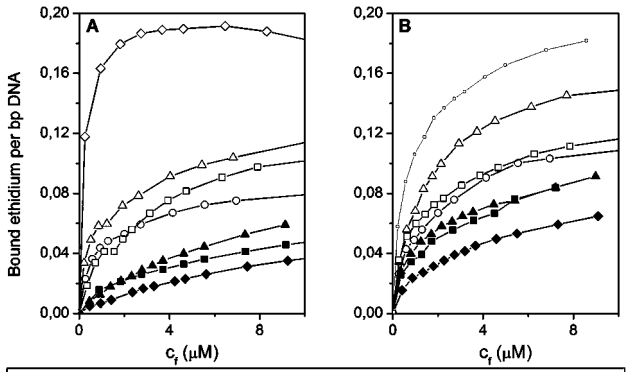


Figure 2. Hydrodynamic diameter of dendriplexes formed at different charge ratios ([N]/[P]), determined by dynamic light scattering. The dendriplexes were prepared in 10 mM Hepes with 10 mM NaCl at $t = 0$ min, and were diluted in medium at $t = 21$ min (indicated by the dotted line). Data represents dendriplexes prepared at charge ratios 2 (▲), 5 (●), 10 (□), and 50 (○). Measurements were performed at least 3 times, and data represents the peak 1 value which was determined from the intensity distribution. Error bars represent the mean peak width, i.e. the polydispersity of the individual samples.

Fect, at least when used at high charge ratios. This indicates that, although these complexes are not sufficiently transfection competent, they do have properties rendering them stable over a wider range of experimental conditions.

In a separate series of experiments dendriplexes were prepared either directly in cell growth medium or in buffer with higher ionic strength (150 mM NaCl). This resulted in the formation of considerably larger and notably unstable dendriplexes, regardless of dendrimer type (data not shown), showing in accordance with previous notions that it is necessary to form dendriplexes at low salt concentrations or in water to obtain a stable preparation of small particles.

Figure 3 shows binding isotherms for ethidium to the DNA in different dendriplexes as well as to uncondensed DNA, at low ionic strength (A) and after exposure to cell medium (B). Calculated apparent binding constants [K_{EtBr}] for ethidium and the number of remaining binding sites [x] in the dendrimer-condensed DNA are shown in panel C. The dendrimers show distinct differences in their abilities to exclude ethidium from DNA, where SuperFect is most efficient closely followed by unmodified PAMAM G4 and G5. The surface-modified dendrimers are relatively poor in excluding DNA with the acetylated dendrimers being somewhat better than the PEGylated ones. Preparation of



C

K_{EtBr}* 10^{-6} (M^{-1}) and x (% remaining binding sites)

	◊ Free DNA	▲G4	■ G5	○ G4-ac	Δ G4-PEG	□ G5-PEG	♦ SuperFect
Low salt (A)	3,72 ± 1,66 (58 ± 11 %)	0,06 ± 0,02 (29 ± 7 %)	0,16 ± 0,10 (22 ± 2 %)	0,42 ± 0,05 (23 ± 2 %)	0,25 ± 0,16 (36 ± 1 %)	0,17 ± 0,03 (27 ± 9 %)	0,03 ± 0,003 (23 ± 3 %)
Medium (B)	0,71 ± 0,30 (48 ± 13 %)	0,34 ± 0,01 (26 ± 3 %)	0,30 ± 0,14 (22 ± 5 %)	0,33 ± 0,16 (39 ± 2 %)	0,37 ± 0,14 (53 ± 1 %)	0,43 ± 0,03 (33 ± 1 %)	0,12 ± 0,01 (22 ± 4 %)

Figure 3. Ethidium binding isotherms for free and dendrimer-condensed DNA ($r = 10$) at 10 mM Hepes + 10 mM NaCl (A) and after dilution in cell growth medium (B). Apparent binding constants and percentage of available binding sites for ethidium are shown in panel C. Data represents dendrimer G4 (▲), G4-ac (60), G4-PEG (Δ), G5 (■), G5-PEG (□), SuperFect (◆), and uncondensed DNA (◇).

dendriplexes at higher charge ratios ($r = 50-100$) results in slightly increased ethidium exclusion for all dendrimer types, but the surface-modified dendrimers never reach the level of exclusion provided by unmodified dendrimers already at low charge ratios indicating that a high surface charge is necessary to provide high levels of DNA condensation (data not shown). Larger dendrimers (G5) provide a higher amount of ethidium exclusion than smaller ones (G4) for both modified and unmodified dendrimers. The calculated binding constants for ethidium are drastically reduced upon condensation of the DNA by dendrimers, from 10-fold for the least efficient, surface modified dendrimers to 100-fold for the most efficient, partially fractured SuperFect dendrimer. The remaining fraction of binding sites is less affected, showing a 2- to 3-fold reduction upon condensation. Exposure to serum-free cell growth medium before the ethidium titrations has opposite effects on uncondensed and dendrimer-condensed DNA (Figure 3B). For uncondensed DNA, a decrease in ethidium binding is observed in accord with previous results showing that an increased ionic strength reduces the affinity of ethidium for DNA.[10] In dendriplexes, on the other hand, the availability of the DNA increases for all dendrimer types. Analysis of data indicate that this is likely due to a change in the apparent binding constant for ethidium, rather than a change in the number of available binding sites. Thus, increased ionic strength has a remarkable effect on the morphology of dendriplexes, rendering them much more favorable for ethidium intercalation. Although the binding constant for ethidium is reduced at least 5-fold in isotonic conditions compared to at low ionic strength, the amount of intercalated ethidium increases in the dendriplexes.

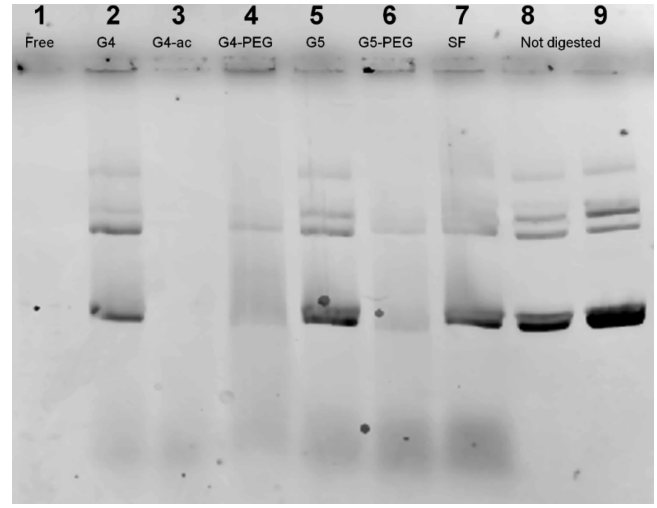


Figure 4. Dendriplex stability in presence of 20% serum. Heparin (0.23 mg/mL) and SDS (1%) were employed to dissociate the dendriplexes before gel electrophoresis in order to visualize the intactness of the DNA. Lane 1: uncondensed DNA. Lanes 2−7: dendriplexes prepared at $r = 5$ using dendrimer G4 (lane 2), G4-ac (3), G4-PEG (4), G5 (5), G5-PEG (6), and SuperFect (7). Lane 8: intact DNA not exposed to serum but purified according to the protocol. Lane 9: intact DNA not subjected to the purification procedure.

Hence, the affinity of dendrimers to DNA is more affected than the ethidium affinity when the ionic strength is increased.

Figure 4 shows the resistance to serum nucleases provided as a result of condensing DNA with dendrimers. Dendriplexes were incubated with 20% bovine calf serum for 3 h, treated with SDS and heparin to dissociate the complexes, precipitated and purified with isopropanol/ethanol and then subjected to gel electrophoresis. For uncondensed DNA, the serum exposure leads to complete degradation (lane 2), while for G4, G5, and SuperFect, intact DNA can be detected after the treatment (lanes 3, 6, and 8). Comparison with intact plasmid DNA, not exposed to serum and purified alongside the dendriplex samples (lane 9), shows that the DNA in the dendriplexes is not totally protected since those bands are fainter, but still the larger part of the DNA survives the serum treatment. Despite the extensive purification aimed to remove the dendrimers from DNA, the dendriplexes were not completely dissociated in all cases, noted by the presence of a dark band in the loading well which shows that some DNA has not been able to enter the gel. This complicates a quantitative analysis of the amount of protected DNA by comparison of band intensities, since some intact DNA molecules still complexed with dendrimers will likely remain in the loading wells. For acetylated dendrimers, no intact DNA is detected, while for PEGylated G4 and G5 some faint bands are seen, showing partial, but not efficient, protection. Preparation of dendriplexes at higher charge ratios resulted in slightly improved protection of the DNA by these dendrimers, but not to the extent of unmodified dendrimers or SuperFect (data not shown).

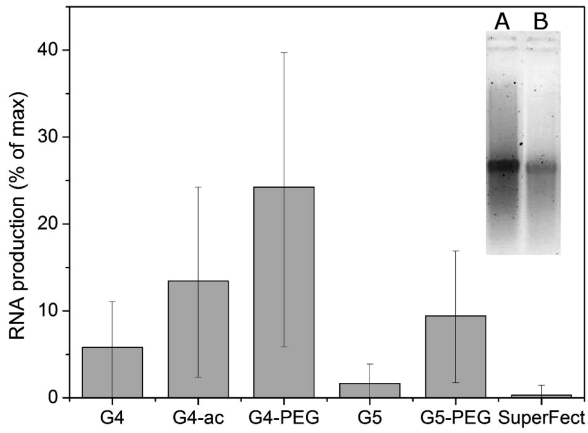


Figure 5. *In vitro* mRNA production from a dendrimer-condensed template DNA ($r = 5$) using T7 RNA polymerase, quantified by UV−vis absorption spectroscopy. The mRNA production from condensed DNA was normalized to the corresponding production from free DNA in each experiment. The presented data is the result of 3 or more independent experiments, and error bars denote the maximum and minimum values. The inset shows a typical gel with RNA bands resulting from transcription of free DNA (lane A) and dendrimer-condensed DNA (lane B). The intensity of the bands varied depending on the amount of produced RNA, but there were never any aborted transcripts detected in the gel for any of the dendrimers.

In Vitro **Transcription Using Dendrimer-Condensed DNA as Template.** Figure 5 shows quantitative data for *in vitro* transcription of dendrimer-condensed DNA ($r = 5$) using T7 RNA polymerase. The amount of produced RNA was determined by UV−vis spectroscopy and characterized using gel electrophoresis. As reported previously by Bielinska et al., condensation of DNA with unmodified PAMAM dendrimers appears to suppress transcription to a high degree.[12] No abortive transcripts are detected in the gel for any of the dendrimers (for an example see inset in Figure 5), indicating that it is the binding of the T7 polymerase to its promoter region that is inhibited by dendrimer-mediated DNA condensation rather than the translocation along the DNA strand. Surface modification renders the dendriplexes much more susceptible to transcription, in agreement with the observed reduced capacity of excluding ethidium (Figure 3) and the increased nuclease sensitivity (Figure 4) of these dendriplexes. SuperFect, on the other hand, is extremely efficient in suppressing transcription, showing close to zero mRNA production.

Discussion

Here we report on the synthesis and design of PEGylated and acetylated PAMAM dendrimers for DNA transfection and drug delivery purposes, and perform a systematic investigation on the effects of these surface modifications on the DNA condensing capabilities and transfection capacity. We show that both types of modification drastically increase the biocompatibility of the dendrimers, with no cytotoxicity observed even at, from a biological perspective, very high concentrations. These results are in agreement with previous studies of dendrimers with similar modifications and add to the notion that decreasing the surface charge of PAMAM dendrimers decreases their cytotoxic effects when directly applied to cells.[30,37,40,44,53] In this study we extended the cytotoxicity investigations to also examine the effect of the corresponding dendriplexes, formed by incubating dendrimers with DNA at low ionic strength. Our data show that both PEGylation and acetylation indeed decrease the cytotoxicity also for dendriplexes. In contrast to a previous study by Brazeau et al., who investigated the toxicity of various cationic gene delivery agents in murine myocytes,[54] we find that complexes of DNA and dendrimers are consistently more cytotoxic than dendrimers alone. It is possible that this enhanced toxicity is due to the larger size of the dendriplexes or altered particle properties that evoke a different response on the cell surface. There are a few studies available that investigate the relationship between size and *in vitro* toxicity of nanoparticles of various types, indicating that intermediate sized particles are generally more toxic than very small ones although the response differs depending on the cell type investigated.[55,56] It is also possible that the increased cytotoxicity is an effect of a high local concentration of dendrimers at the cell surface appearing because of the DNA providing a scaffold for dendrimers to bind to. This could in turn increase the probability of dendrimer pore formation in the cellular membrane, a previously proposed mechanism of toxicity likely requiring cooperative actions of several dendrimers molecules.[33,57] Indeed, increase in surface charge density has been attributed as the reason for the generation-dependent toxicity of unmodified PAMAM dendrimers appearing at higher generations (≥ 6).[28,29] SuperFect has by far the lowest average charge-per-weight ratio of the dendrimers evaluated in this study, but is among the most toxic ones. It is possible that this apparent contradiction is due to the presence of patches with very high charge density remaining from the original unfractured G6 dendrimer.

While the biocompatibility of the surface-modifieded dendrimers was significantly enhanced compared to the

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corresponding unmodified dendrimers, both alone and when complexed to DNA, these dendrimers yielded rather poor results in terms of transfection efficiency. This is initially surprising considering the results from previous studies where PEGylation significantly improved the transfection efficiency of both PAMAM and PLL dendrimers.[36,43] However, the biophysical characterization of the surface-modified dendrimers used in this study indicates that they have a significantly lower capacity of condensing DNA compared to the corresponding unmodified dendrimers and SuperFect. We believe this to be the main characteristic contributing to their reduced transfection efficiency. The ethidium titrations and size measurements together suggest that the surface-modified dendrimers give rise to slightly larger, more loosely condensed complexes, into which ethidium, being a relatively small molecule, can easily penetrate. Increasing the charge ratio to over 10 provides only a small additional increase in the degree of ethidium exclusion for all dendrimers (data not shown). The high availability of DNA in the surface modified dendriplexes is not decreased to any higher extent even at charge ratios over 100. The higher accessibility of DNA condensed with surface-modified dendrimers compared to unmodified ones is also shown by the serum stability experiments, where acetylated dendrimers are clearly totally unable to protect the DNA from nuclease degradation. Also PEGylated dendrimers show only a modest degree of protection, while dendriplex samples made with unmodified dendrimers show a large degree of intact DNA remaining after the exposure to serum. In accord with the lower availability of DNA in these dendriplexes to ethidium and their generally larger hydrodynamic radii it appears as if tight DNA condensation has a protective effect by sterically excluding nucleases. Similarly to the ethidium titration data, using higher charge ratios of the surface-modified dendrimers does not result in any dramatic increase in the degree of condensation of the complexes, with the protective effect against nucleases remaining low to moderate. SuperFect is the only dendrimer that forms small, stable particles with DNA already at charge ratio 2. This cannot be attributed only to its lower surface charge density, but must also be due to the increased flexibility of this dendrimer that is obtained because the branches are cleaved randomly.[22,41] The fractured dendrimer may be able to "wrap" around the DNA molecules providing efficient packing and utilization of all its charges. It has been shown, in a comparative study of linear and dendritic polylysine, that DNA cannot utilize all charges on a spherical, or close to spherical, cationic polymer.[43] Further, in another study the differences in charge density between the DNA and the cationic polymer was shown to affect the morphology of the resulting complexes and in particular their net charge.[58] Thus a fractured dendrimer, having a lower charge density than an intact one, could provide the same amount of DNA condensation and the same net charge of the complexes at lower concentrations, which is preferred from a biocompatibility perspective. The dynamic light scattering data show that all dendrimers evaluated in this study condense DNA sufficiently to allow internalization via endocytosis when used at $r = 5$ or higher. Therefore, the most immediate effect of the reduced DNA condensing capacity of surface-modified dendrimers is likely the observed insufficient protection of the DNA against serum nucleases. It is also possible that these dendriplexes, being more loosely condensed, are more prone to dissociate upon interaction with for example cellular proteoglycans. Interestingly, Männistö et al. found that PEGylation of PLL dendrimers did not alter the binding to DNA unless very high PEG:polymer ratios were used.[43] Our dendrimers have shorter PEG chains and higher surface coverage (PEG550, 25%) than the ones synthesized by Männistö et al. (PEG5000, 5%) or by Luo et al. (PEG3200, 13.6%). Thus, it appears that fine-tuning of these factors is crucial to maintain favorable DNA condensation and transfection properties of dendrimers while reducing cytotoxicity. Kim et al. showed that a relatively low amount (<25%) of surface modification with PEG550 chains suffices to render G3 PAMAM dendrimers biocompatible.[37] Likewise, Jevprasephant et al. showed that the attachment of only 4 chains of PEG2000 to PAMAM G4 dendrimers increased the IC_{50} from 0.13 to 0.79 mM, i.e. a 5-fold increase in biocompatibility.[30] It should be noted that the PEGylated dendrimers synthesized by Luo et al. were used at extremely high charge ratios in the transfection experiments (>100), which we suspect is due to relatively poor DNA condensation capabilities. For our short-chained and more densely grafted PEG dendrimers, increasing the charge ratio up to $r = 150$ did not result in improved transfection efficiency (data not shown). Since little is known regarding the biodegradability and potential long-term toxic effects of PAMAM dendrimers, we believe that such exaggerated charge ratios will not be applicable for use in either *in vitro* or *in vivo* applications. In contrast to a previous speculation that a higher degree of PEG substitution could be beneficial and possibly yield an even higher transfection efficiency,[36] we show here that the interplay between biocompatibility, promoted by low surface charge and hydrophilic surface groups, and DNA condensation capacity favored by high surface charge is much more complex. Based on our results and the aforementioned previous studies, we believe that dendrimers with a lower rather than a higher amount of PEG chains would have an increased affinity for DNA, and therefore could provide sufficient DNA condensation at much lower charge ratios while still having a favorable biocompatibility profile. Further, it appears that dendrimers of too low generation (<4) do not provide sufficient charge for efficient DNA condensation.[16,59] Therefore we believe that an optimal PEGylated PAMAM dendrimer for gene delivery purposes would be

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of intermediate generation (4−5), possibly partially fractured to a low extent, and have a low amount of PEGylation (<10%) using long PEG chains.

Dendriplex preparation in buffer with physiological ionic strength (150 mM NaCl) resulted in formation of significantly larger complexes. These were also in addition unstable, aggregating further over time (data not shown). This is in agreement with a recent study by Perez et al., who investigated the ability of dendrimers to complex siRNA and found complex formation under low ionic strength conditions to be a crucial factor for successful transfection.[26] Although plasmid DNA and siRNA behave very differently when complexed for transfection—whereas plasmid DNA collapses into a tight structure, siRNA behaves more like a rigid rod binding to the surface of the dendrimers[60]—both systems are based on electrostatic interactions between the nucleic acid and the vector. Therefore increasing the ionic strength should have the same effect on both systems, causing a weakened interaction which leads to self-aggregation of complexes and subsequent poor uptake as shown in this study. It is therefore important to control, or at least investigate, the effect of this parameter when studying newly synthesized polymers. Further, we show here that dendriplexes will start to aggregate, but to varying degrees, when diluted in cell growth medium. This is accompanied by a dramatic increase in the availability of DNA for ethidium intercalation, indicating that the dendriplexes become less tightly condensed under these conditions. This may have important implications for transfection efficiency since these effects are immediate and bring about considerable morphological changes to the dendriplexes and also because they inevitably will occur when the complexes are transferred to a cell culture. This could, at least in part, explain the lack of correlation between polymer structure and transfection efficiency in several previous studies.[16,43,61] We show that such morphological changes foremost render dendriplexes more susceptible to DNA degradation (Figure 4), however, in some cases dendriplexes may even dissociate. To investigate whether the effects on dendriplexes in cell growth medium are mainly due to the electrostatics we performed ethidium titrations to dendriplexes in buffer with physiological salt concentration, but without the other components in medium (data not shown). The binding isotherms from these experiments are intermediate to those obtained at low ionic strength (10 mM salt) and those obtained in cell growth medium. This points toward that the morphology changes and increased availability of DNA in dendriplexes exposed to cell growth medum are largely, but not fully, due to the difference in ionic strength. Thus, other more specific interactions could occur and other components in the medium, let alone the effect of specific components on the plasma membranes of the cells to be transfected, may also need to be considered to ultimately understand the molecular characteristics that comprise a dendriplex with high transfection efficiency. Most complexes used in nonviral gene delivery are held together by electrostatic interactions and are therefore likely to undergo a large morphology change when the ionic conditions are altered. Our initial experiments to address these issues indicate that unless this change is characterized, it is difficult, or even impossible, to predict the nature of the dendriplexes that finally reach the cells that are to be transfected. It would be interesting to extend our concept to also characterize the behavior in the presence of serum proteins or extracellular matrix components. Such work is underway in our laboratory. We also want to note the difference between our method of preforming dendriplexes and thereafter probing them with ethidium, and the commonly performed ethidium displacement assay where ethidium is first allowed to intercalate freely into DNA and is subsequently displaced as the DNA condensing agent is added. Although the latter method gives information about the affinity of the condensing agent to DNA, it is not certain that the morphology of these complexes, which might not have reached equilibrium, will be the same as when formed in absence of other molecules. Ethidium intercalation lengthens and unwinds the DNA, changes its torsional and bending flexibility, and reduces its effective charge density, which are all parameters that are likely to influence the DNA condensation process. By allowing the complexes to stabilize before ethidium is added, it is guaranteed that their final morphology is probed rather than some intermediate condensation state. Further, as shown in this study our method allows us to study the effect of changing the surrounding environment.

The *in vitro* transcription data in Figure 5 correlate inversely with the real transfection efficiency (i.e., internalization and subsequent transcription and translation). This implies that efficient DNA condensation is absolutely crucial for the steps in transfection that occur outside the cells and during internalization, whereas the efficiency of the subsequent unpacking events appears to be of considerably less importance. The latter may in fact be rationally understood from our experiments since we show that dendriplexes transferred from low ionic strength buffers to cell growth medium or high ionic strength buffer are largely altered. Thus changes in the surrounding environment affect hugely the unpacking of DNA, and it is likely that the environment inside the cell nucleus, with a large excess of DNA and many other charged molecules, offers naturally a milieu where the dendriplexes can dissociate to a much higher degree than in the test tube. The *in vitro* transcription experiments had to be performed at relatively low ionic strength (40 mM) since the T7 RNA polymerase is very sensitive to salt inhibition. Therefore it is not possible to directly compare these results to the morphological effects on dendriplexes upon exposure to cell growth medium. However, given the sensitivity of dendriplexes to increases in ionic strength, it is reasonable to assume that the morphology of the dendriplexes in the

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transcription buffer is altered although probably not to the same extent as in cell growth medium. To conclude, although it is still of interest to study the vector unpacking event, it appears not to be the factor limiting the transfection efficiency in our system.

Summary

In this work, we have investigated a series of surface-modified dendrimers for potential use as gene-delivery vectors. In contrast to a few previous studies, we find that PEGylation and acetylation, although improving the cytotoxicity profile, significantly decrease the transfection ability of PAMAM dendrimers. We believe that this is due to a decreased potential in condensing DNA, as observed by dynamic light scattering and DNA availability assays. It appears that the exact PEG chain length and degree of surface coverage are parameters of paramount significance for retaining favorable DNA condensing capabilities of PAMAM dendrimers.

We have also investigated the effect on *in vitro* transcription of condensing DNA with dendrimers. This is interesting from a vector-unpacking perspective: does a good gene delivery vector readily allow transcription, or is tight packing of the DNA, providing protection and facilitating uptake, more important? It appears that the latter is true, as SuperFect, one of the most efficient nonviral gene delivery vectors available to date, practically completely inhibits transcription *in vitro*.

Finally, we note that exposure to cell growth medium dramatically alters the morphology of dendriplexes that are formed under conditions of low ionic strength. This might be one reason for previous failures in drawing structure−activity conclusions for various gene delivery systems, and we emphasize the importance of performing biophysical characterization of DNA−vector complexes under conditions as close to the transfection situation as possible. We also suggest it is taken as a role when performing availability studies of DNA to add the compacting agents before introducing the intercalating probe, in order to avoid interference with the complex formation.

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