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Hydrophobically Modified Dendrons: Developing Structure−Activity Relationships for DNA Binding and Gene Transfection

Simon P. Jones,[†] Nathan P. Gabrielson,[‡] Chun-Ho Wong,[§] Hak-Fun Chow,[§] Daniel W. Pack,[‡] Paola Posocco,[∥] Maurizio Fermeglia,[∥] Sabrina Pricl,[∥] and David K. Smith*[,†]

[†]Department of Chemistry, University of York, Heslington, York, YO10 5DD, U.K.

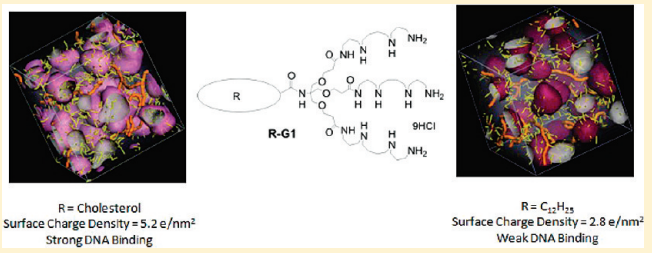
[‡]Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, United States

[§]Department of Chemistry and The Center of Novel Functional Molecules, University Science Centre, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong

[∥]Molecular Simulation Engineering Laboratory, Department of Materials and Natural Resources, University of Trieste, 34127 Trieste, Italy

Supporting Information

ABSTRACT: This paper develops a structure−activity relationship understanding of the way in which surfactant-like dendrons with hydrophilic spermine surface groups and a variety of lipophilic units at their focal points can self-assemble and subsequently bind to DNA with high affinity. The choice of functional group at the focal point of the dendron and the high tunability of the molecular structure have a very significant impact on DNA binding. Mesoscale modeling of the mode of dendron self-assembly provides a direct insight into how the mode of self-assembly exerts its effect on the DNA binding process. In particular, the hydrophobic unit controls the number of dendrons in the self-assembled micellar structures, and hence their diameters and surface charge density. The DNA binding affinity correlates with the surface charge density of the dendron aggregates. Furthermore, these structure−activity effects can also be extended to cellular gene delivery, as surface charge density plays a role in controlling the extent of endosomal escape. It is reported that higher generation dendrons, although binding DNA less strongly than the self-assembling lower generation dendrons, are more effective for transfection. The impact of the lipophilic group at the focal point is less significant for the DNA binding ability of these larger dendrons, which is predominantly controlled by the spermine surface groups, but it does modify the levels of gene transfection. Significant synergistic effects on gene delivery were observed when employing combinations of the dendrons and polyethyleneimine (PEI, 25 kDa), with transfection becoming possible at low loading levels where the two components would not transfect individually, giving practically useful levels of gene delivery.



KEYWORDS: dendron, DNA, gene delivery, mesoscale modeling, self-assembly

INTRODUCTION

In the emergent field of nanomedicine, gene therapy is one of the most widely investigated topics, however, as yet, a general approach to gene therapy *In Vivo* remains elusive.[1] The development of vectors that are capable of delivering genetic material, such as DNA or siRNA, safely and efficiently into cells is therefore an important area of research, and could have a dramatic impact on diseases such as cystic fibrosis.[2] Viral vectors were employed in some of the earliest clinical trials of gene therapy, but it was observed that they can cause problems with immunogenicity and adverse patient response.[3] For this reason, increasing attention has focused on the development of nonviral, synthetic vectors.[4] Nonviral vectors such as Superfect[5] or Lipofectamine[6] are commercially available for in vitro transfections. Furthermore, some nonviral vectors have been investigated in the clinic, with some promising results, for example, in phase 1 trials on cystic fibrosis patients.[7] Nonviral vectors can be divided into two main classes: cationic polymers[8] and cationic lipids.[9] In both cases, cationic charge is required to effectively bind and condense the nucleic acid, which is a polyanion. Cationic polymers have high charge due to their polymeric nature; however this can lead to adverse toxicity.[10] One particularly important class of cationic polymer employed in transfection studies is well-defined branched polymers: dendrimers.[11] Poly(amidoamine) (PAMAM) dendrimers[5,12] and dendrimers based on L-lysine[13] have been widely investigated as DNA delivery agents. The second class of nonviral vector, cationic lipids, achieves high charge via self-assembly, with vector aggregates being responsible for gene delivery; work

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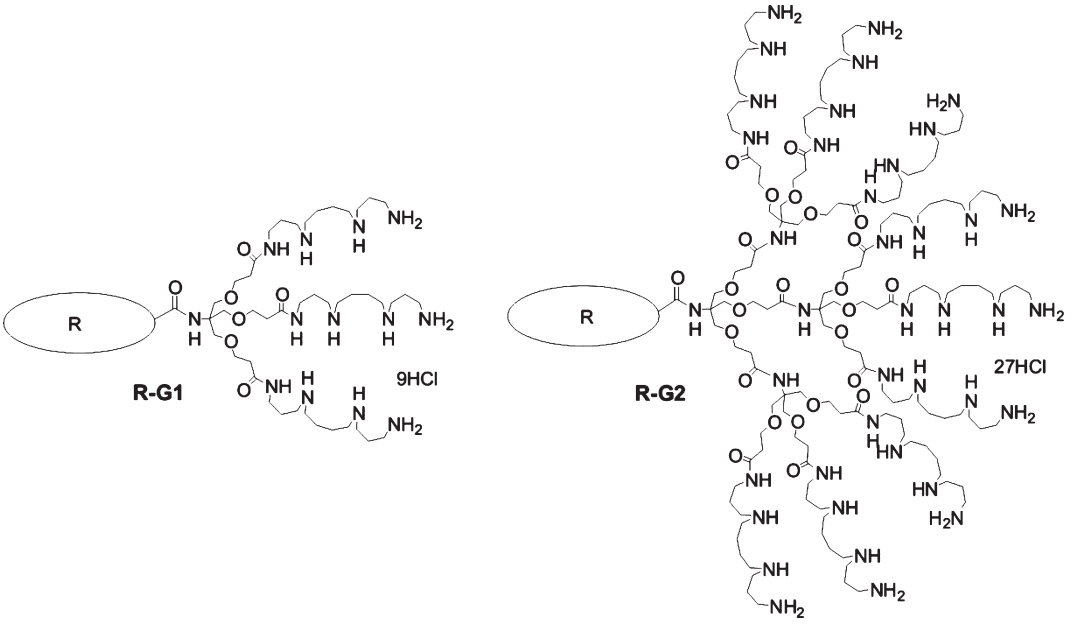


Figure 1. General structure of first and second generation dendrons with spermine surface groups (R-G1 and R-G2).

is ongoing to increase the load of genetic material that can be delivered and decrease toxicity.[9,10]

Developing a detailed structure−activity relationship of transfection is of key importance. For example, Kirby and co-workers demonstrated that gene expression in their cationic lipid derived system was dependent on chain length and amide bond linkage,[14] and since this study, there has been intense interest in developing meaningful structure−activity relationships which correlate molecular-scale modifications with biological gene transfection behavior.[15] In elegant research, the groups of Diederich, Florence and Hammond have explored different series of well-defined dendritic amphiphiles.[16] They revealed that the most active vectors in transfection were able to self-assemble under neutral to endosomal pH. Further studies from Diederich and co-workers showed that their most versatile transfection vectors self-assembled into vesicles or bilayers.[17]

We have developed a class of DNA binder based on a dendritic scaffold with multiple spermine surface groups (Figure 1).[18] Spermine is a bioavailable DNA binder which operates at high micromolar concentrations.[19] We demonstrated that the presence of multiple spermine groups on the dendron led to a multivalency effect, enhancing DNA binding. These dendrons had relatively poor transfection abilities, being several orders of magnitude less effective than polyethyleneimine (PEI), which was used as a positive control.[20] However, we recently reported that modification of the focal point with a lipophilic cholesterol group could lead to significantly enhanced DNA binding and transfection.[21] This was attributed to the lipid-like self-assembly of the dendron into a supramolecular aggregate improving the performance of the system, a hypothesis which was confirmed by mesoscale molecular modeling.[22] As such, we argued that our dendrons combined aspects of both classes of nonviral vector: cationic polymers and lipids. It has also been noted that combining the advantages of both cationic polymers and lipids in a single vector system can give rise to synergistic effects on gene delivery.[23]

We therefore decided to monitor the way in which the structure of the hydrophobic unit modified the DNA binding and gene delivery performance of this class of dendron. Indeed, the high synthetic tunability of dendron structures provides them with a significant advantage over larger spherical dendrimer structures. This can be done in a relatively straightforward manner and allows us to develop structure−activity relationships, in which the structural changes made to the dendron can be correlated with DNA binding and transfection abilities. By using mesoscale molecular modeling, we are able to rationalize our observations. In this way, we employ this combined experimental and theoretical approach to develop effective design principles for smart gene delivery vectors; these results will therefore inform future research in this field.

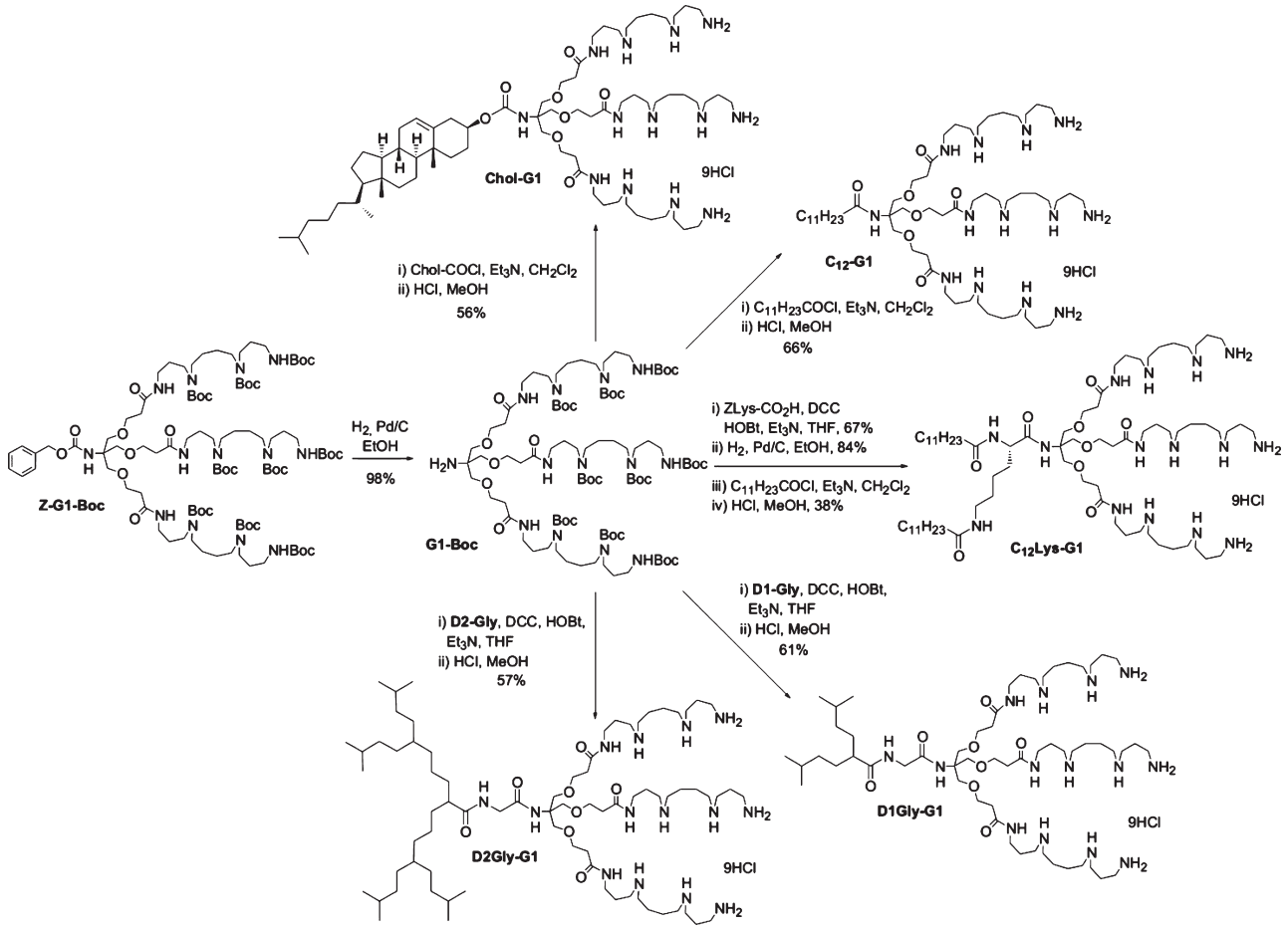
RESULTS AND DISCUSSION

Synthesis of First Generation Vectors. The first generation vectors investigated in this paper were synthesized from previously reported dendron Z-G1-Boc which has a Z-protecting group at the focal point, and has three spermine surface groups protected by multiple *N-tert*-butyloxycarbonyl (Boc) protecting groups (Scheme 1).[18] The Z-protecting group was removed from Z-G1-Boc by hydrogenolysis, to yield G1-Boc, which has a reactive amine group at the focal point. Five different lipophilic units were then selected for attachment to the free amine. The simplest of these were a commercially available dodecanoyl chain and a cholesterol unit. We also selected three synthetic branched systems, one based on lysine functionalized with dodecanoyl chains, the other two being all-aliphatic dendrons previously reported (and provided) by Chow and co-workers.[24]

Dendron Chol-G1 was synthesized in a two step process using the previously reported methodology.[21] First, cholesteryl chloroformate was reacted with G1-Boc in CH_2Cl_2 in the presence of Et_3N, and subsequently the Boc groups were removed by dissolving the product in methanol and bubbling HCl gas through the solution, providing the product in 56% yield.

Novel dendron C_{12}-G1 was synthesized in a similar way by first reacting G1-Boc with dodecanoyl chloride, and then the Boc groups being removed using HCl gas, giving product in a 66%

Scheme 1. Synthesis of First Generation Vectors, Chol-G1, C_{12}-G1, C_{12}Lys-G1, D1Gly-G1 and D2Gly-G1, with Different Hydrophobic Groups at the Focal Point



yield. In order to synthesize the novel dendron C_{12}Lys-G1, we initially reacted G1-Boc with *N*,*N*′-bis(benzyloxycarbonyl) lysine using DCC and HOBt, in anhydrous THF with Et_3N as base, to yield ZLys-G1-Boc. The benzyloxycarbonyl (Z) protecting groups were then removed from the lysine unit by hydrogenolysis, and the unmasked free amines were reacted with dodecanoyl chloride in anhydrous CH_2Cl_2 to yield C_{12}Lys-G1-Boc in 56% yield over the two steps . Finally, the Boc protecting groups were removed with gaseous HCl in methanol to provide the desired C_{12}Lys-G1 in a 38% yield.

We then attempted to conjugate the aliphatic dendrons (D1 and D2) to G1-Boc. However, we were unable to directly couple these dendrons via standard peptide coupling methodologies, presumably due to the steric hindrance associated with reacting together the focal points of two dendritic building blocks. We therefore appended a glycine spacer unit onto Chow's dendrons (see Supporting Information for the methodology).[25] These extended hydrophobic dendrons (D1Gly and D2Gly) could be smoothly coupled to G1-Boc using DCC/HOBt methodology. The Boc groups were finally removed with HCl gas, providing the desired novel products, D1Gly-G1 and D2Gly-G1, in good yields of 61% and 57% respectively.

DNA Binding Studies with First Generation Vectors. Initially we assayed the ability of these dendrons to bind DNA using the standard ethidium bromide (EthBr) displacement fluorescence spectroscopy assay which we have employed in our previous research.[18,26] This method uses competition between the DNA binder and EthBr to assess the concentration at which the DNA binder becomes effective. This can be expressed as the concentration of DNA binder required for half of the EthBr to be displaced from binding to DNA: a C_{50} value. This concentration can more usefully be expressed as a charge excess (CE_{50}) value, better suited for comparing structurally different DNA binders with different molar masses and numbers of amines. To calculate this CE_{50} value, it is assumed that each amine in the DNA binder is protonated, and each phosphate in the DNA is deprotonated. As such, the CE_{50} value is equivalent to a N:P ratio. Lower CE_{50} values represent more effective binding, as a smaller amount of positive charge is required to bind the negative charge associated with the DNA. This assay therefore provides an excellent comparative method for considering the DNA affinities of a family of compounds such as this, in which each member has the same spermine-based DNA binding motif and, as such, is ideally suited for the development of structure−activity relationships. For this study, we compared all of our DNA binders under physiologically relevant salt concentrations (150 mM NaCl).

In our previous studies, we had demonstrated that Z-G1 bound to DNA under the conditions of this assay with a CE_{50} value of 2.7 (Table 1). This was a significant improvement over simple, unfunctionalized spermine, which had a CE_{50} value of 1560, clearly demonstrating the benefits of using a multivalent DNA binding scaffold. Furthermore, we have previously reported

Table 1. Binding Data for First Generation Compounds with DNA Obtained via Ethidium Bromide (EthBr) Displacement Assay[a]

compound	CE_{50} value (N:P ratio)
spermine	1560
D1Gly-G1	4.3
C_{12}-G1	3.3
Z-G1	2.7
D2Gly-G1	1.8
C_{12}Lys-G1	0.85
Chol-G1	0.55

[a] CE_{50} represents the charge excess (N:P ratio) required to displace 50% of the EthBr. [NaCl] = 150 mM.

that modification of the dendron with a cholesterol unit significantly enhanced DNA binding as a consequence of dendron self-assembly, reducing the CE_{50} to just 0.55.[21] This new study therefore allows us to develop an understanding the precise role of the lipophilic unit in mediating the self-assembly, and subsequent DNA binding affinity, of the dendrons. Clearly, modification of the lipophilic unit at the focal point exerts a dramatic effect on the affinity of the dendrons for DNA, even though this group is not itself directly responsible for forming interactions with DNA. Intriguingly, some of the focal point modifications appeared to improve DNA binding, while others had an adverse effect. Dendrons Chol-G1 and C_{12}Lys-G1 had the lowest CE_{50} values of 0.55 and 0.85 respectively, indicating very effective binding indeed, some of the best reported.[27] Compound D2Gly-G1 was somewhat better than Z-G1 having a CE_{50} value of 1.8. On the other hand, compounds C_{12}-G1 and D1Gly-G1 had higher CE_{50} values of 3.3 and 4.3 respectively, indicating they have lower affinities for DNA than the Z-G1 analogue.

In order to support the results of these EthBr assays, we also carried out gel electrophoresis using plasmid DNA. This method has the advantage of directly measuring the binding between the dendrons and DNA, i.e., it is not a competition assay. Furthermore, it is performed under conditions equivalent to the transfection assays reported later in this paper. In the gel electrophoresis experiment, 1 μg of DNA was applied to each well, and the loading of DNA binder required to completely retard the movement of the DNA could then be determined (Figure 2). Pleasingly, the data were in general agreement with the EthBr exclusion assay. The most effective binder was Chol-G1, which proved to be a highly effective DNA binder, retarding DNA mobility above loadings of 0.153 nmoles (N:P ratio, 0.45). Once again, the least effective binders were C_{12}-G1 and D1Gly-G1, which only retarded mobility at loadings above ca. 0.40 nmol (N:P ratio, 1.0). The other dendrons were intermediate in behavior, although C_{12}Lys-G1 was noted to be slightly less effective in this assay than in the EthBr exclusion experiment.

It is therefore clear that although the DNA binding event is located at the spermine surface groups, the unit at the focal point also has a profound influence on binding. In an attempt to rationalize these observations and better understand the structure−activity effects, we employed mesoscale modeling methods.

Modeling Dendron Self-Assembly. Many interesting problems in soft matter science occur at length and time scales sandwiched between the atomistic scale and the macroscopic continuum. Systems such as nanovector/DNA complexes for

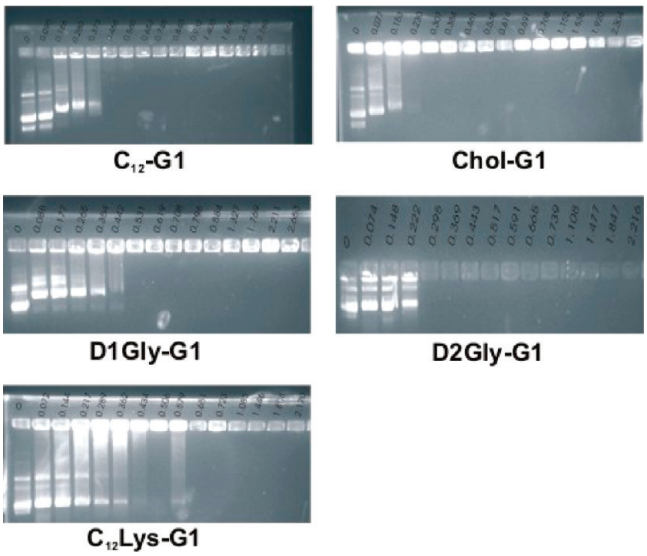


Figure 2. Agarose gel electrophoresis of polyamine/DNA complexes using first generation dendrons. Polyamine:DNA loadings (w:w) are as follows: lane 1, 0:1; lane 2, 0.1:1; lane 3, 0.2:1; lane 4, 0.3:1; lane 5, 0.4:1; lane 6, 0.5:1; lane 7, 0.6:1; lane 8, 0.7:1; lane 9, 0.8:1; lane 10, 0.9:1; lane 11, 1:1, lane 12, 1.5:1; lane 13, 2:1; lane 14, 2.5:1; lane 15, 3:1.

gene transfer can involve spatial inhomogeneities over length scales ranging between 1 and 1000 nm, and exhibit phenomena over time scales of 1 ms or greater. Problems in such length−time space cannot be addressed by traditional atomic-level molecular dynamics, or by conventional finite element approaches that usually deal with phenomena at longer time scale. Rather, one needs to take recourse to computational techniques at the intermediate scale, called the *mesoscale*. Over the past few years, different approaches have been developed to address problems at the mesoscale level, which could be broadly classified as particle-based or density-based. With the purpose of studying the eventual self-assembly morphology of the modified dendron series developed in this work, and their interaction with DNA, we resorted to using a particle-based method called dissipative particle dynamics (DPD).[28]

In DPD, a group of atoms is coarse-grained into a bead, thereby substantially reducing the number of particles to be simulated. Further, rather than interact through Lennard-Jones forces, the beads feel a simple soft pairwise conservative potential which embodies the essential chemistry of the system. This force is of short range, and has a simple analytical form, which results in fast computation per time step and, hence, provides the opportunity to expand the simulation from nanoseconds to real time periods.

Accordingly, we began our DPD-based simulation study by monitoring the dendron self-assembly processes and to gain an insight into the types of aggregates which may be formed. Figure 3 shows that all hydrophobically modified dendrons form supramolecular structures with nanometer dimensions (see Table 2). All of these first generation dendrons form spherical monodisperse micelles, with diameters D_m of 3−5 nm. This is a direct consequence of their conical shape with a relatively large cationic headgroup connected to a comparatively small lipophilic part (*vide infra*).

A simple but effective molecular theory can be invoked to qualitatively describe the evidence inferred from the mesoscale

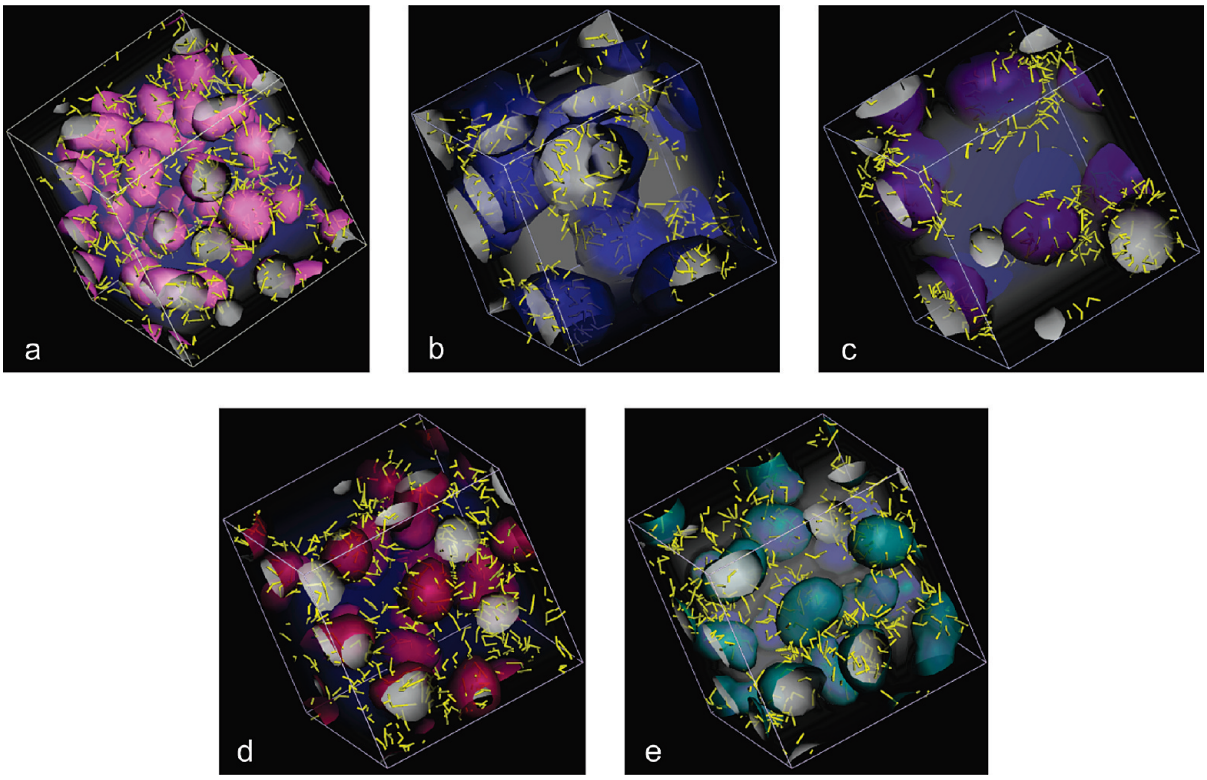


Figure 3. Mesoscale modeling of the amphiphilic dendrons synthesized in this work showing aggregation into spherical micellar objects: (a) Chol-G1; (b) C_{12}Lys-G1; (c) D2Gly-G1; (d) C_{12}-G1; (e) D1Gly-G1. In all pictures, the yellow sticks represent dendron units. Different colored spheres are adopted to represent the various hydrophobic regions. A light gray field is used to represent water.

Table 2. Values of Micellar Diameter D_m (nm), Core Radius R_c (nm), Aggregation Number N_{agg}, Packing Parameter P, and Micelle Surface Charge Density σ_m (e/nm^2) for the Different Modified Dendrons as Obtained from DPD Simulations

compd	D_m	R_c	N_{agg}	P	σ_m
Chol-G1	3.4 ± 0.1	0.8	21	0.24	5.2
C_{12}Lys-G1	4.0 ± 0.2	1.3	24	0.24	4.3
D2Gly-G1	4.9 ± 0.2	1.5	32	0.32	3.8
C_{12}-G1	4.0 ± 0.1	1.3	16	0.28	2.8
D1Gly-G1	4.0 ± 0.2	0.9	12	0.25	2.1

simulations. Indeed, Israelachvili, Mitchell and Ninham proposed the concept of molecular packing parameter and demonstrated how the size and the shape of self-assembled molecules at equilibrium can be predicted from a combination of molecular packing considerations and general thermodynamic principles.[29] To a first approximation, the molecular packing parameter P of a given amphiphile is defined as v_0/al_0 where v_0 and l_0 are the volume and the length of the hydrophobic portion, and a is the surface area of the hydrophobic core of the aggregate expressed per molecule in the aggregate (hereafter referred to as the area per molecule). If we consider a generic micelle with a core radius R_c, made up of N_{agg} molecules, then the volume of the core $V = N_{agg}v_0 = 4\pi R_c^3/3$, the surface area of the core $A = N_{agg}a = 4\pi R_c^2$, and, hence, $R_c = 3v_0/a$, from simple geometrical relations. If the micellar core is densely packed with the hydrophobic moieties without any empty space, then the core radius cannot exceed the extended length l_0 of the hydrophobic part. Introducing this constraint in the expression for R_c, we obtain the well-known condition $0 \leq P \leq 1/3$ if an amphiphile is to form spherical micelles. Simple molecular modeling considerations coupled with the fundamental micellar parameters listed in Table 2 allowed us to calculate the packing parameter for all of the modified dendrons in hydrated conditions. The calculated P numbers listed in Table 2 are between 0.24 and 0.32, in agreement with the observation from modeling studies that all of the dendrons self-assemble into spherical micelles.

A further, key parameter in governing the DNA binding properties of the dendrons and, eventually, the transfection efficiency of the resulting DNA/dendron assembly complexes is the micelle surface charge density σ_m. In practical terms, σ_m can be thought of as a measure of how positively charged a micelle is. Two micelles containing the same number of dendrons, and therefore carrying the same overall charge, may exhibit different values of σ_m, if the micelle sizes, and hence their surface areas, are different. To calculate σ_m one therefore needs to know the aggregation number of the micelle N_{agg}, the charge of each dendron head, and the micellar surface area $S_m = 4\pi R_m^2$, as $\sigma_m = eN_{agg}/S_m$. Using the data reported in Table 2 and the constant value of +9 for the overall charge of each dendron headgroup, the σ_m values shown in the last column of Table 2 can be easily estimated. Summarizing the overall evidence stemming from the analysis of data in Table 2 leads to the following, important considerations. First, the overall series of amphiphilic dendrons synthesized in this work assemble into small, spherical micelles in water and in the presence of physiological ionic strength conditions (150 mM), as experimentally verified for similar systems.[17,30] However, the different architectures of the

Table 3. Predicted Free Energy of Micellization ΔG_{mic} (kJ/mol) and Critical Micelle Concentration CMC (μM) for the Different Modified Dendrons Estimated According to SP5

compd	ΔG_{mic}	CMC
Chol-G1	−87.56	0.021
C_{12}Lys-G1	−80.42	0.089
D2Gly-G1	−77.97	0.15
C_{12}-G1	−55.92	12.5
D1Gly-G1	−49.29	47.6

hydrophobic portion result in differently sized micelles and/or a different number of dendrons per micelle (the aggregation number N_{agg}), and, hence, a different micellar surface charge density σ_m. Pleasingly, the experimentally verified CE_{50} values directly correlate with the surface charge density, σ_m, values estimated from simulation, indicating that the micelles characterized by higher values of σ_m (i.e., Chol-G1, C_{12}Lys-G1, and D2Gly-G1) are tighter DNA binders than their counterparts with lower σ_m values (i.e., C_{12}-G1 and D1Gly-G1). Interestingly, comparing the best DNA binders, Chol-G1, C_{12}Lys-G1 and D2Gly-G1, the former compound assembles into micelles of much smaller diameter than the latter two. This is presumably due to the less sterically demanding nature of cholesterol leading to more effective packing within the micellar interior, compared with the branched hydrophobic units in the latter two dendrons, which will not be able to pack so efficiently. As such, even though the micelles formed by Chol-G1 contain fewer dendron units and have less total positive charge than the micelles formed by C_{12}Lys-G1 and D2Gly-G1, their smaller size means that they have significantly higher surface charge density, and as such, they are therefore much more effective DNA binders.

From an energetic standpoint, the change in Gibbs free energy of transfer of a single amphiphilic molecule from the monomeric state to a micelle of aggregation number N_{agg}, commonly called the free energy of micellization ΔG_{mic}, can be modeled as consisting of a hydrophobic part, $\Delta G_{mic,h}$, and an electrostatic part, $\Delta G_{mic,e}$, so that $\Delta G_{mic} = \Delta G_{mic,h} + \Delta G_{mic,e}$. The hydrophobic part stems primarily from the favorable energy of transfer of the hydrocarbon moieties from the aqueous phase to the micellar phase, and, secondarily, from the unfavorable residual interfacial contact of water with the apolar components within the micelles. The electrostatic part of ΔG_{mic} arises from the repulsion between the ionic head groups within the micellar shell. Following the theory proposed by Tanford[31] and subsequently modified by other authors,[32] we calculated the values of ΔG_{mic} for the five modified dendrons (Table 3). As can be seen, ΔG_{mic} at room temperature has large, negative values, indicating that micellization is a spontaneous and highly favorable process for all amphiphilic dendrons, although ΔG_{mic} decreases on going from Chol-G1 to D1Gly-G1. The headgroup architecture is the same in all amphiphiles, and as such, the main differential contribution to ΔG_{mic} stems from the $\Delta G_{mic,h}$ term, which will reflect differences in the size and structure of the hydrophobic component.

The critical micelle concentration (CMC) is one of the most commonly studied properties of a self-assembled system because it is a direct measure of the thermodynamic stability of the micelles in solution. Basic thermodynamic relationships allow CMC to be directly obtained from ΔG_{mic}; accordingly, the last column in Table 3 lists the predicted CMC data for each system simulated in this work. Typically, micellar aggregates have CMCs of the order of 10^{-3}−10^{-5} M, while lower CMCs, even down to the nanomolar range, are found for amphiphiles that form either membranes or cylindrical aggregates. Recently, however, electron microscopy experiments performed on cholesterol-porphyrin micelles revealed that these amphiphiles could form virtually monodisperse spherical aggregates with a diameter of approximately 7 nm and a CMC value of 11 nM.[33] Amphiphiles showing low CMCs tend to have relatively large hydrophobic segments, and this normally results in an assembly shape with a lower curvature. However, our series of modified dendrons, combine a large hydrophobic portion with a very large headgroup, resulting in a roughly conical amphiphile. The size of the hydrophobic segment is responsible for the low CMCs, while the large size of the headgroup results in the spherical geometry of the assembly. It is interesting to note that the predicted CMC values for C_{12}-G1 and D1Gly-G1 lie above the concentrations of the DNA binding assays (i.e., low μm concentrations); as such, it is possible that the relatively poor DNA binding ability of these compounds reflects the fact that they are not aggregated under the experimental conditions as a consequence of their relatively small hydrophobic segments. Although a word of caution is due about the fact that the calculated values of ΔG_{mic} and CMC are obtained using validated but simplified theoretical approaches, the trends exhibited by these parameters are in line with the experimental data. Indeed, we are currently carrying out full experimental aggregation studies on a closely related set of hydrophobically modified dendrons, and for these systems, the *in silico* predictions of micelle diameters, charge densities and CMC values are closely mirrored by the experimental results, both in terms of trends and absolute values.

We then performed mesoscale simulations of the dendron micelles in the presence of DNA. These investigations reveal that, in all cases, the overall systems consist of parts of free, unfolded, single-chain DNA that connect micelles on which a partial amount of DNA has been adsorbed (see Figure 4). In other words, all dendron/DNA complexes present a typical *beads-on-a-string* structure, made of dendron micelles connected by a DNA thread. Importantly, this predicted morphology is supported by detailed AFM studies between G4 PAMAM dendrimers and DNA,[34] indicative that these self-assemblies of dendrons can be considered to be somewhat like covalently bound higher generation spherical dendrimers. These structures are also somewhat reminiscent of the structure of open chromatin, which consists of an array of nucleosome core particles, separated from each other by up to 80 base pairs of linker DNA.[35] However, in clear contrast to the periodic structure of open chromatin, the dendron micelles appear to be distributed in a nonperiodic, more irregular way.

As a consequence of this model, DNA molecules are partially embedded within the micellar organization and partially exposed to the solution environment, where the Na^+ ions originating from both the DNA and the accompanying ionic strength provide the minimum 90% charge neutralization required for condensation, according to Manning's theory.[36] According to this picture, the cationic micelles act as a matrix in which the DNA chains are partially embedded within the micellar organization and partially free to condense. Figure 4 reveals also that, upon DNA addition, for all systems most of the micelles retain their spherical geometry. However, the appearance of some micelles changes to an elliptical cross-section, resulting from a stretching of the micelles induced by the linear geometry of DNA to maximize mutual electrostatic contacts.

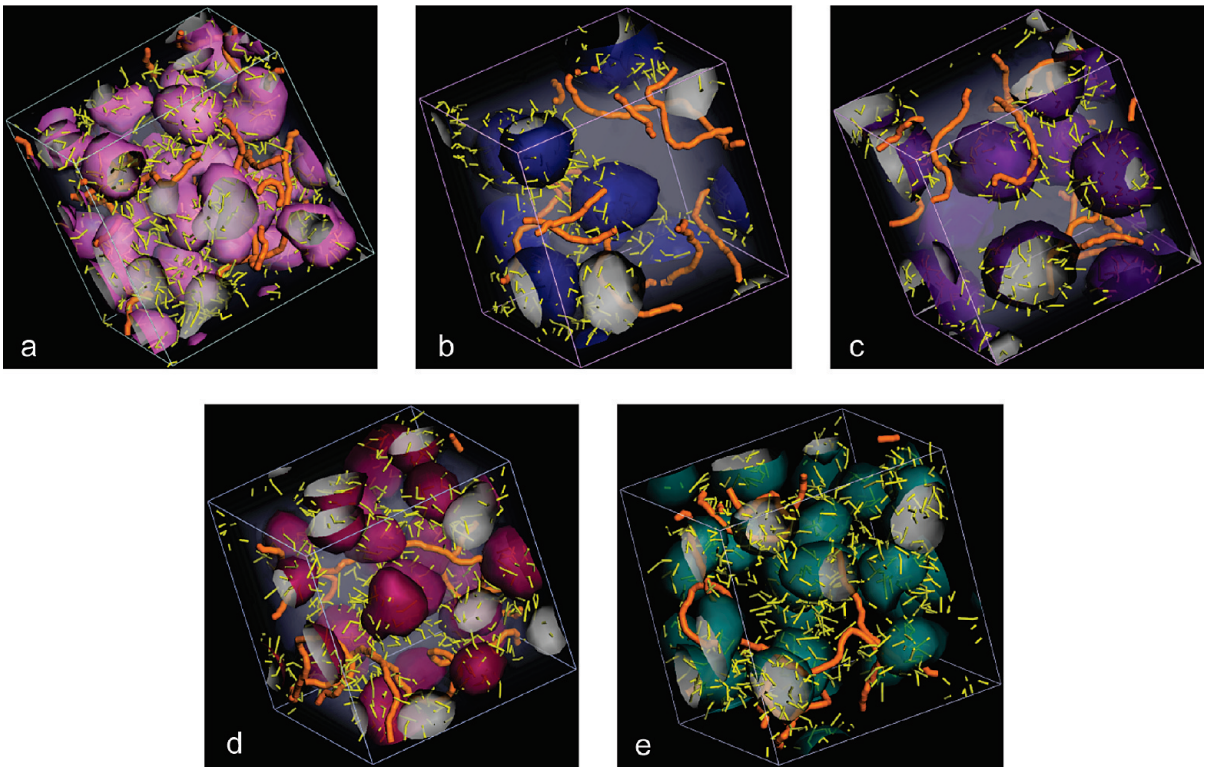


Figure 4. Mesoscale modeling of the interaction of DNA with the amphiphilic dendrons synthesized in this work: (a) Chol-G1; (b) C_{12}Lys-G1; (c) D2Gly-G1; (d) C_{12}-G1; (e) D1Gly-G1. In all pictures, yellow sticks represent dendron units. Different color spheres are adopted to represent the various hydrophobic regions along the series. A light gray field is used to represent water. DNA molecules are depicted as orange sticks.

In all systems, but particularly for C_{12}-G1 and D1Gly-G1, DNA localizes in the interstitial space and gives rise to small bundles. At first sight the bundling of DNA helices may appear to be unexpected, however, these DNA bundles are formed by the interplay of the salt-induced screening of the electrostatic interactions and the depletion-attraction caused by the dendron micelles. Depletion-attraction is a somewhat underappreciated force associated with the aggregation of two large colloidal objects as a consequence of the osmotic pressure generated by the exclusion of smaller objects from their interacting interface.[37] While depletion-attraction has previously been reported for like charges or neutral objects, the screening of the electrostatic interactions enables this effect to be observed also between DNA and the oppositely charged dendron micelles, for which the electrostatic interactions are attractive. The presence of salt therefore facilitates bundling of DNA by reducing the electrostatic repulsion between DNA molecules, and also reduces the electrostatic attraction between positively charged micelles and negatively charged DNA. The existence of DNA bundles and condensed regions can also be understood by taking into account the small dimensions of the micelles formed by all these dendrons and the relevant values of σ_m. Indeed, extensive wrapping of DNA around small micelles will result in a quite high cost of DNA bending (or elastic) free energy; accordingly, this is not observed in any of the systems considered. Furthermore, a decrease of the DNA adsorbed amount per particle is observed as the surface charge density, σ_m, decreases. This is only partly compensated by the fact that some of the free part of DNA adsorbs on new particles, and therefore the bundling of unbound DNA becomes more favored.

In aqueous solutions both DNA and dendron micelles are associated with their respective counterions. The high charge density of DNA actually results in *counterion condensation*: in its solution structure, the base length between negative phosphate groups on the DNA backbone is equal to l_0 = 1.7 Å. This is significantly less than the Bjerrum length in water $l_B = e^2/e_w k_B T$ = 7.1 Å, where ε_w is the dielectric constant of water (=80), k_B is the Boltzmann constant, and T is the temperature. The Bjerrum length corresponds to the distance where the Coulomb energy between two unit charges is equal to the thermal energy $k_B T$. Under these conditions, it has been shown that positive counterions will condense on the DNA backbone until the Manning parameter $\xi = l_0/l^*$ approaches unity, l^* being the renormalized distance between the negative charges after counterion condensation.[38] A similar analysis shows that, near the surface of a positively charged micelle, almost half of the negative counterions are contained within the Gouy−Chapman length $l_{G-C} = e/2\pi l_B \sigma_m$. Combining DNA and our amphiphilic dendron micelles allows the charges of the spermine head groups to neutralize the phosphate moieties on DNA. This replaces and releases the tightly bound-counterions of both micelle and nucleic acid in solution. The resulting gain of translational entropy by the counterions is a driving force for higher order self-assembly into micelle/DNA complexes.[39] It should be pointed out that the term *bound counterions* is used in a loose form: indeed, the counterions near the DNA or a micelle surface are bound and yet remain in their fully hydrated state. This implies no change in the entropy of water molecules upon release of bound counterions into solution. The driving force of the counterion release mechanism is reduced by added salt, as in the

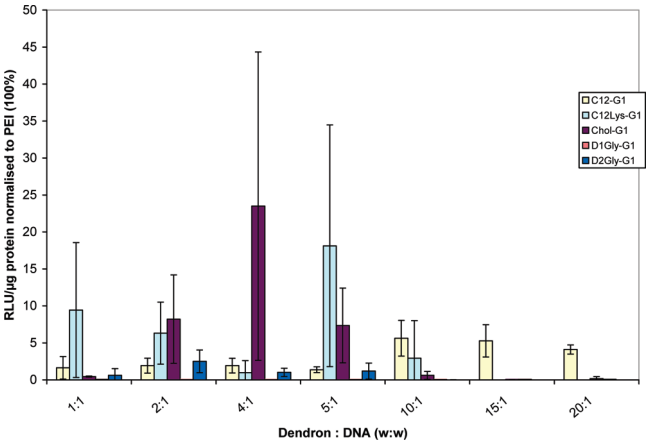


Figure 5. Transfection efficiency of first generation dendritic vectors in HEK 293 cells. Luciferase expression was normalized by total cellular protein; i.e., data were calculated in RLU/mg of protein and are then quoted as percentages of the transfection efficiency of PEI ($N = 6$, error bars represent standard deviation). Transfection is performed in the presence of chloroquine (100 μM).

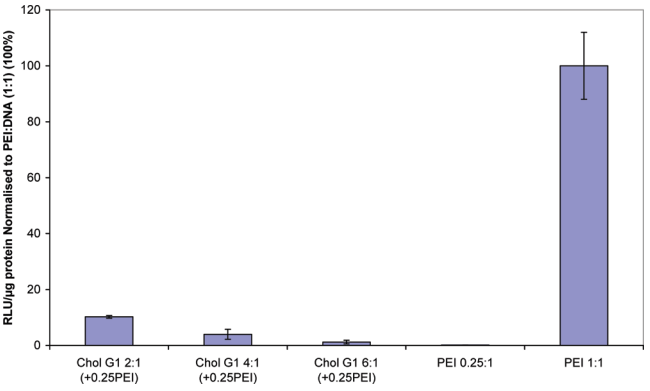


Figure 6. Transfection efficiency of Chol-G1 when mixed with PEI (*no chloroquine*). For comparison, transfection efficiencies with PEI (0.25:1) and PEI (1:1) are also presented.

experiments and simulations described in this work. This is particularly true for counterion release from the dendritic micellar assembly, which relies on a concentration gradient between the layer of ions confined close to the micelle and the bulk solution. Since l_{G-C} scales with l/σ_m, the concentration of counterions next to each micellar entity scales with σ_m. Therefore, the addition of salt to a solution in which the complex between DNA and micelles are formed has a stronger effect on those complexes for which the micelles are characterized by lower values of σ_m. This is in agreement with the present experimental evidence, for which the DNA binding ability of the amphiphilic dendrons, as quantified by the ethidium bromide assay, decreases with the decreasing charge surface density of the micelles.

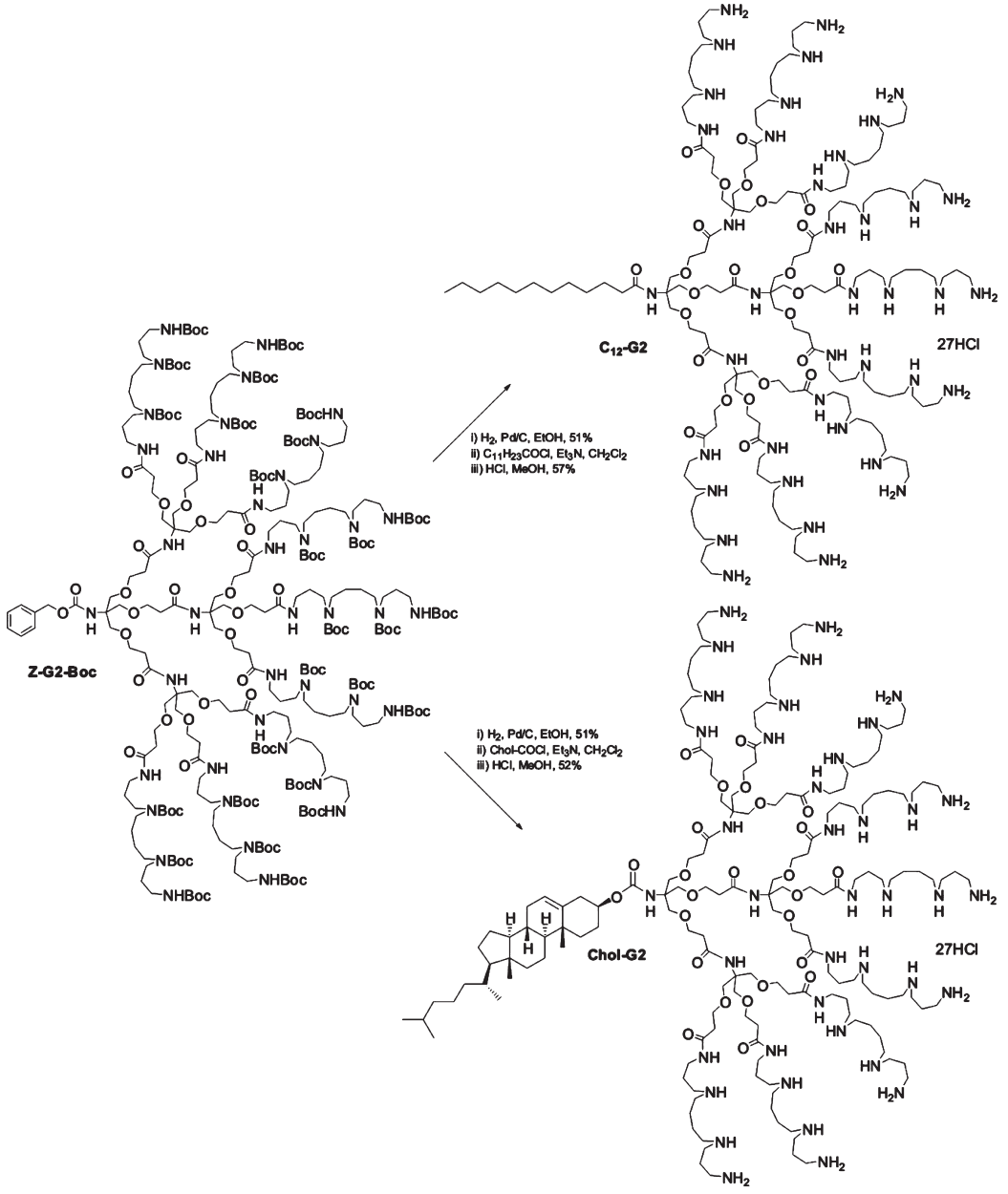
In Vitro Transfection with First Generation Vectors. A number of studies have previously demonstrated that vector aggregation could be correlated with gene transfection ability,[14−17] and we therefore decided to monitor the transfection potential of our dendrons, in order to determine the effect of hydrophobic modification on gene delivery. In order to determine the transfection efficiency of these dendrons, we transfected HEK293 cells with polyplexes comprising 1 μg of a luciferase-encoding plasmid (pGL3) and varying amounts of each dendron. After 4 h, the medium was changed and the cells were left for 20 h before measuring luciferase activity in cell lysates. We employed PEI (polyethyleneimine, 25k, branched) as a positive control in these studies.[40] Unfortunately, none of our first generation (G1) vectors were able to transfect cells directly; it was necessary to add chloroquine (100 μM) in order to achieve some transfection. Chloroquine can act as a weak proton sponge in endosomes, raising endosomal pH, and ultimately leading to endosome rupture.[41] We monitored the data from these chloroquine-enhanced experiments in order to determine the effect of structural modifications on transfection (Figure 5).

Pleasingly, Chol-G1 and C_{12}Lys-G1 gave the most effective chloroquine-assisted tranfection: these were the compounds which demonstrated the most effective DNA binding. For Chol-G1 the optimum transfection, which reached 24% of PEI, was observed at a dendron:DNA ratio of 4:1 (w/w). For C_{12}Lys-G1, the optimum transfection of 17% of PEI was at a 6:1 (w/w) ratio. Compound C_{12}-G1 exhibited lower levels of transfection: up to ca. 5% of PEI, as would have been predicted based on its lower affinity for DNA and lesser extent of hydrophobic functionalization. Surprisingly, however, compounds D1Gly-G1 and D2Gly-G1 exhibited very poor transfection over all mass ratios. We had thought that this type of compound, in particular D2Gly-G1 which bound DNA reasonably well, may be fairly effective in transfection assays, in analogy with the research of Diederich and co-workers.[16] It is possible that the branched aliphatic dendron plays some other role in the cell which effectively inhibits DNA transfection; the biological behavior of these hydrophobic dendrons in cellular systems is clearly worthy of further investigation. In summary therefore, Chol-G1 exhibited the most promising DNA binding and the best transfection data; however, it must be remembered that transfection could only be obtained in the presence of chloroquine.

In order to determine whether endosomal escape really was the limiting factor in the transfection, we decided to investigate transfection mediated by mixtures of Chol-G1 and PEI. It is argued that PEI can act as a proton sponge and therefore assists in endosomal rupture.[42] We therefore employed Chol-G1 and PEI as a mixture in the absence of chloroquine, at concentrations such that no transfection would be observed with either individual component. Specifically, we employed a mixture of 2:0.25:1 Chol-G1:PEI:DNA (wt/wt/wt). When used individually, 2:1 Chol-G1:DNA and 0.25:1 PEI:DNA showed no transfection at all, but we observed that the mixed delivery system gave significant levels of transfection; >10% of positive control (Figure 6). We argue that the small amount of PEI present in the mixture is sufficient to assist endosomal rupture, while the dendron is effectively able to bind DNA and act as the carrier molecule. We reason that PEI cannot act as the major source of DNA binding in this mixed system because it shows significantly weaker DNA binding ability than Chol-G1 and is only present in low amounts.

This demonstrates the potential of mixed systems to enhance gene delivery. In particular, PEI enhances the delivery potential of our dendritic vector, allowing it to operate at low concentrations in the absence of chloroquine. Conversely, our dendritic vector may allow toxic cationic polymers, such as PEI, to be employed while decreasing the loading required for transfection, hence reducing potential toxicity. It is our belief that this kind of synergistic approach to gene transfection holds considerable future potential, in agreement with other studies in which

Scheme 2. Synthesis of Second Generation Vectors, Chol-G2 and C12-G2



mixtures of cationic lipids and cationic polymers have shown beneficial synergistic effects.[23]

Understanding the pathways and mechanisms governing the interactions of modified-dendrons/DNA complexes and cells is crucial to making dendron-mediated gene delivery therapeutically viable. The complexity of the transfection process—from initial attachment of a nanovector/DNA complex to the plasma membrane to internalization of the complex via endocytosis, its release form the endosome followed by the dissociation of the vector from the DNA, and finally the transport of DNA into the nucleus followed by successful gene expression—suggests that an interplay of many critically important parameters needs to be considered in order to achieve transfection. The nanoparticle surface charge density σ_m discussed in the previous modeling section is one such parameter, controlling at least some of the aspects outlined above. If cellular attachment and uptake were the only limiting transfection efficiency factors via a σ_m-dependent mechanism, a linear increase of the transfection efficiency with σ_m would be predicted. Furthermore, for complexes with low σ_m, the transfection ability is also limited by endosomal escape. This is substantiated by transfection experiments performed in the presence of chloroquine, as illustrated in Figure 5. These experiments indicate that complexes with higher σ_m should be more able to escape from endosomes. The escape from endosomes likely occurs via an activated fusion process of the oppositely charged endosome membrane and the micelle/DNA complex. The activation energy of this process can be written as $\delta E = ak - b\sigma_m$, where a and b are positive constants. The parameter k is the bending rigidity of the micelle, which is mainly determined by the hydrophobic portion of the molecule and the area per hydrophobic moiety. Bending or deformation of the micelle, as required by fusion, results in an energy cost

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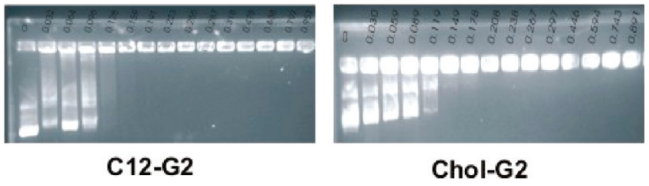

Figure 7. Agarose gel electrophoresis of polyamine/DNA complexes using second generation dendrons. Polyamine:DNA loadings (w:w) are as follows: lane 1, 0:1; lane 2, 0.1:1; lane 3, 0.2:1; lane 4, 0.3:1; lane 5, 0.4:1; lane 6, 0.5:1; lane 7, 0.6:1; lane 8, 0.7:1; lane 9, 0.8:1; lane 10, 0.9:1; lane 11, 1:1, lane 12, 1.5:1; lane 13, 2:1; lane 14, 2.5:1; lane 15, 3:1.

proportional to k. Since the interacting entities during fusion are oppositely charged, the activation energy decreases with increasing σ_m, making fusion more likely, in keeping with the current experimental results. In these ways the modeling studies are in agreement with the experimental results of the transfection assays.

Synthesis of Second Generation Vectors. Having explored structure activity relationships in our first generation vectors, we then went on to synthesize and experimentally investigate some second generation (G2) dendritic systems. We opted to synthesize Chol-G2 and C_{12}-G2, as the syntheses were straightforward (Scheme 2), and for the first generation vectors, these two modifications had been at either extreme of DNA binding and transfection behavior. Compound Z-G2-Boc was deprotected at the focal point by hydrogenolysis, and then coupled with dodecanoyl chloride or cholesteryl chloroformate to yield C_{12}-G2-Boc and Chol-G2-Boc respectively. The overall yields were somewhat lower than those for the first generation system, reflecting the greater steric hindrance of the amine group at the focal point of the more highly branched dendron. The Boc groups were then removed by treatment with HCl gas in methanol to yield the target compounds C_{12}-G2 and Chol-G2 in excellent yield.

DNA Binding Studies with Second Generation Vectors. Once again, we employed the EthBr exclusion assay in order to monitor the binding of these vectors to DNA. In this case, unlike the first generation system, there were minimal differences between the CE_{50} values observed with Chol-G2 (CE_{50} = 1.4) and C_{12}-G2 (CE_{50} = 1.7). For these second generation compounds, the hydrophilic dendron block is much larger than in the G1 systems, and clearly dominates the molecular structure and shape. As such, these second generation dendrons should have less potential to self-assemble in a surfactant-like manner. We propose that the nine spermine surface groups, which contain 27 amino groups, dominate the DNA binding process, and the functional group at the focal point therefore exerts much less of an effect. In this way, at least for DNA binding, the data indicate that focal point modification is less important in larger G2 dendrons than in their smaller analogues.

In order to probe DNA binding further, we performed gel electrophoresis (Figure 7). This confirmed the observation that there was little difference between Chol-G2 and C_{12}-G2. In the case of Chol-G2, DNA mobility was retarded at a loading of 0.119 nmoles (N:P ratio of 1.1). For C_{12}-G2, DNA retardation was observed at a very similar loading level (0.123 nmoles, N:P ratio of 1.1).

Interestingly, the CE_{50} values (from EthBr exclusion) and N:P ratios (from gel electrophoresis) for Chol-G2 binding DNA are significantly higher than those observed with Chol-G1. This is perhaps surprising given that Chol-G2 has many more spermine groups, and may have been be expected to be a more effective binder. Indeed, in our previous studies, Z-G2 was, as expected, more effective than Z-G1 in terms of its CE_{50} value.[18] These observations clearly reinforce the concept discussed above that the cholesterol unit can, in the case of Chol-G1, play a proactive role in encouraging and promoting self-assembly of the dendron, generating a high density of spermine groups, with this process having a major positive effect on the ability of Chol-G1 to interact with DNA. Clearly this does not occur for the G2 dendron, and the spermine surface groups alone control binding.

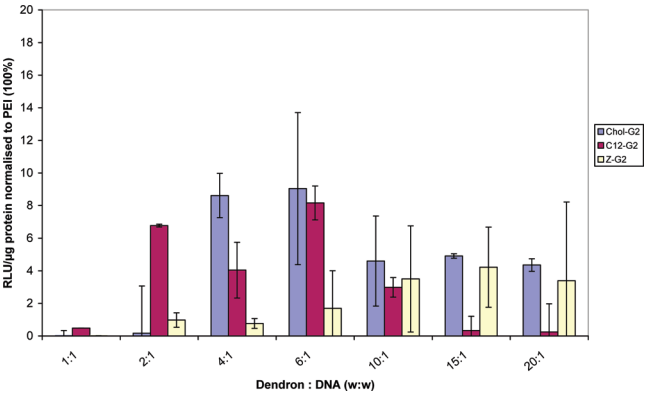

Figure 8. Transfection efficiency of second generation dendritic vectors in HEK 293 cells. Luciferase expression was normalized by total cellular protein; i.e., data were calculated in RLU/mg of protein and are then quoted as percentages of the transfection efficiency of PEI (N = 6, error bars represent standard deviation).

In Vitro Transfection with Second Generation Vectors. We then went on to test the second generation vectors for their ability to transfect cells using the methodology described above. Given that Chol-G2 showed less effective DNA binding than Chol-G1, we were unsure whether any transfection would be observed. However, significant transfection with this vector was observed, even in the absence of chloroquine (Figure 8). Indeed transfection of up to ca. 10% of that obtained with PEI (1:1) as positive control was observed. Furthermore, the behavior of compound C_{12}-G2 was broadly similar to Chol-G1, as may have been anticipated by the similar, but slightly diminished DNA binding affinities. It should be noted that both of these vectors were most effective at a ratio of 6:1 (w/w).

Intriguingly, however, both Chol-G2 and C_{12}-G2 were significantly more effective than Z-G2 at lower dendron/DNA ratios. Dendron Z-G2 only exhibited transfection efficiencies of ca. 1−2% of PEI levels. This indicates that the functional group at the focal point does play some role in enhancing transfection, even though it does not appear to have a major effect on DNA binding. It is possible that because self-assembly is disfavored with these larger dendrons, the hydrophobic unit (cholesterol group or aliphatic chain) is better able to disrupt membranes and enhance endosomal escape. This observation of effective transfection with hydrophobically modified G2 dendrons is in agreement with our investigations of these dendrons modified at the focal point with *hydrophobin* proteins, in which we observed that the combination of a G2 branch and a hydrophobic protein was required for effective transfection to be observed.[43]

In the comparison between G1 and G2 systems, it should also be noted that the larger G2 dendrons contain additional amine groups, and it is therefore likely that they are better able to play a buffering role within the endosome, further enhancing endosomal

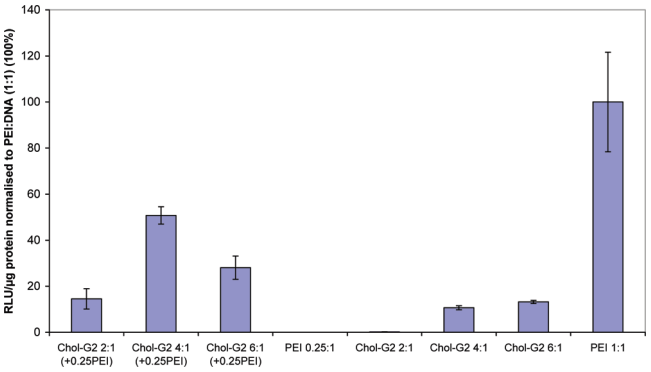

Figure 9. Transfection efficiency of Chol-G2 when mixed with a small amount of PEI (no chloroquine present). For comparison, transfection efficiencies with PEI (0.25:1), PEI (1:1) and Chol-G2 in the absence of PEI, are also presented.

escape. This may help explain why some transfection is observed, even in the absence of chloroquine.

In order to probe transfection further, we once again applied our most effective vector (Chol-G2) as a mixture with PEI in order to determine whether the mixed vector exhibited synergistic transfection effects (Figure 9). Remarkably, even though a ratio of 0.25:1 PEI:DNA (w/w) exhibits no transfection, the addition of Chol-G2 to this mixture (4:0.25:1, Chol-G2:PEI:DNA) gave rise to very good levels of transfection (ca. 50% of that observed with positive control). Once again, this demonstrates the potential of PEI to enhance the transfection potential of these dendrons (the level of transfection was enhanced 5-fold) and also allow them to be employed at lower loadings (the optimal loading of Chol-G2 decreased from 6:1 to 4:1). Furthermore, the PEI, which presumably acts by assisting buffering in the endosome, can be employed at lower concentrations, where it is unable to transfect at its own right, allowing it to be employed in a transfection vector at much lower loadings than previously realized.

CONCLUSIONS

In this paper, we have reported a structure−activity relationship for a set of dendritic vectors with DNA binding spermine surface groups. In particular, we report that the hydrophobic/hydrophilic balance plays a subtle role in controlling DNA binding and transfection ability. The easy synthetic tunability of these dendron structures can therefore be readily exploited to maximize their biological activity. For the first generation system, the choice of the group at the focal point of the dendron had a significant impact on the DNA binding. The binding affinity for DNA was enhanced by the presence of significant hydrophobic group. Mesoscale modeling demonstrated that these systems were better able to pack into tighter micellar aggregates, which have higher surface charge density and as such, show enhanced electrostatic interactions with DNA. In this way, self-assembly of the dendrons enhances DNA binding. Furthermore, the differences in DNA binding affinity appeared to correlate with the transfection ability of the dendrons, although chloroquine had to be added to assist endosomal escape. In contrast, the second generation dendrons bound DNA in a manner which was relatively independent of the hydrophobic group, presumably because the large hydrophilic spermine surface area dominates the binding. These dendrons were able to transfect in the absence of chloroquine, presumably due to the greater number of amines providing some buffering effect. The cholesterol functionalized dendron was a significantly more effective transfection agent than the dendron with a Z-protecting group, possibly due to the ability of the cholesterol group to disrupt endosomal membranes. Finally, we observed significant synergistic effects when employing combinations of our dendrons (Chol-G1 or Chol-G2) and PEI. Indeed, transfection was possible using mixtures at loading levels which were so low that the individual components did not exhibit significant transfection ability on their own. Using this approach, we were able to achieve gene transfection at practically useful levels.

ASSOCIATED CONTENT

Supporting Information. Details of synthetic methods and characterization data for all dendrons, details of the biological assays performed in the work, and methods used for modeling. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author
*E-mail: dks3@york.ac.uk.

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