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Peptide Dendrimer/Lipid Hybrid Systems Are Efficient DNA Transfection Reagents: Structure–Activity Relationships Highlight the Role of Charge Distribution Across Dendrimer Generations

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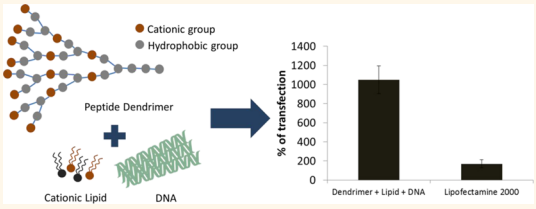
ABSTRACT Efficient DNA delivery into cells is the prerequisite of the genetic manipulation of organisms in molecular and cellular biology as well as, ultimately, in nonviral gene therapy. Current reagents, however, are relatively inefficient, and structure–activity relationships to guide their improvement are hard to come by. We now explore peptide dendrimers as a new type of transfection reagent and provide a quantitative framework for their evaluation. A collection of dendrimers with cationic and hydrophobic amino acid motifs (such as KK, KA, KH, KL, and LL) distributed across three dendrimer generations was synthesized by a solid-phase protocol that provides ready access to dendrimers in milligram quantities. In conjunction with a lipid component (DOTMA/DOPE), the best reagent, G1,2,3-KL ($(LysLeu)_8(\mathit{Lys}LysLeu)_4(\mathit{Lys}LysLeu)_2\mathit{Lys}GlySerCys\text{-}NH_2$), improves transfection by 6–10-fold over commercial reagents under their respective optimal conditions. Emerging structure–activity relationships show that dendrimers with cationic and hydrophobic residues distributed in each generation are transfecting most efficiently. The trigenerational dendritic structure has an advantage over a linear analogue worth up to an order of magnitude. The success of placing the decisive cationic charge patterns in inner shells rather than previously on the surface of macromolecules suggests that this class of dendrimers significantly differs from existing transfection reagents. In the future, this platform may be tuned further and coupled to cell-targeting moieties to enhance transfection and cell specificity.



KEYWORDS: peptide dendrimers · dendrons · gene delivery · DNA transfection · nonviral gene therapy

A major hurdle in genetic manipulation of organisms in molecular and cellular biology (*e.g.*, gene knock-outs or knock-ins and ultimately nonviral gene therapy) is the inefficiency of current DNA delivery systems. While viral vectors are the most effective gene delivery systems and have been used in several clinical trials,[1−6] their intrinsic immunogenicity, potential side effects (*e.g.*, insertional mutagenesis), limited nucleic acid packaging capacity, and difficulties of mass production will restrict their application in gene therapy. Nonviral gene delivery carriers include cationic lipids and polymers that are nonimmunogenic, flexible in packaging different kinds and sizes of nucleic acids, relatively easy to handle, and have potential for mass production.[7] This is why they remain an attractive long-term option for gene delivery, despite their currently relatively poor transfection efficiency.[8−11] Systematic exploration and understanding of the structure–activity relationships of nonviral transfection reagents are therefore necessary for the development of efficient and safe alternatives as gene transfer tools.

Molecules synthesized for the purpose of transfection usually include some or all of

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the following molecular recognition features: cationic groups for electrostatic DNA binding, a hydrophobic part to facilitate transmembrane transfer, and specific recognition entities.[12−15] Among the systems used for nucleic acid delivery are lipid-based systems, polymers, and lipid−polymer hybrid vectors. Lipid small molecules include Gemini surfactants,[14] liposomes,[16] or lipidoids[17] that associate DNA cargo with a lipid package to facilitate membrane transfer. Cationic polymeric systems have been used in gene delivery based on their electrostatic interactions with negatively charged DNA that are enhanced by multivalency.[18] Oligo- or polymeric materials include linear or branched structures such as polyethylene imine (PEI),[19−21] cationic peptides[22] such as polylysine or polyarginine[23−29] (sometimes in combination with a targeting sequence[30]), and also dendrimers[31] such as polyamidoamine (PAMAM),[32−38] degraded PAMAM,[38] PAMAM-poly(ethylene glycol) block copolymers,[39,40] polypropyleneimine (PPI),[41−43] and polylysine dendrimers.[24,44] The intrinsic ability of a transfection reagent to bind and deliver nucleic acids can be changed by attachment of lipid groups,[45−48] amino acids (*e.g.*, arginine[36,37,42,49−51] and phenylalanine[52]) on the macromolecule surfaces. In addition, some surface modifications were shown to improve the vector stability,[37] membrane permeability,[53] and/or endosomal escape.[54,55] Combinations of lipids and polymeric systems that are jointly administered have also been employed and can be synergistic; that is, the transfection effects of combinations of reagents can exceed those of individual species. Such multicomponent vectors[12,21,30,56,57] combine multivalent interactions of the polymer with the nucleic acid and the negatively charged cell surface with the ability of a lipid to facilitate membrane crossing.[30,58−60] The wealth of structures used for transfection raises the question whether the functional properties can be modified in a rational way. However, there are relatively few instances in which it was possible to correlate combinatorial or systematic changes in the molecular structure of the transfection reagents to a change in their properties.[14,45,61−63] This is partly due to the limits of the synthetic protocols: variation of the backbone to allow incorporation of heterogeneous building blocks is difficult in dendrimers, so that the character of the interior and surface of the dendrimers is difficult to vary in a differential manner. The limits of the synthetic degrees of freedom had the consequence that previous analyses of transfection ability focused mainly on surface modifications.[36,42,53,64−66]

As a result, systematic studies of the effect of backbone components on their gene delivery properties of dendrimers have not been explored, and a more rational understanding of design principles for dendrimers capable of efficient transfection reagents is lacking.

Our peptide dendrimers can be readily prepared by solid-phase peptide synthesis and allow the incorporation of alternative building blocks.[67−73] The robust synthesis procedure allows incorporation of hydrophobic, charged, or polar residues into the dendritic scaffold to explore chemical and functional space. In contrast to other transfection reagents, the character of our transfection reagent can be varied in dendritic layers, so that not only the surface of the transfection reagent but also its interior is affected. Tapping the potential of interactions of inner shell residues becomes possible to tune the molecular recognition properties to support transfection. Many widely used transfection reagents, such as PEI,[57,74] are polydisperse and often ill-defined (notwithstanding their clear utility in everyday use), which may limit the stringency of any rules even from systematic studies. By contrast, our synthetic protocol allows the synthesis of well-defined, fully characterized compounds at spectroscopic quality (see Supporting Information). Ready access to the compounds allows a unique opportunity to develop a molecular understanding and systematic study of related transfection reagents. Dendrimers were administered together with the structurally well-defined lipid combina;tion of DOTMA/DOPE (D/D) that further enhances transfection.[30,60]

In this study, we exploit the modularity of dendritic structures with different amino acid building blocks and charge distributions within the dendrimers to obtain structure−activity relationships. Figure 1 shows the structures of a series of third generation dendrimers incorporating a branching Lys and the dyad LysX (where X is leucine, histidine, or alanine; Figure 1, X_1, X_3, and X_5). The 11 dendrimers have similar sizes and architectures but varying constituent parts. Their structures vary in the charge per dendrimer, the ratio of molecular weight and charge (M_w/charge), and the charge distribution within the dendrimers, and the data for this collection suggest that these parameters influence gene delivery in a systematic way. The dendrimer/lipid formulations show marked improvements in transfection compared to commercial reagents such as PEI (up to 8-fold) and Lipofectamine L2000 (6-fold better).

RESULTS AND DISCUSSION

Design and Synthesis of the Peptide Dendrimer Transfectants. To test whether the peptide dendrimer scaffold would be suitable for transfection, third generation dendrimers with two amino acids between branching units were synthesized by fast and reliable solid-phase peptide synthesis to yield milligram quantities of water-soluble dendrimers (Figure 1 and Table S1).

Within the dendrimer scaffolds, two molecular recognition elements, cationic and hydrophobic groups that are recurring features in a variety of transfection reagents,[12−14] were introduced. Specifically, the

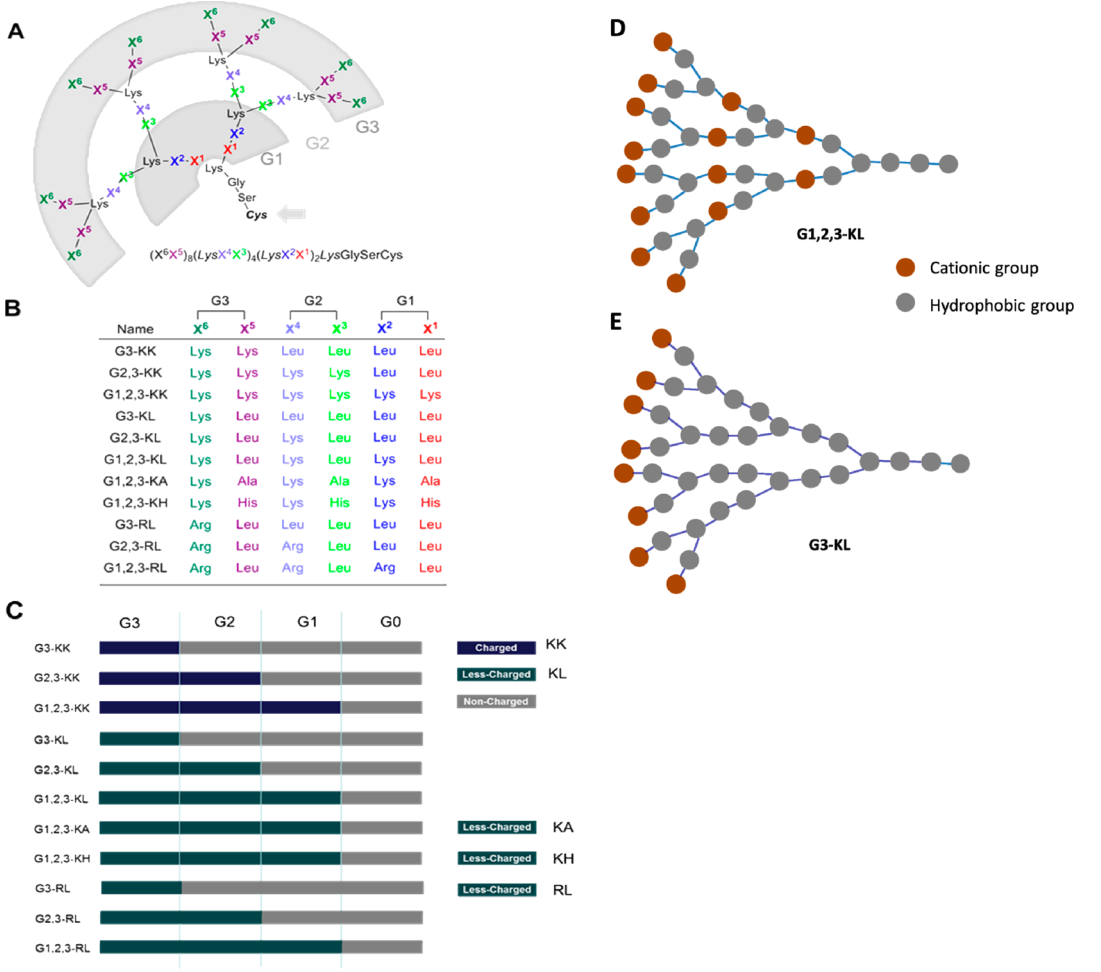

Figure 1. Schematic representation of the peptide dendrimers studied. (A) General structure of the peptide dendrimers used in this study highlighting the three generations, the attachment point for dimerization (a Cys marked by a gray arrow), and the general formula for this collection of dendrimers. The table in (B) shows the specific amino acids distributions in each dendrimer. The nomenclature describes dendrimers by the generation G and the amino acid pattern in these generations, where the hydrophobic amino acids motif LL is substituted by cationic amino acid motifs, such as KA, KH, KA, or KL. For example, the most charged dendrimer G1,2,3-KK contains 28 cationic lysines (16 in G3, 8 in G2, and 4 in G1) plus 7 neutral branching lysines. The dimers of G1,2,3-KL, G2,3-KL, and G3-KL are $[G1,2,3\text{-}KL]_2$, $[G2,3\text{-}KL]_2$, and $[G3\text{-}KL]_2$, respectively, and other dimers are named analogously. (C) Overview of the charge patterns in this dendrimer collection, showing the arrangements of double-charged (KK), charged (KL, KA, KH, RL), and uncharged patterns (LL). The C-terminal Cys of the dendrimers (marked by a gray arrow) is a primary amide ($-RCONH_2$), and the N-termini are free amines. The structures in (D,E) show the scaffolds of the most (G1,2,3-KL) and least effective (G3-KL) transfecting agents, respectively. These two examples highlight the key structural differences in the charge distribution: the cationic charges are distributed across the dendrimer in G1,2,3-KL. In G3-KL, the cationic charges are only present on the surface (*i.e.*, in the third generation). The chemical structures of these two dendrimers are shown in Figure S2 in the Supporting Information.

general requirement for cationic and hydrophobic functionality was addressed by incorporating permutations of the molecular recognition elements KK, KA, KH, KL, and LL in different spheres of the dendrimer framework: either on the periphery or distributed across the three dendrimer generations (see Figure 1B for the sequences). These groups represent combinations of hydrophobic groups of different sizes (A, L), with a positive charge (K, pK_a ~9.5) and a group with buffering capacity around neutral pH (H, pK_a ~6.5) that has been shown to facilitate endosomal escape.[54,55] Given the multitude of potential combinations, the following considerations were made to limit the number of compounds, yet still cover substantial functional diversity. In each of the eight tested dendrimers, the outermost layer (generation 3) contains at least eight lysines to provide a cationic interaction with anionic cell-surface groups and DNA, paired up with either Lys, Leu, Ala, or His. Figure 1C shows the different charge patterns that our collection covers. To address the question of size dependence on transfection, three peptide dendrimers were tested as dimers.

Peptide dendrimers were initially used for transfection alone (Figure 2A) and then with the lipids, DOTMA and DOPE (w/w 1:1, Figure 2B), that synergistically enhanced the transfection efficiency. Enhancements for lipid/dendrimer/DNA complexes (compared to dendrimer/DNA complexes alone) ranged between 1 and 2 orders of magnitude for the better transfection

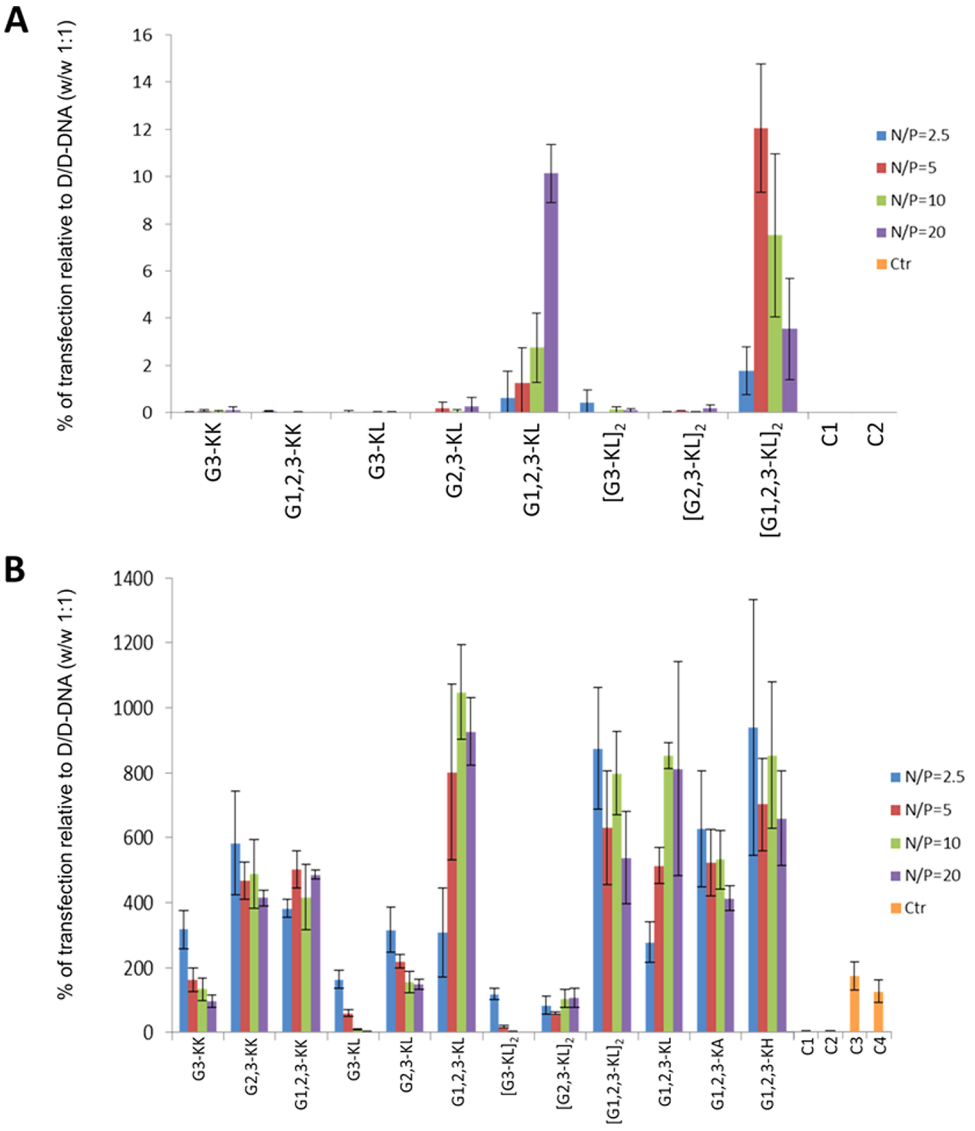

Figure 2. Transfection of HeLa cells mediated by dendrimer–DNA complexes with or without the addition of a lipid component (DOTMA/DOPE). The addition of the lipid component within the dendrimer DNA complexes improved transfection by at least 30-fold. HeLa cells (1×10^4) were transfected with a luciferase-expressing plasmid (pCI-Luc; 0.25 μg) using dendrimers at different N/P ratios and DOTMA/DOPE (D/D) (also known as lipofectin) (0.25 μg). The luminescence of cells was analyzed 24 h post-transfection. The luminescence of the cells treated by the dendrimer complexes was normalized by dividing by the luminescence of cells treated with a D/D DNA complex (w/w 1:1) to yield the percentage (%) of transfection. (A) Transfection using dendrimer and DNA. (B) Transfection using D/D, dendrimer, and DNA. Controls: (C1) Untreated cells; (C2) cells treated with the plasmid pCI-Luc alone; (C3) cells treated with L2000; (C4) cells treated with PEI ($n \geq 3$; mean $\pm$ SD). The N/P ratio refers to the ratio between protonated nitrogen atoms of the dendrimer and DNA phosphate groups. The optimal N/P ratio for transfection may vary between dendrimers; therefore, it is important to test different N/P ratios to identify the optimal transfection formulations of dendrimers in our collection. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

reagents, for example, 50-fold in $[G1,2,3\text{-}KL]_2$ (at an N/P ratio of 5) or 30-fold in G1,2,3-RL (at an N/P of 10) (see Figures 2 and 6 and Figure S1 in the Supporting Information). A further increase beyond one weight equivalent of the lipid component D/D in the formulation diminished the transfection efficiency (see Supporting Information, Figure S2). Most of the lipid–peptide dendrimer hybrids were shown to be at least as efficient in luciferase transfection experiments when compared with other reagents (*e.g.*, PEI and L2000) in common use that also present cationic and hydrophobic features.

In HeLa cells, the best formulation, G1,2,3-KL/lipid, shows clear improvements in the expression of the luciferase transgene compared to commercial and widely used reagents: 10-fold compared to D/D alone (w/w to DNA 4:1), 8-fold more than polyethylene imine (PEI), and up to 6 times as much as Lipofectamine 2000 under their respective optimal conditions recommended by the manufacturers.

Exploration of Structure–Activity Relationships in a Family of Peptide Dendrimers. To allow a systematic quantitative analysis of the role of charge within the dendrimers,

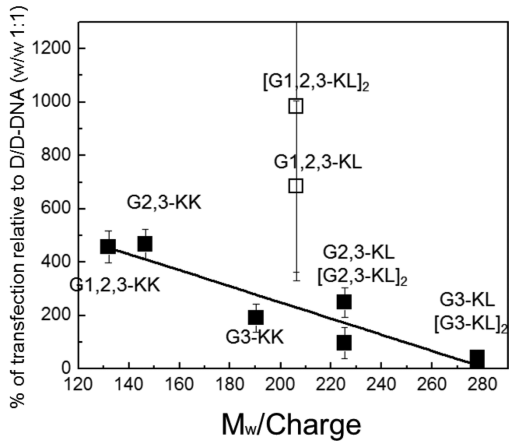

Figure 3. Molecular weight (M_w) to charge ratio governs the transfection efficiency of dendrimers. The majority of the dendrimers tested (■) exhibits a broad trend: a change of 100 in the M_w/charge ratio brings about a ~3-fold increase of the luciferase transgene readout. A subset of the dendrimers (□) exhibits further enhancement (by ~2−10-fold) above this trend, and these outliers are the most efficient transfection reagents in this study. These compounds share a structural feature, namely, one charged lysine in each branching unit of each generation, suggesting that this distribution contributes to an improved transfection efficiency. Luminescence values for HeLa cells transfected with a luciferase-expressing plasmid (pCI-Luc) in the presence of peptide dendrimers and D/D (measured 24 h after transfection). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (w/w 1:1, 0.25 μg) to generate the % of transfection. The percentage of transfection at an N/P ratio (*i.e.*, the ratio between protonated nitrogen atoms of the dendrimer and DNA phosphate groups) of 5:1. Figure S4 shows data for three additional N/P ratios for transfection that exhibit similar trends. Conditions: 1 × 10^4 HeLa cells transfected with 0.25 μg pCI-Luc. Error bars refer to the mean ± SD for experiments carried out in triplicate in three independent experiments except for G2,3-KK, where n = 3 (see Figure S1 for a comparison of the dendrimer formulations and commercial reagents).

transgene expression, DNA binding, and cell toxicity were correlated to the M_w/charge ratio and the charge distribution in the three dendrimer shells. One or two lysines were incorporated into each branch of the first, second, and third shells, and the total number of protonatable lysines varied between 8 and 28 (and thus a maximum total dendrimer charge between 16 and 36, including the free N-termini in addition to Lys side chains).

Variation of Dendrimer Charge. Figure 3 shows luciferase reporter gene expression data for dendrimers in which the cationic charges of lysine have been replaced by the hydrophobic group leucine bearing an identical number of carbon centers. Plotted against the M_w/charge ratio, the dendrimers fall into two groups: 6 of 8 data points are linearly correlated. Although the range of M_w/charge values is necessarily limited, the corresponding change in transfection is more than 10-fold. The data suggest that dendrimers with *more* cationic character are better able to transfect. The slope of this curve is shallow; that is, a change of 100 in M_w/charge brings about only a 3-fold increase of the luciferase transgene readout. By contrast, the total absolute charge per dendrimer was less well-correlated to transfection efficacy (as evidenced by more scattered data, especially at high N/P ratios; see Supporting Information, Figure S3). To probe whether the observed structure−activity relationship of the M_w/charge ratio and transfection is an intrinsic property or due to the effect of a specific formulation, transfection experiments were carried out at varying ratios of dendrimer to DNA (*i.e.*, N/P ratios). The optimal N/P ratio between protonated nitrogen atoms from the dendrimer and phosphate groups from the DNA is known to vary between structurally modified transfection reagents. A comparison of transfection trends at a range of N/P ratios (2.5−20) allows the separation of intrinsic effects as a function of the structure of the transfection reagents from those that are brought about by adjustable external factors. Here identical behavior was observed, namely, that more cationic dendrimers transfect more efficiently, suggesting that the effects are intrinsic properties of the dendrimers (see Supporting Information, Figure S4). The transfection efficiency was measured by luciferase expression in HeLa cells (see Supporting Information, Figure S4) and shows a similar trend, with lower M_w/charge ratios exhibiting better transfection efficiencies. Overall, this analysis suggests that the M_w/charge ratio is a significant parameter governing transfection by peptide dendrimers.

Charge Distribution of the Dendrimers Affects Transfection Efficiency. The correlation described in the previous section serves to distinguish dendrimers obeying an apparent broad trend from dendrimers for which transfection is further enhanced (albeit by only 2−10-fold). Indeed, these outliers share a structural feature, namely, one charged lysine in each branching unit of each generation (*i.e.*, G1,2,3-KL, $[G1,2,3\text{-KL}]_2$). To further investigate the effect of charge distribution of the dendrimers on transfection, the transfection efficiency of the dendrimer with charged lysines in the third generation (G3-KL) was compared against dendrimers with charged lysines in both the second and third generations (G2,3-KL) and all generations (G1,2,3-KL) (Figure 4). G1,2,3-KL transfects up to 5 times more effectively than dendrimer G2,3-KL, whereas the dendrimer bearing charged lysines *only* in the third generation (G3-KL) transfects least effectively. Uniform distribution of the KL dyad in each dendrimer generation gave better transfection agents than grouping hydrophobic residues in one or two generations (Figure 4).

This effect is independent of the partner group of lysine. Replacing leucine by histidine or alanine (in the context of identical amino acid composition, *e.g.*, in dendrimers G1,2,3-KA and G1,2,3-KH) gave dendrimers with transfection efficiency comparable to G1,2,3-KL and 5-fold better than G2,3-KL and G3-KL (Figure 5).

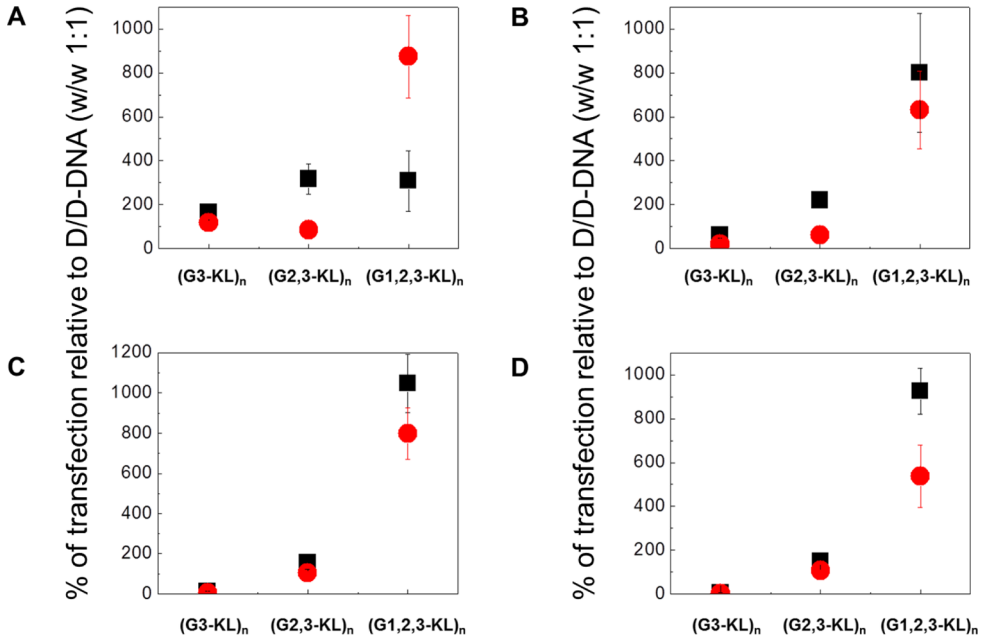

Figure 4. Effect of the charge distribution and size of the dendrimers on transfection. Dendrimers with one hydrophobic and one cationic amino acid within each generation are more effective in transfection. Luminescence values for HeLa cells transfected with a luciferase-expressing plasmid (pCI-Luc) in the presence of peptide dendrimers G3-KL, G2,3-KL, and G1,2,3-KL (black, n=1) and their respective dimers (red, n=2) with D/D (measured 24 h after transfection). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (w/w 1:1, 0.25 μg) to generate the % of transfection. The figure contains data for the N/P ratios of (A) 2.5:1, (B) 5:1, (C) 10:1, and (D) 20:1. Conditions: 1 $\times$ 10^4 HeLa cells transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

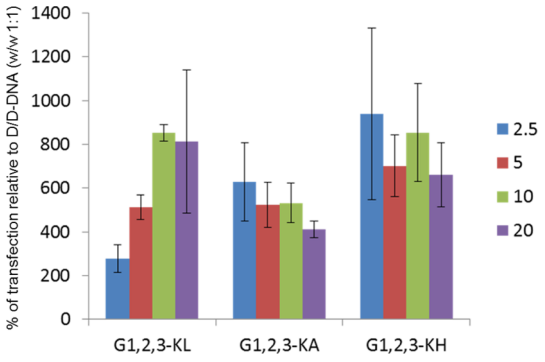

Figure 5. Comparison of the transfection efficiency of dendrimers composed of a KL dyad against dendrimers with a KH or KA dyad. Substituting the hydrophobic amino acid leucine with alanine or histidine does not affect the overall transfection efficiency. Luminescence values for HeLa cells transfected with a luciferase-expressing plasmid (pCI-Luc) in the presence of peptide dendrimers G1,2,3-KL, G1,2,3-KA, and G1,2,3-KH with D/D (measured 24 h after transfection). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (w/w 1:1, 0.25 μg) to generate the % of transfection. The figure contains data for the N/P ratios of (A,blue) 2.5:1, (B,red) 5:1, (C,green) 10:1, and (D,violet) 20:1. Conditions: 1 $\times$ 10^4 HeLa cells were transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

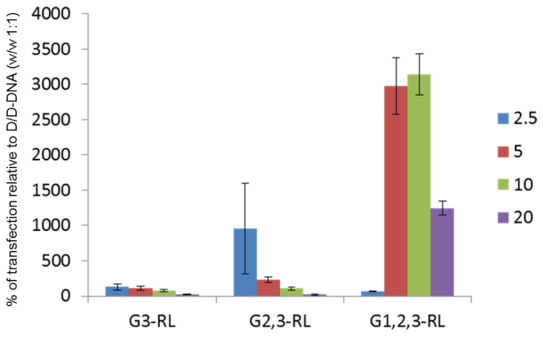

Figure 6. Effect of the charge distribution of the RL-based dendrimers on transfection. The distribution of charges over multiple generations of a dendrimer scaffold leads to a higher transfection efficiency with arginine instead of lysine as the cationic residue. Luminescence values for HeLa cells transfected with a luciferase-expressing plasmid (pCI-Luc) in the presence of peptide dendrimers G3-RL, G2,3-RL, and G1,2,3-RL with D/D (measured 24 h after transfection). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (w/w 1:1, 0.25 μg) to generate the % of transfection. The figure contains data for the N/P ratios of (A,blue) 2.5:1, (B,red) 5:1, (C,green) 10:1, and (D,violet) 20:1. Conditions: 1 $\times$ 10^4 HeLa cells were transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

Dendrimers in which the identity of the cationic group was changed (swapping lysine for arginine in dendrimers G3-RL, G2,3-RL, and G1,2,3-RL) transfected similarly well and allowed insight into whether the distribution effect would be specific for lysine or apply more generally to any cationic charge (Figure 6), overriding the features (the higher pK_a of the guanidinium

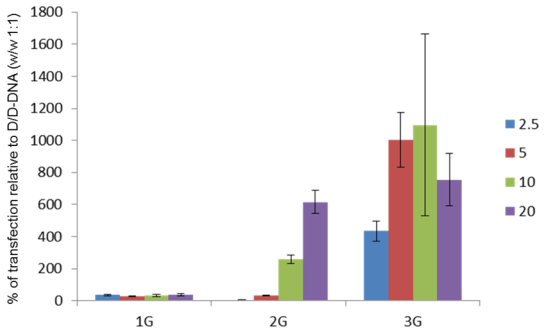

Figure 7. Relationship between the number of generations of dendrimers and transfection. An increase in dendrimer generations invariably leads to an increase in the transfection efficiency (regardless of the N/P ratio). Luminescence values for HeLa cells transfected with a luciferase-expressing plasmid (pCI-Luc) in the presence of peptide dendrimers of one (1G), two (2G), and three generations (3G) with D/D (measured 24 h after transfection). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (w/w 1:1, 0.25 μg) to generate the % of transfection. The figure contains data for the N/P ratios of (A,blue) 2.5:1, (B,red) 5:1, (C,green) 10:1, and (D,violet) 20:1. Conditions: 1 $\times$ 10^4 HeLa cells were transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

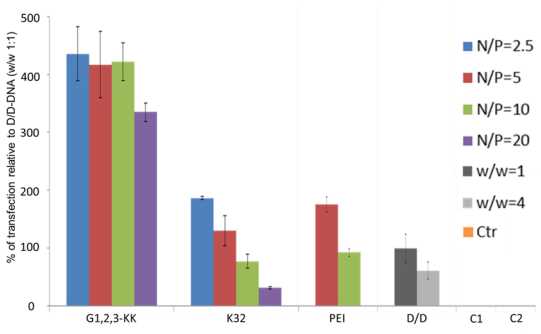

Figure 8. Transfection by peptide dendrimer G1,2,3-KK in comparison to other relevant systems. The dendrimer G1,2,3-KK (bearing 28 protonable lysines) was compared to a linear peptide composed of 32 lysines (K32) at four N/P ratios (left to right: 2.5, 5, 10, and 20). Depending on the N/P ratio, the dendrimeric system transfects 4–10-fold more efficiently than a linear analogue, suggesting that structural features influence transfection. Further controls are 25K PEI (N/P ratios of 5 (left) and 10 (right), D/D (w/w ratios of 1 and 4)), a transfection experiment in the absence of transfection reagent (C1), and the luminescence of untreated cells (C2). The luminescence values were normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (1:1 w/w, 0.25 μg) to generate the percentage of transfection. Conditions: 1 $\times$ 10^4 HeLa cells transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

side chain (~12) compared to the primary amino acid lysine and its charge delocalization). In this series, the best transfection agent is the G1,2,3-RL, followed by G2,3-RL and G3-RL as the least efficient structure. Dendrimers in which the cationic charge is distributed in each generation are clearly better than those with the same number of charges at the surface only. These results underline the importance of placing charges in the inner shells and suggest that the charge *distribution* in the dendritic scaffold affects efficiency in addition to the overall charge and the M_w/charge ratio.

Size Dependence. To study the effect of the size on transfection, the transfection efficacies of dimeric dendrimers (prepared and purified as single compounds) were compared to their monomers (Figure 4). Dimerization improved transfection about 3-fold at a lower N/P ratio of 2.5 (Figure 4A), although this effect disappears at higher N/P ratios (5, 10, and 20, Figure 4B–D). Thus, a simple increase in charge (by increasing the number of monomer equivalents) is not necessarily effective, whereas charge position and density are independent of the N/P ratio. Together with previous studies that had shown that an increase in generations of lysine dendrimers leads to better transfection[24,44] these observations lead to the conclusion that it is not an increase of the dendrimer size by dimerization that improves transfection, but rather the number of charges, their location, and the number of dendrimer generations that govern transfection efficiency instead.

However, a critical size appears to be reached with the three-generational dendrimer: first and second generation analogues are weak candidates. The first generation dendrimers have shown a very low transfection ability and do not bind DNA efficiently. A second generation analogue shows detectable transfection only at high N/P ratios (Figure 7).

A further control addresses whether there is participation of the cysteine thiol group in the core. Dendrimer Ala-G1,2,3-KL (in which alanine replaces the core cysteine) shows similar transfection to dendrimer G1,2,3-KL, suggesting that this cysteine is not participating in the creation of larger aggregates or affecting transfection *via* other effects (Supporting Information, Figure S10).

Dendrimers Transfect More Efficiently than Their Linear Counterparts. To compare the transfection effects of the structural differences between linear and dendritic structures, a linear peptide consisting of 32 lysines (K32) was compared against an all-lysine dendrimer (G1,2,3-KK) (Figure 8). At N/P ratios of 2.5, 5, 10, and 20, G1,2,3-KK transfected 2–10-fold better than the complexes made of K32 at the respective N/P ratios. The G1,2,3-KK-DNA complexes also transfected 2.5 and 4 times better than the PEI- or D/D-DNA complexes, respectively. Thus, the data suggest an advantage for a dendritic structure over a linear structure for transfection worth up to an order of magnitude. This observation reinforces the conclusion from comparisons of dendrimers with their linear analogues (specifically a linear polylysine polymer with 170 amines *vs* a sixth generation dendrimer with 128 amines and 126 lysine residues) that indicated a 100-fold advantage for dendrimer-mediated gene expression.[75] However, in a more recent study of

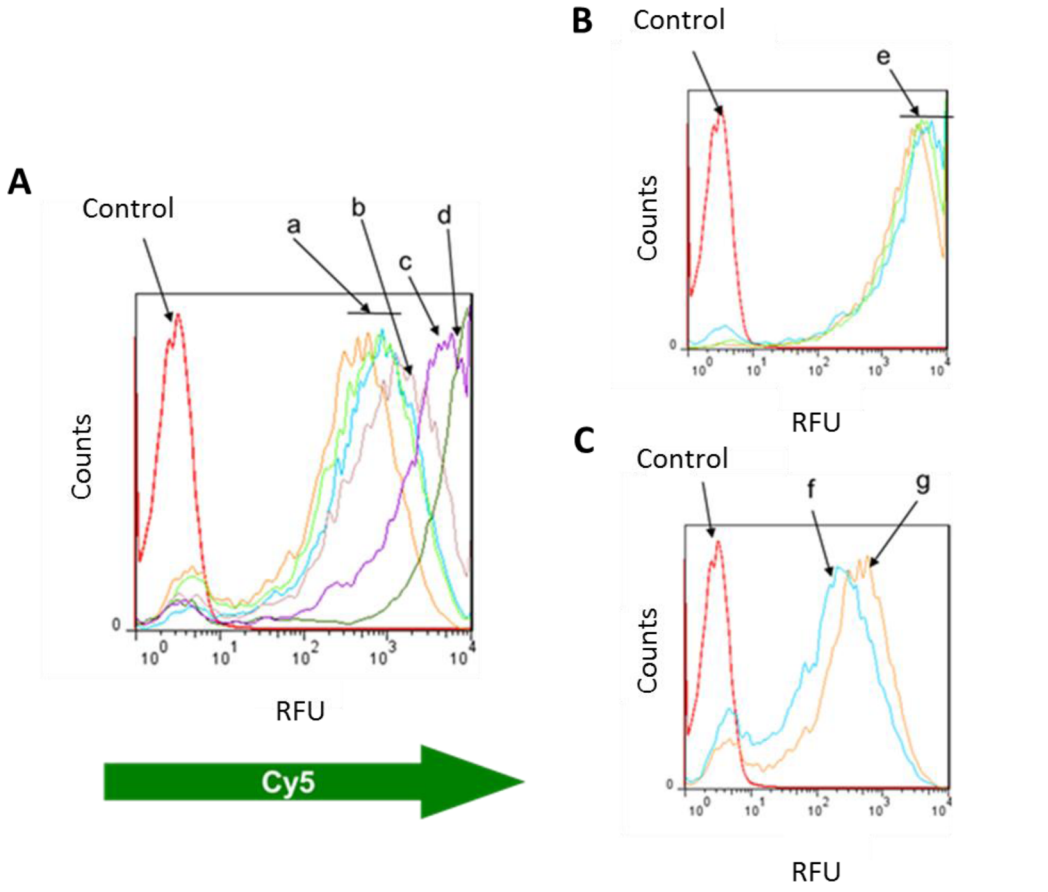

Figure 9. Cellular uptake of lipid-dendrimer-DNA complexes. More than 90% of cells take up the lipid-dendrimer-DNA complexes, indicating that their internalization is not the limiting factor for transfection. (A) Dendrimers containing the motif KL; (B) dendrimers containing the motif KL, KH, and KA; (C) uptake of linear and dendritic KK peptides (Compounds: a, G1,2,3-KK, G2,3-KK, and G3-KK; b, G2,3-KL; c, G1,2,3-KL; d, G3-KL; e, G1,2,3-KL, G1,2,3-KA, and G1,2,3-KH; f, K32; and g, G1,2,3-KK). Transfected cells were washed (3×, 1 h) in heparin solution (2 mg/mL) to remove surface-bound DNA. The cells were then treated with trypsin, and the internalization of the DNA complexes was measured by flow cytometry (RFU: relative fluorescence units). Control: untreated cells. Conditions: 5×10^4 HeLa cells were transfected for 4 h with Cy5-labeled, 0.75 μg of pCI-Luc in a 5:1 N/P ratio and in the presence of D/D (0.75 μg).

polymeric systems, a linear and a dendrimeric polylysine structure transfected similarly well, while a hyperbranched polylysine performed best.[76]

Cellular Uptake and Internalization of the Lipid-Dendrimer-DNA Complexes. The uptake of Cy5-labeled dendrimer-DNA complexes was quantified by flow cytometry. To distinguish cell-surface-bound complexes from internalized complexes, the transfected cells were washed with a heparin salt that should compete with DNA-cell-surface interactions removing the fluorescent label (see Supporting Information, Figure S5). Thus, a Cy5 signal should arise only from the cells that had internalized their cargo. Uptake was studied under conditions similar to transfection. Here, all complexes were internalized by more than 90% of the cells (Figure 9), but much less are transfected and transfection efficiency varied considerably (10−100-fold) between dendrimers. Thus, uptake is necessary but not sufficient for successful transfection. The G3-KL complexes were most effectively internalized, with a mean fluorescence intensity (MFI) 2-fold higher than the best transfectant, G1,2,3-KL (Figure 9A), despite their poor transfection efficiency. G1,2,3-KK DNA complexes were taken up more efficiently than the linear lysine-DNA complexes. The observation that uptake appears to be uniformly good for peptide dendrimer complexes is remarkable as uptake of PAMAM/DNA into brain capillary endothelial cells had to be enhanced (to 70%) by modification with transferrin, although this may be partly due to cell-specific differences.[77]

Role of the Lipid Component. The addition of a lipid component (D/D) to our dendrimer-DNA complexes leads to a slightly more compact nanoparticle (∼10−20% smaller) than in the absence of lipid and the resulting complexes fall in a range between 38 and 50 nm in hydrodynamic size (see Supporting Information, Table S2).

The functional properties are affected in the following way:

(a) *Transfection efficiency.* D/D is known to promote transfection,[60] and we accordingly observed an enhancement over transfection of DNA in the absence of lipid. When dendrimer-DNA complexes were supplemented with D/D lipid, the total enhancement of

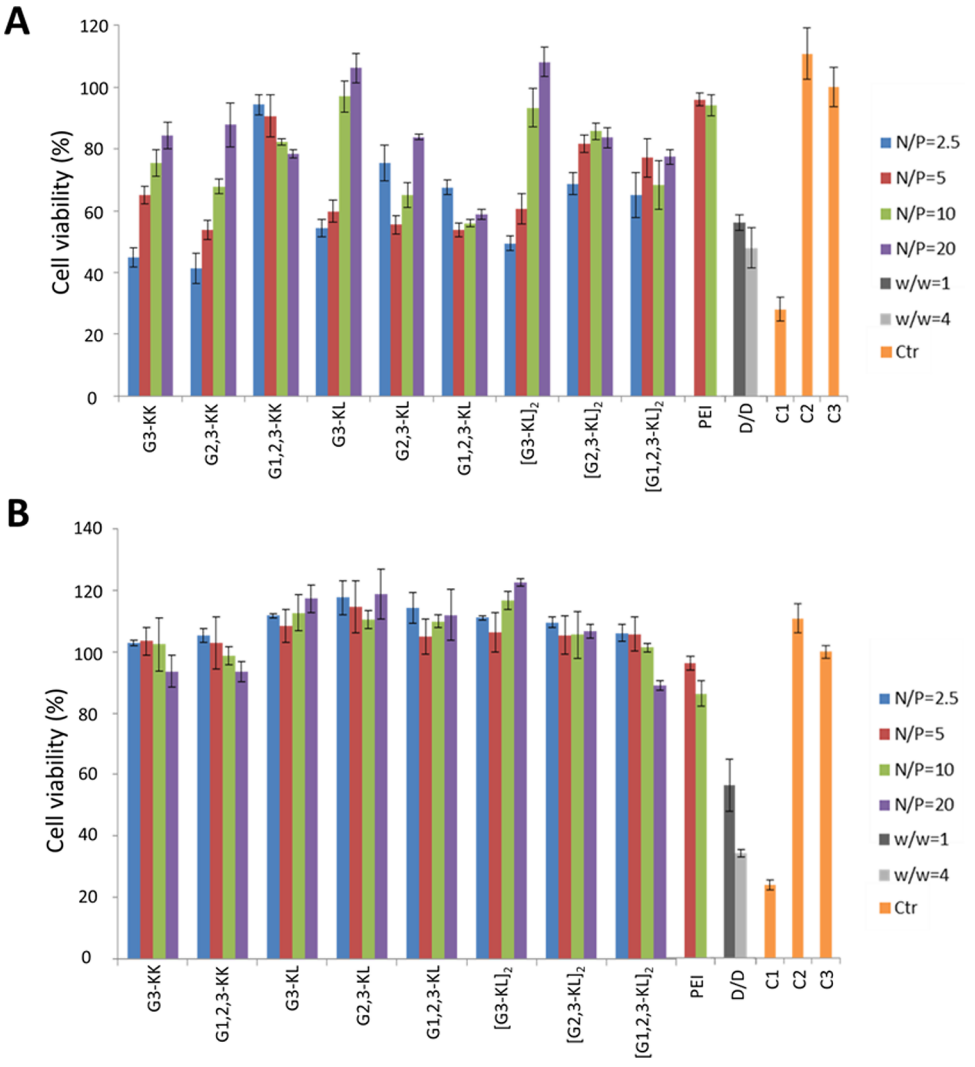

Figure 10. Cytotoxicity of dendrimer-DNA complexes in comparison to other systems. The lipid-dendrimer complexes are less toxic than commercial reagents such as L2000. Removal of the lipid from the system decreases the toxicity drastically, suggesting that toxicity stems from the lipid component. Cell viability was measured 24 h after transfection using the nuclear stain crystal violet at four N/P ratios (left to right: 2.5, 5, 10, and 20). Uptake of the nuclear stain was taken to indicate viable cells, so that the absorbance of stain is proportional to the number of viable cells remaining following transfection. The crystal violet absorbance of the cells treated by the complexes was compared to that of the untreated cells and cell viability is reported as a percentage compared to untreated cells. (A) Viability of *lipid*-dendrimer-DNA complexes (with 0.25 μg D/D) and (B) dendrimer-DNA complexes in the absence of a lipid component. For comparison, data on cells transfected with PEI (M_w 25 kDa, N/P ratios, 5 and 10), D/D, Lipofectamine 2000 (C1), DNA (but no transfection reagent) (C2), and untreated cells (C3) are included. Conditions: 1 $\times$ 10^4 HeLa cells were transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

transfection was typically increased by 30−50-fold (Figure 2).

(b) *Cytoxicity*. Crystal violet assays showed some toxicity caused by the lipid-dendrimer-DNA complexes in HeLa cells (Figure 10A). The majority of dendrimers showed high cell viability: 27 out of 36 formulations allowed 60% or more cells to survive after transfection with dendrimer-D/D, despite the cytotoxicity of D/D alone (~50% cell death). Thus, the dendrimers partly offset (or at least do not increase) the inherent toxicity of D/D. For the best transfecting dendrimers, toxicity was greater, reaching ~60% cell viability for G1,2,3KL and 60−80% for the dimer $[G1,2,3KL]_2$. In addition to inherent toxicity, it is possible that efficient luciferase overexpression itself (or related effects) indirectly leads to cell death. When comparing the structure, M_w/charge ratio, dimerization, and charge distribution of the dendrimers to the toxicity, no correlation was found (see Supporting Information, Figures S6 and S7). These comparisons imply that the molecular determinants for transfection do not coincide with those for toxicity. Importantly, these values are similar to or lower than equivalent experiments with commercial reagents such as L2000 (~30% cell viability) and D/D alone (~50% cell viability).

(c) *Endosomal escape*: In the absence of lipids, the dendrimer-DNA complexes caused either <10% or insignificant cell toxicity (Figure 10B), further indicating

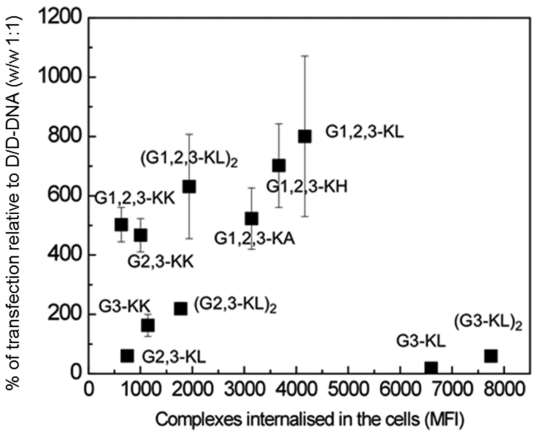

Figure 11. Relationship between complex internalization and transfection efficiency. The comparison of transfection efficiency (measured by luciferase transgene expression which was normalized by dividing them by the analogous values for cells treated with a D/D DNA complex (1:1 w/w, 0.25 μg) to generate the % of transfection (see Figure 2) and complex internalization (measured by the mean fluorescence intensity, MFI, after treatment with Cy5-labelled DNA; see Figure 8) suggests that better internalization leads to better transfection. However, this loose correlation is not obeyed by all peptide dendrimers used in this study. The two outliers (*i.e.*, mono- and dimeric G3-KL) show slow release of DNA cargo (Figure S10), suggesting that this step has become limiting for transfection. The identity of the dendrimers is annotated, and their composition can be decoded using Figure 1B. Conditions: For internalization (*x*-axis), 10^5 HeLa cells were exposed to 0.75 μg pCI-Luc; for transfection (*y*-axis), 10^4 HeLa cells were transfected with 0.25 μg pCI-Luc. Error bars refer to the mean $\pm$ SD for experiments carried out in triplicate. The data shown represent three sets of independently reproducible experiments.

that the cell toxicity observed with lipid-dendrimer-DNA complexes was mainly due to the lipid component. It is possible that replacement of the lipid component by a less toxic lipid will further reduce toxicity. Strategies to reduce cell toxicity of our system can be implemented by substituting the lipid component to less toxic lipids[78] and/or conjugating endosomal-cleavable peptide.[79,80] It has been shown that good transfection is achieved as a trade-off between low toxicity (including non-specific binding to nucleic acids) and sufficient affinity for DNA.[45]

Helper lipids are often used to promote internalization or endosomal escape.[81] To distinguish between these alternatives, we compared the uptake and internalization of Cy5-labeled dendrimer-DNA complexes by flow cytometry (FACS) in the absence and presence of D/D. The uptake of the D/D-dendrimer-DNA complexes was similar to the uptake of the dendrimer-DNA complexes alone (see Supporting Information, Figure S8), suggesting that any effect of D/D is not related to internalization and implying that its role must instead lie in improvement of endosomal escape.

Binding and Dissociation Properties of the Complexes. Unloading of DNA cargo is an important feature of a successful transfectant, and inefficient unloading can contribute to poor transgene expression.[45,82] To address the binding and dissociation properties of the lipid-dendrimer-DNA complexes, PicoGreen-labeled DNA was used to detect binding by the fluorescence decrease of encapsulated DNA. Addition of negatively charged heparin leads to DNA release and restores PicoGreen fluorescence. The heparin concentration necessary to release a fraction of DNA from the complex serves as a relative measure of DNA affinity in the complex. Figure S9 shows how similar most dendrimer complexes were in the stability of their DNA complexes and DNA unloading capabilities, dissociating at only slightly different heparin concentrations. Only G3-KL and $[G3\text{-}KL]_2$ stand out: their complexes were not able to release the DNA under the heparin challenge at all, consistent with their poor transfection performance.

CONCLUSIONS

We have discovered a new transfection system, consisting of cationic three-generational peptide dendrimers and a lipid component that rivals commercial agents in their ability to transfect mammalian cells. Structure–activity relationships point to an underlying tendency for dendrimers with lower M_w/charge ratios (Figure 3) and charges distributed over the whole dendritic structure (third, second and first generations) to be the best transfection reagents. By contrast, charges concentrated on the peripheral layer only (third generation) lead to poor transfection, and dendrimers with charges in the two outer layers (third and second generations) have intermediate activity. The dendrimers with highest charge (*e.g.*, the all-lysine dendrimer G1,2,3-KK) are not the best reagents (being surpassed by G1,2,3-KL, for example), suggesting that the presence of Lys-Leu repeats over rides charge. These comparisons suggest that structure and charge *distribution* are significant additional criteria to be considered—the more distributed the charge is, the better is the transfection reagent—and thus the total charge is not the dominant feature for transfection with peptide dendrimers.

Such trends as a result of charge organization have not been observed previously because dendrimer structures with heterogenerational features were not easily available. Instead, dendrimer-like structures assembled from identical monomers (and the same repeating structure in all generations), such as PAMAM,[83] PEI,[84,85] or hyperbranched polyamines[86] were used. These structures needed to be significantly bigger to be useful; for example, triazine dendrimers required 4–5 generations[87] and PAMAM dendrimers 5–10 generations to be similarly effective. Indeed, increasing the size of our peptide dendrimers by dimerization had virtually no effect. While modulation of internal hydrophobicity can help (*e.g.*, by Leu replacing Lys in G1,2,3-KL), the precise identity of relevant hydrophobic residues appears to be immaterial (*e.g.*, the transfection efficiencies of G1,2,3-KL, G1,2,3-KA, and G1,2,3-KH are similar).

The uniformly excellent uptake suggests that the differences in the transfection performance mainly depend on events inside the cell (Figure 11), such as unloading of DNA, endosomal escape, or better nuclear localization, which are still to be addressed in future work. Consistent with this view, the peptide dendrimer with the worst transfection performance (G3-KL) was shown not to release its cargo.

Systematic studies addressing the effects of the inner architecture of macromolecules, such as the backbone components (and the associated parameters such as charge density and charge distribution) on transfection have not, hitherto, been extensively explored. We use an efficient synthetic protocol that provides ready access to such deeply buried cationic groups, making "deep modification" in inner shell positions possible.[77] Modifications to the interior of dendrimers have also been reported in heterogeneous dendrimeric hybrid structures[88] or dendrimers incorporating orthogonal functional groups.[89] Given that exploration of the potential of interactions of inner shell residues has only just begun,[90] our methodology sets the scene for further systematic exploitation of transfection efficacy or DNA binding. In addition, further work may address the downstream cellular effects, for example, by integration of cell-targeting sequences[30,79,80] and/or nuclear localization signals[91−94] together with further modifications, involving sugars and other potential ligands. Our reagents are easy to prepare and purify as defined molecules by a well-established synthetic methodology. By combining amino acids in a dendritic fashion, transfection reagents were developed with properties not found in previously studied dendrimers or linear peptides. Peptide dendrimers are water-soluble, do not form aggregates, and show by themselves no cytotoxicity. Many other reagents, such as polymers and commercial dendrimers, are polydisperse, while our monodisperse compounds based on natural amino acids will make the approval process for therapeutic use and the definition of further structure−activity relationships simpler. The current level of efficiency is encouraging and the insight into generational charge arrangement is novel. We conclude that exploitation of the chemical degrees of freedom in making novel peptide dendrimers has led to more efficient, useful reagents for transfection.

MATERIALS AND METHODS

Cell Lines, Transfection Reagents, and Plasmids. HeLa cells were maintained in RPMI (Invitrogen, Paisley, UK) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a humidified atmosphere in 5% carbon dioxide. The plasmid pCI-Luc is derived from plasmid pCI (Promega, Southampton, UK) with the luciferase gene inserted.[82] Branched polyethylenimine (PEI) (25 kDa) was purchased from Sigma-Aldrich (Gillingham, UK). Lipofectamine 2000 (L2000) and lipofectin, a 1:1 (w/w) formulation of *N*-[1-(2,3-dioleyloxy)-propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and the neutral helper lipid dioleoyl phosphotidylethanolamine (DOPE), were obtained from Invitrogen. PEI, L2000, and lipofectin were used as a positive control transfection agent, in accordance with the manufacturer's instructions.

Strategy of Dendrimer Synthesis and Characterization. A collection of 11 peptide dendrimers consisting of three generations was prepared by solid-phase synthesis using Fmoc/Boc protection,[67,70] followed by acid cleavage from the resin and HPLC purification to give single compounds in good yields (4−26% after purification) with analytical data (HPLC, ESI-MS) consistent with the target structures (Table S1).

Introduction of hydrophobic residues (leucine or alanine) or histidine generated dendrimers with different charges and M_w, while the dendrimeric structure was preserved. Three dendrimers were also dimerized to investigate the size dependence of gene delivery.

General Synthetic Details[58,61]**.** Reagents were purchased in the highest quality available from Fluka and Sigma Aldrich. PyBOP-(benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) and Fmoc(fluorenylmethyloxycarbonyl)-protected amino acids were obtained from NovaBiochem and Advanced ChemTech (Giessen, Germany). Fmoc TentaGel S RAM resin was supplied by Rapp Polymere (Tübingen, Germany). All solvents used in reactions were bought in analytical quality or distilled and dried prior to use. Solvents for chromatographic purifications were distilled from commercial preparation of technical quality.

Solid-Phase Peptide Synthesis (SPPS). Solid-phase peptide synthesis was performed manually in polypropylene syringes fitted with a polyethylene frit and a Teflon stopcock and stopper. Fmoc-protected amino acid (3.0 equiv) and PyBOP (3.0 equiv) were added in *N*-methylpyrrolidone (NMP) to the deprotected TentaGel S RAM resin (loading 0.24 and 0.22 mmol g^{-1}). *N*,*N*-Diisopropylethylamine (DIEA, 5.0 equiv) was added, and the reaction was stirred for 60 min (reaction times were prolonged for the amino acids after one branching unit (120 min), after two branching units (180 min), and three branching units (240 min)); the equivalents of amino acid, PyBOP, and diisopropylamine were accordingly increased. The resin was then washed with *N*-methyl-2-pyrrolidone, methanol, and dichloromethane (three times each). The effectiveness of the coupling was monitored by the trinitrobenzenesulfonic acid (TNBS) test.[95] Capping of unreacted peptide chains was carried out with a solution of acetic anhydride/dichloromethane (DCM) (1:1 v/v) for 15 min before washing the resin again with NMP, methanol, and DCM (three times each). The Fmoc-protecting group was removed with a solution of piperidine/NMP (1:4 v/v) for 10 min (applied twice). The resin was washed with NMP, methanol, and DCM (three times each). The cleavage was carried out using a mixture of trifluoroacetic acid/H_2O/triisopropylsilane/1,2-ethanedithiol (94:1:2.5:2.5 v/v/v/v) solution for 4.5 h. After filtration, the peptide was precipitated in *tert*-butyl methyl ether, centrifuged, and dried under vacuum. The crude peptide was purified by preparative RP-HPLC.[67]

Homodimerization. The monomeric dendrimer (5 mg, 1 equiv) was dissolved in water (120 μL). A methanolic solution of 2,2-dithiodipyridine (aldrithiol) (45.5 mM, 0.5 equiv) was added to the solution. After thiol-activation was completed, a second portion of monomeric dendrimer (5 mg, 1 equiv) was added to the reaction mixture. A solution of $(NH_4)HCO_3$ (20 mM) was added to adjust the pH to 8. The solution was stirred for 3 h at room temperature and then acidified with water containing 0.1% TFA. The homodimer was purified by preparative RP-HPLC.[96,97]

Fluorescence Quenching Assay for DNA Binding. PicoGreen (Invitrogen) was added to DNA and diluted in TE buffer (10 mM Tris/HCl at pH 7.5; 1 mM EDTA; final [DNA] = 0.002 μg/μL). PicoGreen was added to DNA in a ratio of 1:150 (v/v) so that every 100 μL of the solution contained 0.2 μg of nucleic acid and incubated for 10 min at room temperature. During the incubation, different amounts of

transfection reagents were diluted in TE buffer. Then D/D (50 μL; 0.2 μg) and dendrimers (50 μL, the amount depending on the chosen N/P ratio that was varied from 0.625:1 to 40:1) were added per well of flat-bottom 96-well plates, and DNA (100 μL; 0.2 μg) containing the PicoGreen was added to the transfection reagents. As a control, DNA (100 μL; 0.2 μg) labeled with PicoGreen was added to TE buffer (1×, 100 μL per well). Following 30 min incubation at room temperature, TE buffer (1×, 100 μL) was added to each well. The PicoGreen signals were then detected with a fluorescent plate reader, FLUOstar Optima (BMG Labtech, Aylesbury, UK). The PicoGreen signals from the complexes were normalized against a "DNA only" control to yield the percentage of the PicoGreen signal detected.

Complex Dissociation Assay. DNA (100 μL; 0.2 μg) labeled with PicoGreen was added to D/D (50 μL; 0.2 μg) and dendrimers (50 μL, the amount depending on the N/P ratio) in a well of flat-bottom colorless 96-well plates. As a control, DNA (100 μL; 0.2 μg) labeled with PicoGreen was added to TE buffer (1×, 100 μL). Following incubation at room temperature (30 min), different concentration of heparin (0.2 to 1.4 U/mL, Sigma-Aldrich) diluted in TE buffer (1×, 100 μL) was added to DNA complexes or DNA alone (0.2 μg, 200 μL) and incubated for a further 30 min. The fluorescent signals from PicoGreen were recorded using a microplate reader (FLUOstar Optima, BMG Labtech). DNA labeled with PicoGreen was used to normalize the signal.

Cellular Uptake of the Transfection Complexes. In a well of 24-well plates, 10^5 cells were seeded 24 h prior to transfection. The cells were transfected for 4 h with Cy5-labeled DNA prepared by using the Mirus DNA labeling kit (Mirus, Cambridge, UK) following the manufacturer's instructions. Each well of the cells were transfected with complexes (500 μL) of lipofectin (0.75 μg) and dendrimers (an N/P ratio of 5; typical stock concentration = 2.85−4.5 μg in 500 μL) or the positive control and Cy5-labeled DNA (0.75 μg). Following the transfection, the cells were washed three times with heparin (200 μL; 2 mg/mL) for 1 h to remove the DNA complexes bound at the cell surface. The cells were then trypsinized, resuspended in full growth medium, and transferred to flow cytometry tubes. The cells were analyzed by flow cytometer using CyAn ADP (Beckman Coulter, High Wycombe, UK).

Transfection Procedure. Twenty-four hours before transfection, 1×10^4 HeLa cells were seeded in 100 μL of full growth medium in order to reach 70% confluence/well in 96-well plates. Plasmid transfection complexes were formed by mixing the dendrimers (100 μL, from 60 to 105 μg, dependent on N/P ratios) with D/D (4 μg; 100 μL) and incubating this mixture with a luciferase-expressing plasmid (pCI-Luc; 4 μg; 100 μL) in different N/P ratios in OptiMEM (Invitrogen) for 30 min at 25 °C. Transfection control complexes (*e.g.*, PEI, lipofectin, or Lipofectamine 2000) (total volume = 100 μL in OptiMEM) were mixed with the luciferase-expressing plasmid (pCI-Luc) (4 μg; 100 μL) at the respective manufacturers' recommended concentrations. Before overlaying the DNA complexes on the cells, OptiMEM was added to dilute the complexes so that each complex contained 0.25 μg of DNA in a total volume of 100 μL in one well of a 96-well plate. After removing complete medium from the cells, the complexes were added to the plates. The plates were incubated with the cells for 4 h at 37 °C, 5% carbon dioxide. The complexes were replaced by full growth medium for 24 h before luciferase activity was assayed.

Transgene Expression Assay. The cells were washed twice with PBS and incubated with reporter lysis buffer (20 μL, Promega) for 20 min at 4 °C then at −80 °C overnight. After the cells were defrosted at room temperature, a luciferase assay buffer (Promega; prepared according to the manufacturer's protocol, 100 μL) was added to each well. The luminescent product was measured by relative light units (RLU) in a FLUOstar Optima luminometer (BMG Labtech). The protein content of each cell lysate was determined as follows: the lysate (20 μL) was mixed with a Bio-Rad protein assay reagent (180 μL, Bio-Rad, Hemel Hempstead, UK), incubated for 10 min, and the absorbance at 590 nm was measured and converted to protein concentration using a BSA standard curve. RLU per milligram of protein represented luciferase activity. The ratio of these two values is the activity per protein unit (in RLU/mg). The values displayed in Figures 2−4 and 8 are represented after normalization against a control transfection experiment with D/D and are shown as percentages.

Cell Viability. Cells were transfected as described in Transfection Procedure. Following 24 h of transfection, the medium was removed and the cells were washed twice with PBS. The PBS was then removed, and the cells were left to dry for 1 h at room temperature (to allow permeation of the nuclear stain). Crystal violet solution (50 μL of a stock solution supplied by Sigma−Aldrich) was added to the cells and incubated for 15 min at room temperature. Following washing with distilled water (five times), the cells were left to dry (approximately 30 min at room temperature to remove water), methanol (200 μL) was added, and the suspension was incubated for 1 h at room temperature. The relative amount of the crystal violet stain retained by viable cells was determined by the absorbance of the methanolic solution at 550 nm.[98]

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: A scheme describing the solid-phase synthesis of peptide dendrimers, details of the analytical methods and analytical data (yield, measured molecular weight, HPLC data) on all compounds studied. Furthermore, additional data on transfection efficiency, cell viability, complex uptake, and DNA unloading as a function of various parameters are provided. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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