Transfection Agents

Amphiphilic Dendrimers: Novel Self-Assembling Vectors for Efficient Gene Delivery**

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Nonviral gene delivery remains an outstanding challenge to the scientific community.[1] Fifteen years of interdisciplinary effort since the introduction of cationic amphiphiles for DNA and RNA transfection by Felgner et al.[2] compare to millions of years of evolutionary optimization of the competing viral vectors. While actual progress has been moderate, some key issues for efficient delivery have been identified.[3] Biocompatibility, charge/receptor-mediated uptake, tissue-specific targeting, endosomal escape, nuclear tropism, and vector unpacking all contribute to the canon of requirements. The hunt for a "magic bullet" has led to a stunning multitude of synthetic vector systems, including small molecule amphiphiles,[4] linear, branched, and more sophisticated block copolymers,[5] as well as dendrimers.[6,7] As a recent development, polymeric controlled-release drug-delivery systems

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[**] Financial support by the NCCR "Nanoscale Science" is gratefully acknowledged.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

  DOI: 10.1002/ange.200250284

have also shown promising features.[8] While members of all classes have been commercialized, and some structure–activity relationship (SAR) data compiled, no breakthrough has yet been achieved. It seems that "magic potions" rather than "magic bullets"[9] will provide the three orders of magnitude of efficiency that the nonviral systems are still lacking.

However, classic architectures can still be optimized in surprising ways.[10] Nevertheless, the above observations have induced a search for new modular self-assembling platforms that try to combine features of the first-generation vectors.[11]

With the aim of producing programmable supramolecular architectures of defined and controllable composition in space, it seems advantageous to introduce a rigid scaffold. Drawing on the low toxicity, geometric tunability, and ease of multiple functionalization of cationic dendrimers on the one hand and the classic self-assembly of amphiphilic molecules on the other, we have developed a set of molecular building blocks to prepare amphiphilic dendrimers with rigid cores which feature a variety of geometries and substitution patterns. Self-assembly is of central importance, as it permits remaining at relatively low molecular weight, which has been shown to be advantageous in other systems.[12] High cationic charge density at the dendrimer surface should favor both DNA binding and endosomal escape once internalized. Here we describe the series of amphiphilic dendrimers **1**–**4** (for the synthesis, see the Supporting Information), based on a rigid tolane (diphenylethyne) core and describe their remarkable activity as transfection agents with a surprising structure–activity relationship.

Preliminary DNA-binding experiments were carried out by a dye-exclusion assay (see Experimental Section).[13] As a measure for binding affinity, c_{50} and CE_{50}, the concentration and charge excess ratio,[14] respectively, at which 50% of the dye complexed to DNA was displaced by added compounds **1**–**4**, were determined (Table 1). Ranking compounds based on their CE_{50} values gives an idea of the per-charge affinities, that is, the affinities based on the c_{50} value divided by the nominal charge of the molecule in question. In this way, a binding affinity ranking based on c_{50} was found to be $\mathbf{3} > \mathbf{2} > \mathbf{1} > \mathbf{4}$ (based on CE_{50}: $\mathbf{2} \cong \mathbf{3} > \mathbf{1} \gg \mathbf{4}$). These data suggest that the number of C_{12} chains in the lipophilic dendron has an important influence on binding affinity. The 2.5-fold increase in the c_{50} value from **3** to **2** is easily explained by the concomitant threefold decrease in nominal charge. As expected, the difference disappears when the ranking is based on the CE_{50} value. In contrast, going from three lipophilic side chains in **2** to one in **1** results in a twofold higher c_{50} value, and changing from three chains in **3** to nine in

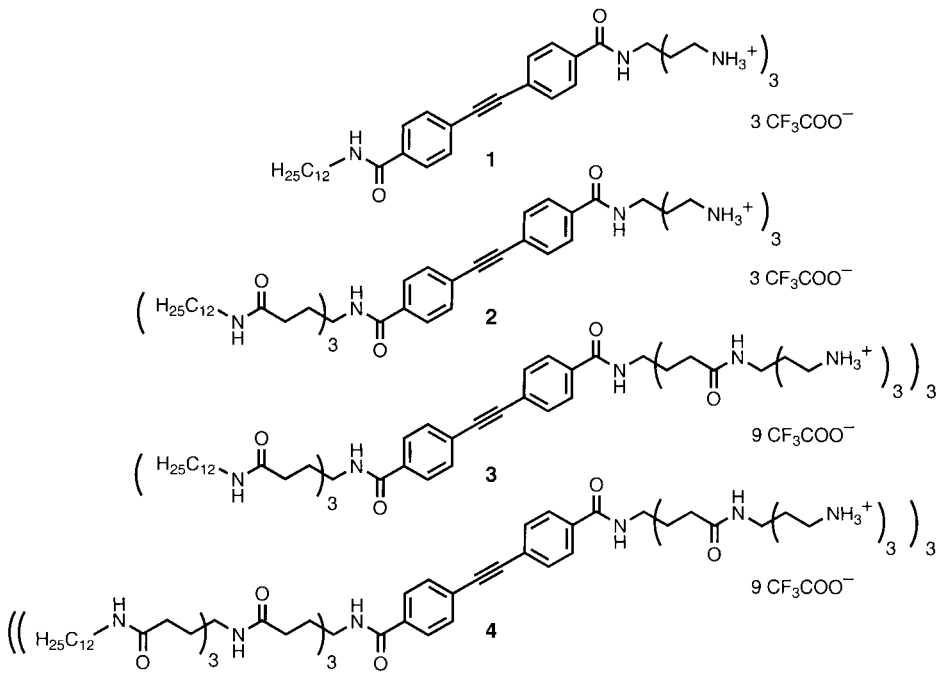

Table 1: Dye-exclusion assay.[13][a]

	Number of C_{12} chains	Nominal charge	M_W [g M^{-1}][b]	c_{50} [μM][c]	CE_{50}[d]
1	1	3+	917.9	5.4	5.4
2	3	3+	1481.8	2.5	2.5
3	3	9+	2679.7	1.0	3.0
4	9	9+	4371.4	9.0	26.9

[a] GFP reporter plasmid (1 μg mL^{-1}) and Picogreen dye[18] concentrations were kept constant. The PicoGreen–DNA complex fluorescence intensity was monitored at $\lambda_{em} = 530$ nm ($\lambda_{exc} = 485$ nm) and was plotted against the concentration (*c*) or charge excess ratio[14] (*CE*) of compounds **1**–**4**. [b] Molecular weight including trifluoroacetate counteranions. [c] Concentration at which the fluorescence intensity was reduced to 50%. [d] *CE* value at which the fluorescence intensity was reduced to 50%.

4 even gives a ninefold increase. Clearly, a structure with three lipophilic chains is strongly favored. However, **4** can still almost completely displace the dye, that is, compete for all the binding sites on the DNA. This result gives rise to the assumption that **4**, with its large lipophilic segment, may form stable aggregates that interact with DNA on a small part of their surface only. Clearly, the self-assembly[15] properties play a much more important role on binding than anticipated.

In a study of the transfection efficiency (TE), systematic variation of the *CE*[14] gave clear and strong maxima for **2** (CE_{max} = 8–12, c_{max} = 20–30 μM) and **3** (CE_{max} = 12–16, c_{max} = 10–14 μM; Figure 1). Dendrimers **1** and **4** did not show

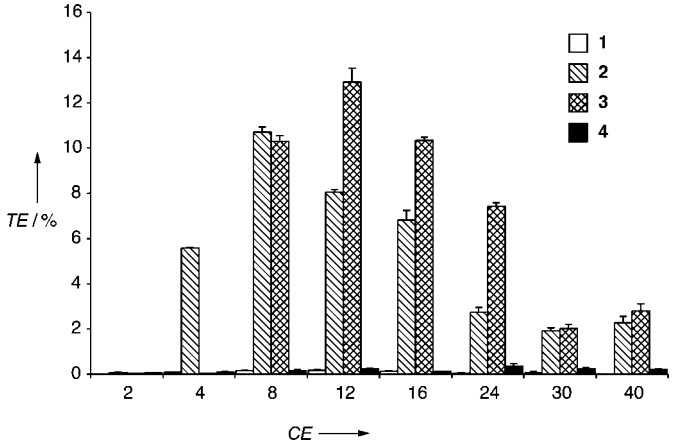

Figure 1. Transfection efficiency (TE) of **1**–**4** is given as a function of the charge excess ratio *CE*. Standard deviation (SD, n = 2) within an experiment was less than 10%. The SD (n = 3) became much larger (up to 30%) between experiments, primarily because of the fact that the absolute position of the maxima varied (CE_{max} = 8–12 for **2**, CE_{max} = 12–16 for **3**). However, the separation between the maxima of **2** and **3** was always $\Delta CE_{max} = 4$. The data shown are from one experiment carried out in duplicate.

significant transfection activity. Again the geometry and thus the aggregation properties determined by the lipophilic dendron in the molecule dominate the activity, as indicated by the comparison between dendrimers with identical hydrophilic dendrons (**2** versus **1** and **3** versus **4**, respectively). While the size and nominal charge of the hydrophilic part affects the optimum concentration (c_{max} of **3** is one half that of **2**), the absolute TE is not significantly affected by it. The small difference that was observed could be attributed to the fact that the surface charge density of **3** is higher, thus the pK_a values of some of the terminal ammonium centers should drop and some buffer capacity be gained. This effect is consistent with the proton sponge endosomal escape hypothesis.[16]

To assess the overall quality of our vectors we used commercially available compounds as a reference. A clear ranking could be determined from the maximum TE values observed (Figure 2): **3**≅**2** > DOTAP > PEI≅SuperFect > **4** > **1**. Only very basic optimization has been carried out so far, and even at this unrefined state the activities of our low generation amphiphilic dendrimers **2** and **3** surpass those of traditional medium-sized poly(amidoamine) (PAMAM) dendrimers (SuperFect) by a factor of 3 to 5.

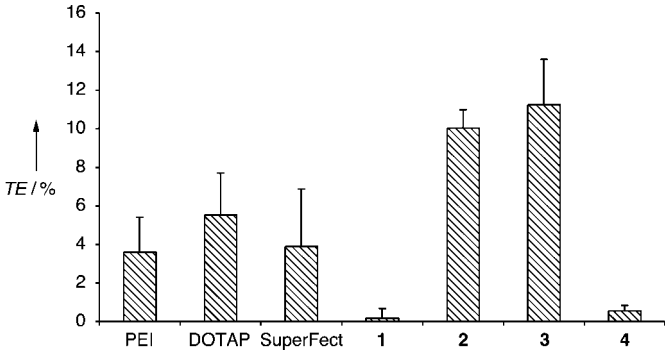

Figure 2. Maximum TE of **1**–**4** at optimum conditions are compared to reference compounds PEI, DOTAP, and SuperFect. Error bars represent SD (n = 3) between different experiments, each carried out in duplicate.

All the compounds in the optimum *CE* range (8–16) were found to have low cytotoxicity (Figure 3). Performance was similar to the reference compounds, the ratio of dead cells (R_{dead}, see Experimental Section) being less than 20%. Toxicity increased at a disproportionate rate for $CE > 16$ ($CE > 30$ for **4**). This trend coincides with the drop in

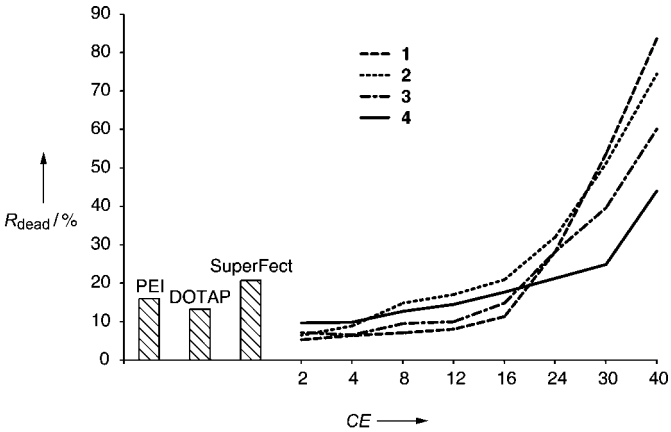

Figure 3. The ratio of dead cells (R_{dead}) after exposure of the cells to the vector–DNA constructs of **1**–**4** (lines) is given as a function of *CE*. R_{dead} is also given for the reference compounds DOTAP and SuperFect under the conditions according to the manufacturer's instructions (columns).

transfection activity at the same *CE* values. Lethal concentrations that would kill 50% of the exposed cells (LC_{50}) were estimated (**1**: 75, **2**: 75, **3**: 30, and **4**: 35 μM). These values are at least three times higher than the optimal concentration range for transfection with the respective compound. It is clear that the tripling in nominal charge from **2** to **3** is responsible for a 50% decrease in the LC_{50} value. The effect of the lipophilic part of the molecules seems to be of less importance. Toxicities at optimum concentrations for transfection do not show a recognizable correlation with structural features.

The presence of serum is known to impede transfection in the case of small molecular amphiphiles. Thus, exposure of the cells to vector–DNA constructs is usually carried out in a medium with reduced serum, or completely without serum.

To assess the effect of serum on the TE we carried out a *CE* series in the presence of fetal bovine serum (FBS) during the transfection step (see Experimental Section).

To our surprise, compounds **2** and **3** that performed so similarly in all other experiments did exhibit a significant difference in this regard (Figure 4). While **3** lost almost all of its transfection activity, **2** was much less affected, and

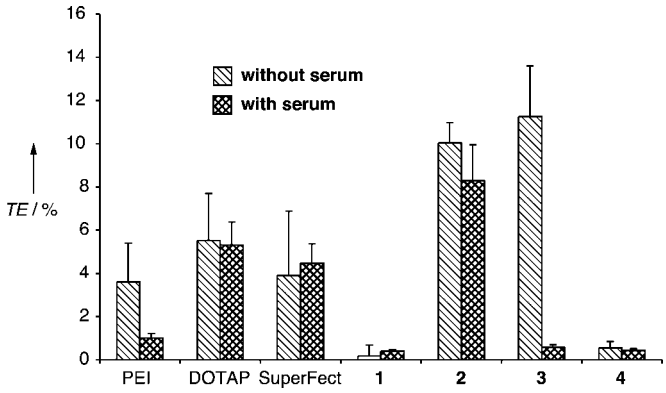

Figure 4. TE of **1**–**4** as well as the reference compounds PEI, DOTAP, and SuperFect are contrasted for the absence (hatched columns) and presence (crosshatched columns) of fetal bovine serum (FBS) during the incubation of the vector–DNA construct with cells. Error bars represent SD ($n = 3$) between different experiments, each carried out in duplicate.

maintained an activity comparable to DOTAP. The *CE* series for **2** (as in Figure 1) revealed a lower, broad maximum (data not shown) centered around $CE = 8$. The strongly reduced activity of **3** in the presence of serum probably results from the presence of anionic serum proteins. These could significantly alter the structure of the DNA complex of **3** with its high nominal charge (9+) in its hydrophilic part. This result warrants a detailed study on the aggregation states in aqueous buffers, which is currently underway.[15]

Assembly of DNA–dendrimer complexes was studied by tapping mode atomic force microscopy (AFM). Single adsorbed plasmids became clearly visible (Figure 5A) when pure DNA was deposited by drop casting on freshly cleaved mica in the absence of **2** (see Experimental Section). Deposition of **2** without DNA gave homogenous thin layers

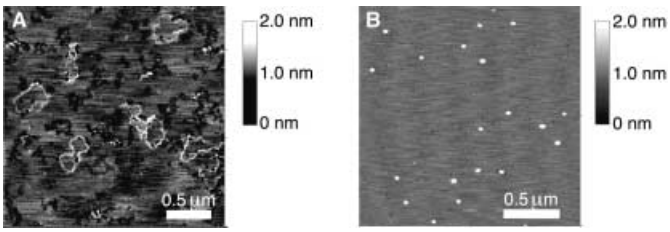

Figure 5. A) AFM image of pGFP on mica in the absence of dendrimer. No divalent cations were added, such that only a small amount of DNA is adsorbed on the surface. Individual plasmids are visible as white loops. B) AFM image of pGFP and **2** mixed as described in the Experimental Section and deposited on freshly cleaved mica. The structures observed resembled flattened spheres (height: 5–10 nm, diameter: 60–80 nm). No free plasmid was found. This is a clear indication of efficient condensation of DNA by compound **2**.

of about 1.5 nm thickness (not shown). However, a large number of flattened spheroidal or slightly oblong complexes were observed when DNA and **2** were mixed at $CE = 5.6$ prior to deposition (Figure 5B). No free plasmid could be detected. This result clearly indicates that **2** not only binds to DNA, as demonstrated by the dye-exclusion assay, but also very efficiently condenses DNA into well-defined structures.

To conclude, a series of amphiphilic dendrimers featuring extended rigid cores has been prepared, which not only showed high transfection activity, but also brought a couple of surprises. A sharp maximum in SARs, a much stronger influence of the hydrophobic part on DNA binding and transport than anticipated, low toxicity, and an unusual serum effect warrant more detailed analysis of the factors at hand. In contrast to classical cationic dendrimers, where there is a minimum size for transfection, our dendrimers do not show that limitation. However, while an optimum M_W value greater than 116000 was found for PAMAM dendrimers,[7] we determined an optimum activity with rather small dendrimers ($M_W = 1500$–2700). A similar effect has been noted for lysine oligopeptides, which reached maximum efficiency at $M_W = 2800$.[17] While the biological characterization is far from complete, we are confident that the synthetic building blocks and the modular vector assembly strategy will enable us to explore this new class of amphiphilic dendrimers in breadth and depth.

Experimental Section

Green fluorescent protein reporter plasmid (pGFP) was generated by cloning the green fluorescent protein (GFP) gene into the VR1012 vector. Plasmid DNA was prepared with Qiagen (Basel, Switzerland) endotoxin-free buffers according to the manufacturer's instructions and resuspended in sterile Millipore 18.2 MΩ water at 1 mg mL^{-1}. The bacterial β-galactosidase (LacZ) containing plasmid (placZ) was generated accordingly.

DNA binding study: Binding affinity was monitored by a dye-exclusion assay.[14] GFP reporter plasmid (pGFP) was used for all experiments. The pGFP (1 µg mL^{-1}) and dye concentration where kept constant in the presence of increasing amounts (1–20 µM) of compounds **1**–**4**. Expulsion of the PicoGreen dye[18] from the complex with pGFP was followed by the reduction of its fluorescence intensity I_{530} ($\lambda_{exc} = 485$, $\lambda_{em} = 530$ nm). The change in fluorescence intensity (ΔI_{530}) was plotted against the concentration (or *CE*). The concentrations (or *CE*) at which 50% of the dye are displaced from the complex with pGFP (c_{50} and CE_{50}) were determined from the titration curves.

Transfection efficiency study: For transfection experiments, the human embryonic kidney HEK293 model cell line (ATCC CRL-1573) was cultured as previously described.[19] Cells were transferred into 96-well plates to give approximately 2×10^5 cells per well at 90% confluency. pGFP was mixed with different volumes of thoroughly sonicated solutions of dendrimers **1**–**4** (1.0 mg mL^{-1} in 20 mM HEPES[20] buffer at pH 7.4). Equilibration of the samples was allowed for at least 30 min before vortexing and adding Dulbecco's modified Eagle medium (DMEM[21]) growth medium containing penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}). The growth medium was removed from the cells before aliquots of 0.5 µg pGFP per well were added in a total volume of 200 µL. Cells were then incubated (37 °C, 5% CO_2) for 2 h. The plasmid-containing medium was then replaced by DMEM (penicillin (100 U mL^{-1}), streptomycin (100 µg mL^{-1}), 10% FBS) and the cells incubated (37 °C, 5% CO_2) for another 24 h. Transfection efficiency[22] (TE) was determined by

manual counting of green-fluorescent cells under a fluorescence microscope. The commercially available transfection agents DOTAP[23] and SuperFect[24] were used as reference compounds according to the manufacturer's instructions (scaled to 0.5 μg pGFP per well). For two wells, 24 μL of a solution (42 $\mu g\,mL^{-1}$) of poly(ethyleneimine) (PEI)[25] were mixed with 1 μL of pGFP stock solution and incubated for 30 min before diluting to 400 μL with DMEM. All experiments were carried out in duplicate or triplicate and repeated at least once.

Cell viability study: Cells were transfected with placZ according to the experimental details given for the transfection study at *CE* values between 2 and 40. The ratio of dead (R_{dead}) cells was determined after application of a "Live/Dead" kit[26] according to the manufacturer's instructions. Readout of the data was performed with a fluorescence plate reader (transfection with pGFP would have given false high readings for live cells). LC_{50} values were estimated by linear interpolation of the R_{dead} values (extrapolation in the case of **4**).

Serum effect study: Exactly the same conditions as for the transfection study were used, with the sole difference that, after equilibration of vector and DNA, DMEM containing 10% FBS was added and incubation of cell with vector and DNA was thus carried out in the presence of serum.

AFM: Studies were performed with a Nanoscope 3100 (Digital Instruments, Santa Barbara, CA) operated in noncontact (tapping) mode using NSG01 probes (NT-MDT, Moscow) with a spring constant of 2.5–5.5 $N\,m^{-1}$ and a resonance frequency of 120–150 kHz. Measurements were done under ambient conditions (20 °C). Image analysis was performed with the Nanoscope III Control (Digital Instruments, Santa Barbara, CA) software package. Unless otherwise stated, data processing was limited to first- or second-order plane fitting and zeroth or first-order flattening.

Drop casting: 10 μL of a solution of pGFP (0.2 $ng\,\mu L^{-1}$) were mixed with 2 μL of a solution of **2** in Millipore water (8 $ng\,\mu L^{-1}$). After equilibration (30 min), 2 μL of this mixture were deposited on freshly cleaved mica. The water was then allowed to evaporate (1 h at 20 °C, ambient pressure). Imaging was performed immediately afterwards.

Received: October 2, 2002 [Z50284]

Keywords: amphiphiles · cross-coupling · dendrimers · DNA recognition · gene transfection

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[15] Self-assembly of **1**–**3** was observed by dynamic light scattering measurements in aqueous HEPES buffer after sonication. Preliminary data show a particle size distribution with a maximum centered around 100 nm for all compounds. Both **1** and **3** exhibit one additional maximum centered around 5 nm; D. Joester, S. Bufali, P. Walde, P. L. Luisi, F. Diederich, unpublished results.
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[20] HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
[21] GIBCO high-glucose DMEM (4.5 $g\,L^{-1}$ glucose) from Invitrogen life technologies (Basel, Switzerland).
[22] TE is defined as the number of transfected cells divided by the total number of cells.
[23] DOTAP is an aqueous dispersion of *N*-[1-(2,3-dioleoyl-oxy)-propyl]-*N,N,N*-trimethylammonium methyl-sulfate from Roche Diagnostics. It carries one permanent charge and is a classic small-molecule amphiphile.
[24] SuperFect is an activated (that is, fractured) PAMAM dendrimer formulation from Qiagen (Basel, Switzerland).
[25] PEI $M_W = 600\,000$–$1\,000\,000$ from FLUKA (Buchs, Switzerland) is a randomly branched cationic polymer.
[26] Live/Dead Kit from Molecular Probes (Lucerne, Switzerland).

