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Efficient Transfection of siRNA by Peptide Dendrimer-Lipid Conjugates

--Manuscript Draft--

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Abstract:	Efficient small interfering RNA (siRNA) delivery into cells is the basis of target gene specific silencing and, ultimately, gene therapy. However, current transfection reagents are relatively inefficient and very few studies provide the sort of systematic understanding based on structure-activity relationships that would provide rationales for their improvement. This work establishes peptide dendrimers (administered with cationic lipids) as siRNA transfection reagents and records structure-activity relationships that highlight the importance of positive charge distributed in the two outer layers and a hydrophobic core as key features for efficient performance. These transfection reagents work as well as highly optimized commercial reagents, yet show less toxicity and fewer off-target effects. More generally, the degrees of freedom in the synthetic procedure will allow the placing of decisive recognition features to enhance and fine-tune transfection and cell specificity.
Response to Reviewers:	Response to reviewers => Please note that changes are highlighted in red for the eyes of the reviewers (in extra attached copies under the heading "additional material - authors") . Reviewer 1 The authors might like to address the following minor points on preparing a definitive version: 1.- The authors have chosen to limit the introductory paragraph to siRNA delivery systems. Whereas I agree that the small size of siRNA as compared to pDNA or mRNA often translate in differences on the performance of cationic carriers, in practice most of them are polyvalent. I would rather suggest putting the focus on the molecular and monodisperse character of PDs, analogous to other molecular nucleic acid vectors reported in the literature and equally offering the possibility to conduct structure-activity studies (which is rare in the field). It is worth it to explicitly mention this analogy in the Introduction. Examples of those include calixarene, fullerene, pillarene, cyclodextrins and other macrocyclic derivatives. The following references might be incorporated to illustrate this point: Nat. Commun., 2013, 4, 1721-1727; Sci. Rep. 2014, 4, 4916; Angew. Chem. Int. Ed. 2014, 53, 13126-13130 (note that the two later are examples of siRNA delivery) ; Org. Biomol. Chem. 2015, 13, 1708-1723; Chem. Commun. 2016, 52, 10117-10120. The introduction was changed accordingly and the references incorporated – p. 1, l. 4-14/right “Furthermore, in many cases the molecular basis of the observed effects is hard to delineate. Structure-activity relationships that would provide means to rationally

manipulate the observed effects are rare, emerging sometimes from polydisperse systems [4c], but mainly from analysis of monodisperse reagents [2b, 11a, 12]."

2.- Page 1, column 1, line 37-38: the definition of the abbreviation siRNA should be moved to line 30, first time it is mentioned.

Changed.

3.- Page 2, column 1, line 30: RL should be KL, I gather.

We could not locate this suggested correction. If the sentence "The best candidates, G2,3-KL and G2,3-RL exhibited 70% and 65% gene knock down, respectively." is meant, this statement is correct (see Table 1 – here residual GAPDH activity is quoted as the corresponding 30% and 35%.

4.- Page 3, column 2, line 2: strong uptake can be (remove , which). Line 44: that it is (remove the).

Changed.

5. Note that the references format does not fit ChemBioChem's style and that some journal names are not properly abbreviated (e.g. Angewandte Chemie)

Changed.

Reviewer 2

1.While the manuscript is generally well written, the discussion is a bit hard to follow. The authors jump directly into the biological results (figure 2) first followed by the biophysical evaluations in fig 4. As a result, the authors start with figure 2A and B, then jump to fig 4 and then come back to figures 2E and F. A number of times, the results and analysis and discussions are intertwined which make the narrative not as intuitive. Why not discuss biophysical properties first, followed by cell uptake and finally the biological results?

We have taken up the suggestion of the reviewer and changed the text to show first the most important biological data and then the biophysical properties that give inside into the possible mode of action of the dendrimers. Accordingly the data were rearranged, leading to the splitting up of one figure.

2. What is DOTMA and DOPE - this should be clarified at first mention in manuscript.

Changed.

3. In general, the transfection agents do not outperform the gold standard lipofectamine 2000 from the literature. The authors claim that their formulation is less toxic based on minimal data showing greater knockdown of a control gene with L2000. That bit of data is not very convincing and the formulation does not cause off-target effects (it is toxic to cells) but the siRNA does. It just as likely that the enhanced activity with L2000 (80% knockdown versus 70% knockdown with G2,3KL) is responsible for these "off-target" effects.

We agree with the reviewer that the differences in cytotoxicity are small, but we still show that they are significant: our conclusion is based on sextuplicate experiments which were repeated 3-times, with the statistical analysis indicating significance. It is possible that the "off-target" effects are responsible for this, because the cell viability in Fig. 2B was measured after transfection.

We have added this idea to the text (p. 2, l. 24-29/right): "Apparently a non-specific interference effect is observed …. and possibly causing the observed greater cytotoxicity (compared to G2,3-KL and G2,3-RL)"

	4.Why did the authors not compare the best dendrimer to L2000 in a dose-response to really characterize the differences in potency. We have now added data on optimisation the best L2000 formulation (Supplementary Figure S3). We have chosen the best L2000 formulation to compare with our PD formulation for the gene knockdown efficacy. Similarly, why wasn't L2000 used a control for the cell uptake and biophysical studies. We have now included cell uptake and biophysical data of L2000 in supplementary Figures S3 and S4. 5.Introduction - what is an siRNA with 2'-overhangs? Sorry – we meant 3'-overhangs (now also highlighted in the SI in 'Experimental procedures', p. S13). 6.Introduction - the LNP platform being used for siRNA delivery in the clinic is the clear front-runner We have added two references to highlight how advanced these formulations are. (p. 4, l. 19-22/left: "While clinical use (as for the much more advanced lipid nanoparticles [19]) remains so far just a long-term ambition, PDs join the arsenal of siRNA delivery reagents as a new class")
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Question	**Response**
Submitted solely to this journal?	Yes
Has there been a previous version?	No
Dedication	
Animal/tissue experiments?	No

Efficient Transfection of siRNA by Peptide Dendrimer-Lipid Conjugates

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Abstract: Efficient small interfering RNA (siRNA) delivery into cells is the basis of target gene specific silencing and, ultimately, gene therapy. However, current transfection reagents are relatively inefficient and very few studies provide the sort of systematic understanding based on structure-activity relationships that would provide rationales for their improvement. This work establishes peptide dendrimers (administered with cationic lipids) as siRNA transfection reagents and records structure-activity relationships that highlight the importance of positive charge distributed in the two outer layers and a hydrophobic core as key features for efficient performance. These transfection reagents work as well as highly optimized commercial reagents, yet show less toxicity and fewer off-target effects. More generally, the degrees of freedom in the synthetic procedure will allow the placing of decisive recognition features to enhance and fine-tune transfection and cell specificity.

The therapeutic potential of gene silencing by RNA interference (RNAi) is based on downregulation of a target gene. This is achieved by the intracellular action of double-stranded short interfering RNA (siRNA; in this work a 21 base-paired RNA with 3'-overhangs) that promotes the degradation of complementary mRNA via the RISC complex. In order to reach the place of action, siRNA needs to be delivered into the P-body in the cell. Delivery into the cytosol is crucially dependent on chemical transfection reagents that transport siRNA efficiently and safely to its intracellular target site.[1] The success of transfection is determined by the efficiency of transporting the negative cargo into the cell, avoiding RNA degradation and minimizing the toxicity of transfection complexes. Despite enormous activity in the area, extracellular and intracellular barriers are still significant bottlenecks, render transfection inefficient and thus need to be overcome. A variety of transfection reagents, usually combining hydrophobic and cationic features, have been tested,[2] yet a systematic understanding of how to manipulate and control the efficiency and mode of cellular uptake, intracellular transport and endosomal release remains challenging.

Cationic lipids [2a, 3], polymers [4] dendrimers [5], dendrimer-like structures [5g, 6], peptides [7], nanocarriers [8], nanoparticles [9] nanocapsules [10] and supramolecular complexes [11] are examples of carrier vehicles that transport siRNA into cells. However, efficiency, toxicity and stability in serum need to be further improved. Furthermore, in many cases the molecular basis of the observed effects is hard to delineate. Structure-activity relationships that would provide means to rationally manipulate the observed effects are rare, emerging sometimes from polydisperse systems [4c], but mainly from analysis of monodisperse reagents [2b, 11a, 12].

Peptide dendrimers (PDs)[13] are a new class of monodisperse transfection reagents, but have so far only been used for the transfection of plasmid DNA [12c]. They have been shown to be efficient DNA transfection reagents and obeyed systematic structure-activity relationships [12c]. A key determinant of efficiency was shown to be the location of cationic charges across three dendrimer generations, a feature that had not been accessible in other transfection reagents (see Figs. 1 and S1, SI for the structural formulae). Double stranded siRNA is more compact than DNA and acts in the cytosol rather than in the nucleus. Therefore efficient reagents for plasmid delivery are not necessarily active in siRNA transfection.[14] We now show that members of the same group of PDs originally shown to be active on DNA also transfect RNA. However, variation of the amino acid building blocks throughout the dendrimer suggests that transfection is governed by different rules for RNA *vs* DNA. Thus, a library of third generation PDs [12c] displaying lysine (K) or arginine (R) as cationic residues and leucine or alanine as ~~a~~ hydrophobic moieties and dimerized KL (=LysLeu) dendrimers (shown in Table 1; see the SI for detailed synthetic procedures and characterization of the PDs) was tested in a gene knock-out assay. Table 1 shows the 14 structures of the PDs tested, which are referred to in the text by abbreviations that highlight the pattern in the dendrimer generational layers (e.g. G2,3-KL has the motif KL in generation 2 and 3 and LL in generation 1; see Figure 1).

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The success of siRNA transfection was measured by experiments in which HeLa cells were treated with an siRNA designed to target the enzyme GAPDH (siGAPDH)[14] and a combination of the PDs (at an N/P ratio of 10) with cationic lipid/ neutral helper lipid DOTMA/DOPE (DOTMA: 1,2-dioleyloxy-3-trimethylammonium propane chloride, DOPE: 1,2-dioleoyl-glycero-3-phosphatidylethanolamine; administered in a w/w ratio of 2:1 to siRNA). The transfection efficiency was quantified by monitoring the decrease in GAPDH enzyme activity (evaluated with a fluorescence assay).[14] To rule out that gene silencing is due to a non-specific effect, a validated siRNA (siNC) was used as a control (see SI). All fourteen PDs showed (when administered in combination with DOTMA/DOPE) some degree of transfection. None of them showed activity in the absence of the cationic lipids (SI Figure S2). The best candidates, G2,3-KL and G2,3-RL exhibited 70% and 65% gene knock down, respectively. This represents significant gene silencing when compared to the DOTMA/DOPE-siRNA complex ($p<0.05$) and also to untreated cells (being comparable to the highly optimized commercial reagent L2000; Figure 2A). L2000, however, suffers from strong off-target effects: we observed 35% GAPDH knockdown even when using a *non-specific* siRNA (siNC, see SI Figure S3), where no knock-down of GAPDH should be observed. Apparently a non-specific interference effect is observed for L2000-siRNA, affecting the expression pattern of a number of genes[15] and possibly causing the observed greater cytotoxicity (compared to G2,3-KL and G2,3-RL). In particular for molecular therapy approaches the imprecision caused by such non-specific effects would be detrimental and the avoidance of off-target activity gives PDs an advantage.

The physically associated dendriplexes were prepared by simply mixing dendrimer with DOTMA/DOPE and siRNA (see the SI for a typical experimental procedure). When the transfection efficiency of PDs with different cationic patterns in different generations was compared (KK vs KL vs RL, i.e. LysLys vs LysLeu vs ArgLeu), the dendrimers with KL (=LysLeu) or RL (=ArgLeu) were consistently more efficient than the ones with two lysines (e.g. G2,3-KK vs G2,3-KL or G2,3-RL; G1,2,3-KK vs G1,2,3-KL or G1,2,3-RL, $p<0.05$). The dendrimer G1,2,3-KK, a monodisperse polylysine dendrimer with a lysine diad within all the generations, performed worst in siRNA transfection, when compared to alternating charged and hydrophobic residues (G1,2,3-KL or G1,2,3-RL, $p<0.05$; see the SI p.2 for statistical analysis). Indeed, the KK series of PDs was less effective in gene silencing when compared to DOTMA/DOPE alone (Figure 2A). All PDs performed significantly better at silencing of the target gene than linear polylysine sequences ($p<0.001$): complexes of K24 and K32, respectively, siRNA and DOTMA/DOPE mediated no significant gene knock-down and caused higher cytotoxicity (Figures 2B). This observation suggests that the globular dendritic structure is beneficial, possibly by providing better encapsulation or protection to facilitate cellular siRNA uptake (Figures 3 and 4), when compared to the more rigid linear lysines, which are not as effective.[16] At the same time PD/lipid combinations showed low or barely measurable toxicity, as determined by crystal violet viability assays (Figure 2B).

A clear trend was identified by comparison of dendrimers with the same amino acid composition, but different structural arrangements of these same amino acids in the dendrimer: the best transfection reagents have hydrophobic residues in the first generation and alternating hydrophobic/charged residues in the second and third generations, which induced significant gene silencing compared to all other PD complexes, DOTMA/DOPE, the respective siNC and untreated cell controls ($p<0.05$) (e.g. G2,3-KL or G2,3-RL). The correlation of transfection efficiency with the alternating pattern is independent of the type of charged residue (e.g. G2,3-KL or G2,3-RL). These rules differ from those derived for DNA transfection: spreading the charge throughout the dendrimer contributed to efficient transfection of DNA, while concentration of charges at the surface seemed to be more important for siRNA. Increasing the size of the dendrimers by dimerization tended to be detrimental to activity for siRNA (e.g. G2,3,-KL vs $[G2,3,-KL]_2$), but was enhancing DNA transfection.[12c] The charge density (molecular weight to charge ratio) of PDs had been shown to be important in governing DNA delivery[12c] while there is no such correlation for siRNA. This difference suggests that the delivery of siRNA depends on physical dendrimer characteristics that remain to be specified. CD spectroscopy showed that the more active G2,3-KL, G2,3-RL, G1,2,3-KL and G1,2,3-RL differ from the more hydrophobic G3-KL and G3-RL in their conformation in solution (Fig. 3A), while diffusion 1H NMR indicated that the less active G3-KL and G3-RL are less compact than the active dendrimers (Table 2). Dynamic light scattering measurements demonstrated that the KL, RL series form smaller siRNA complexes with increased charges and more compact complexes than DOTMA/DOPE alone (Fig. 3B). These empirical correlations indicate structural effects, even though they may not necessarily be the direct cause of variations in gene silencing efficiency.

The properties of the dendriplex consisting of PD, lipid and RNA were analyzed in the following experiments that track the stages of the transfection process, namely assembly of the dendriplex and its stability, possible dissociation and cell penetration

(i) *Complex stability*. Differences in binding and unloading properties of these new complexes were tested with PicoGreen-labelled siRNA, which was mixed with combination of DOTMA/DOPE (2:1 to siRNA, w/w) and PDs at different N/P ratios (from 0.6 to 20). Binding of siRNA to PD results in quenching of the fluorescence of PicoGreen: thus a low signal indicates complex formation. Figures 4A and B show which dendrimers formed stable complexes with siRNA (starting from N/P ratios of 5 and 10). Apparently the charge distribution on the dendritic skeleton influences the binding ability. The three PDs containing a KK (LysLys) motif bound the siRNA similarly well. PDs with LysX (X = Leu, Ala, His) units showed a wider range of siRNA binding: G3-KL gave the largest decrease in PicoGreen signal, whereas G2,3-KL, G1,2,3-KL, G1,2,3-KA and G1,2,3-KH showed lower siRNA binding. The trend was similar for the corresponding homodimers of the KL (LysLeu) series. G2,3-RL and G3-RL, bind to siRNA similarly, suggesting the RL (ArgLeu) dendrimers formed stable complexes with the siRNA mixture with a less pronounced dependence on the total charge or number of charged residues with the generations, compared to the KL (LysLeu) and KK (LysLys) species.

(ii) *Dissociation*. Further insight into dissociation of siRNA/PD/lipid complexes was obtained by a competition assay with the negatively charged heparin as a challenger. The siRNA dendriplexes (N/P ratio of 10:1) were treated with heparin at different concentrations (Figs. 4C and 4D), so that a threshold concentration could be determined at which heparin out-competed the RNA. The effect of charge distribution on the binding strength was again evident (in Figure 2E and F): the most effective siGAPDH silencing complexes G2,3-KL and G2,3-RL were neither the strongest or weakest siRNA binders (60% and 35% of siRNA release at 1.5 U/mL of heparin, respectively). It may be possible that dendrimers that bind siRNA too strongly hinder its release in the cells, while weaker siRNA binding results in untimely dissociation of the constructs, lowering the window for complex uptake and activity.

(iii) *Uptake*. The uptake of the short interfering RNA into HeLa cells was evaluated by flow cytometry. Cy3-labelled RNA, with the PD (N/P ratio of 10) and DOTMA/DOPE (2:1 to siRNA, w/w) and siGAPDH were added to cells. After transfection (4 hours), the cells were washed with heparin to remove any surface-bound siRNA, prior to trypsinization and analysis by flow cytometry (Figure 5).

For the KL series, G1,2,3-KL clearly showed lower cell uptake, whereas G3-KL and G2,3-KL gave similar RFU signals. The same trend was observed for their corresponding homodimers (Fig. S4 SI). The RL PDs also showed a similar pattern for the three different dendrimer scaffolds, in which the overall fluorescence signal for the individual complexes was stronger compared to the analogous KL compounds, suggesting better uptake into HeLa cells. Overall, the observation that G1,2,3-KL and G1,2,3-RL did not show strong uptake can be explained by the weaker siRNA binding. The observation that G3-KL and G3-RL did not show better transfection can be rationalized by their weak siRNA dissociation, i.e. an inability to set their cargo free. The analogues, in which leucine was replaced by alanine (G1,2,3-KA) or histidine (G1,2,3-KH), gave similar Cy3 signals, indicating that the nature of the hydrophobic residue does not influence the transfection ability of the complexes. The uptake of the lipid-dendrimer-siRNA complexes evidently correlates with the transfection efficiency (see Figure S4, SI, for the remaining dendrimers and controls).

Taken together the results indicate that the interplay of charged and hydrophobic residues is necessary for efficient transfection: the PD with lysine residues in the branches only (and consequently the highest total charge) is the least effective reagent. The same observation was made with the linear sequences K24 and K32, both not performing well in transfection assay. Placing positive charges only on the peripheral layer is also not sufficient for efficiency. There is a subtle balance between the number of positive charges, the nature of the cationic residue and the distribution of charges through the dendritic structure that jointly determine the efficiency of a transfection reagent (when administered with DOTMA/DOPE). The right combination is achieved in the dendrimers G2,3-KL and G2,3-RL, which also appear to provide a less compact conformation in solution that favors complexation, a unique property of the dendritic nature of this new transfection reagents. Overall the activity of dendrimers with lysine and arginine are similar: G2,3-KL and G2,3-RL are the most active PDs (70% and 65 % GAPDH activity silencing respectively, Table 1). Changing the hydrophobic residue from leucine to alanine or histidine in the PDs did not influence the GAPDH activity substantially, consistent with the idea that it is the pattern of hydrophobic and cationic residues rather than a specific feature of the dendritic

structure that plays an important role in the transfection ability of dendrimers.

PDs with a branched and flexible architecture are well suited to form stable complexes with short and rigid siRNA molecules. The requirement for flexibility has already been highlighted for the related polyamidoamine dendrimers (PAMAM), which are too rigid for efficient binding and delivery. Degraded PAMAM dendrimers were shown to be more suitable transfection agents[17] and our work provides a possible explanation, by showing that structures with greater flexibility can be more effective. However, in other systems (such as triazine dendrimers) rigid structures were more effective. This discrepancy could be due to the very different chemical composition and the resulting intrinsic properties of the scaffold that influences siRNA binding.[5c, 18]

These guidelines could be drawn up only because - in contrast to the overwhelming majority of transfection reagents described previously – systematic variation of PD structure was possible and gave rise to structure-function relationships. While clinical use (as for the much more advanced lipid nanoparticles [19]) remains so far just a long-term ambition, PDs join the arsenal of siRNA delivery reagents as a new class in which exploration of chemical diversity can help rationalize efficiency gains to provide a framework for the design of effective and safe transfection reagents in the future. Well-defined PD structures are easily synthesized by solid phase peptide synthesis (SPPS) from natural or unnatural amino acids,[20] which will make this new class of RNA transfection reagents readily accessible.

Experimental Section

Synthesis. The peptide dendrimers were prepared by solid phase peptide synthesis and isolated by HPLC to give pure compounds showing the expected mass by ESI/MS (see Supporting Information, SI).

siRNA binding and dissociation assays. To assess the binding properties of the siRNA to the dendrimers and DOTMA/DOPE, PicoGreen labeled siRNA was incubated with the reagents for 30 minutes at room temperature. The PicoGreen signals were then detected with a fluorescent plate reader, FLUOstar Optima. To assess the dissociation properties of the siRNA from the dendrimers and DOTMA/DOPE, complexes were formed as described above and were then subjected to a challenge with heparin for 30 minutes at room temperature. The PicoGreen signal was recorded using FLUOstar Optima. The PicoGreen signals from the complexes were normalized against a 'siRNA only' control to yield the percentage of the PicoGreen signal detected. L2000 was used as a control.

siRNA transfection and cell viability measurement. HeLa cells were transfected with siRNA transfection complexes formed from dendrimers, DOTMA/DOPE and siRNA for 4 hours. The transfection complexes were replaced by full growth medium following the transfection for 48 hours, and the GAPDH activity was then assayed. To measure cell toxicity that may cause by transfection, the cells were transfected as described, washed and stained with the nuclear dye Crystal Violet. The remaining dye was then dissolved in methanol and quantified by its absorbance at 550 nm. Validated non-targeting siRNA and L2000 were used as controls. Experiments in the absence of DOTMA/DOPE showed inefficient transfection (as in our previous work with DNA transfection[12c]) and non-specific knockdown effects.

Flow cytometry. To analyze siRNA complex uptake in the HeLa cells, Cy3-labelled siRNA were mixed with dendrimers and DOTMA/DOPE, and the complexes were used to transfect the cells for 4 hours. The cells were then washed with heparin and PBS before analyzed by a flow cytometer CyAn ADP.

Statistical analysis. The data presented in this study was analyzed using a two-tailed, unpaired Student t-test and one-way or two way ANOVA followed by a post-hoc test when appropriate.

The *Supporting Information* contains details of peptide dendrimer synthesis and characterization (^{1}H NMR, DOSY-NMR, RP-HPLC chromatograms, hydrodynamic radii, CD spectra, mass spectra, transfection protocols, DNA binding assays, complex dissociation assay, cell viability assay, flow cytometry and statistical analysis.

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[1] (a) J. Kurreck, *Angew Chem* **2009**, *48*, 1378-1398; (b) R. Kanasty, J. R. Dorkin, A. Vegas, D. Anderson, *Nat Mater* **2013**, *12*, 967-977; (c) A. Wittrup, J. Lieberman, *Nat Rev Genet* **2015**, *16*, 543-552.

[2] (a) X. Guo, L. Huang, *Acc Chem Res* **2012**, *45*, 971-979; (b) A. J. Kirby, P. Camilleri, J. B. Engberts, M. C. Feiters, R. J. Nolte, O. Soderman, M. Bergsma, P. C. Bell, M. L. Fielden, C. L. Garcia Rodriguez, P. Guedat, A. Kremer, C. McGregor, C. Perrin, G. Ronsin, M. C. van Eijk, *Angew Chem* **2003**, *42*, 1448-1457; (c) J. Hoyer, I. Neundorf, *Acc Chem Res* **2012**, *45*, 1048-1056; (d) J. P. Behr, *Acc Chem Res* **2012**, *45*, 980-984; (e) M. A. Mintzer, E. E. Simanek, *Chem Rev* **2009**, *109*, 259-302; (f) I. Nakase, H. Akita, K. Kogure, A. Graslund, U. Langel, H. Harashima, S. Futaki, *Acc Chem Res* **2012**, *45*, 1132-1139.

[3] (a) S. C. Semple, A. Akinc, J. Chen, A. P. Sandhu, B. L. Mui, C. K. Cho, D. W. Sah, D. Stebbing, E. J. Crosley,

E. Yaworski, I. M. Hafez, J. R. Dorkin, J. Qin, K. Lam, K. G. Rajeev, K. F. Wong, L. B. Jeffs, L. Nechev, M. L. Eisenhardt, M. Jayaraman, M. Kazem, M. A. Maier, M. Srinivasulu, M. J. Weinstein, Q. Chen, R. Alvarez, S. A. Barros, S. De, S. K. Klimuk, T. Borland, V. Kosovrasti, W. L. Cantley, Y. K. Tam, M. Manoharan, M. A. Ciufolini, M. A. Tracy, A. de Fougerolles, I. MacLachlan, P. R. Cullis, T. D. Madden, M. J. Hope, *Nat Biotechnol* **2010**, *28*, 172-176; (b) A. Akinc, A. Zumbuehl, M. Goldberg, E. S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D. N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J. R. Dorkin, K. Narayanannair Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K. G. Rajeev, D. W. Sah, J. Soutschek, I. Toudjarska, H. P. Vornlocher, T. S. Zimmermann, R. Langer, D. G. Anderson, *Nat Biotechnol* **2008**, *26*, 561-569.

[4] (a) D. J. Gary, N. Puri, Y. Y. Won, *J Contr Rel* **2007**, *121*, 64-73; (b) S. An, D. He, E. Wagner, C. Jiang, *Small* **2015**, *38*, 5142-5150; (c) L. D. Van Vliet, M. R. Chapman, F. Avenier, C. Z. Kitson, F. Hollfelder, *ChemBioChem* **2008**, *9*, 1960-1967.

[5] (a) K. Brunner, J. Harder, T. Halbach, J. Willibald, F. Spada, F. Gnerlich, K. Sparrer, A. Beil, L. Mockl, C. Brauchle, K. K. Conzelmann, T. Carell, *Angew Chem* **2015**, *54*, 1946-1949; (b) M. Wang, H. Liu, L. Li, Y. Cheng, *Nat Commun* **2014**, *5*; (c) O. M. Merkel, M. Zheng, M. A. Mintzer, G. M. Pavan, D. Librizzi, M. Maly, H. Hoffken, A. Danani, E. E. Simanek, T. Kissel, *J Contr Rel* **2011**, *153*, 23-33; (d) T. Yu, X. Liu, A. L. Bolcato-Bellemin, Y. Wang, C. Liu, P. Erbacher, F. Qu, P. Rocchi, J. P. Behr, L. Peng, *Angew Chem* **2012**, *51*, 8478-8484; (e) O. F. Khan, E. W. Zaia, H. Yin, R. L. Bogorad, J. M. Pelet, M. J. Webber, I. Zhuang, J. E. Dahlman, R. Langer, D. G. Anderson, *Angew Chem* **2014**, *53*, 14397-14401; (f) X. Liu, C. Liu, E. Laurini, P. Posocco, S. Pricl, F. Qu, P. Rocchi, L. Peng, *Mol Pharmacology* **2012**, *9*, 470-481; (g) Y. Inoue, R. Kurihara, A. Tsuchida, M. Hasegawa, T. Nagashima, T. Mori, T. Niidome, Y. Katayama, O. Okitsu, *J Contr Rel* **2008**, *126*, 59–66; (h) H. Liu, H. Chang, J. Lv, C. Jiang, Z. Li, F. Wang, H. Wang, M. Wang, C. Liu, X. Wang, N. Shao, B. He, W. Shen, Q. Zhang, Y. Cheng, *Sci Rep* **2016**, *6*, 25069.

[6] (a) W. Fischer, M. Calderon, A. Schulz, I. Andreou, M. Weber, R. Haag, *Bioconjug Chem* **2010**, *21*, 1744-1752; (b) H. Zeng, H. C. Little, T. N. Tiambeng, G. A. Williams, Z. Guan, *J Am Chem Soc* **2013**, *135*, 4962-4965; (c) P. Ofek, W. Fischer, M. Calderon, R. Haag, R. Satchi-Fainaro, *FASEB J* **2010**, *24*, 3122-3134; (d) H. Zeng, M. E. Johnson, N. J. Oldenhuis, T. N. Tiambeng, Z. Guan, *ACS Centr Sci* **2015**, *1*, 303-312; (e) C. Chen, P. Posocco, X. Liu, Q. Cheng, E. Laurini, J. Zhou, C. Liu, Y. Wang, J. Tang, V. D. Col, T. Yu, S. Giorgio, M. Fermeglia, F. Qu, Z. Liang, J. J. Rossi, M. Liu, P. Rocchi, S. Pricl, L. Peng, *Small* **2016**, *12*, 3667-3676.

[7] Z. Tai, X. Wang, J. Tian, Y. Gao, L. Zhang, C. Yao, X. Wu, W. Zhang, Q. Zhu, S. Gao, *Biomacromolecules* **2015**, *16*, 1119-1130.

[8] K. Motoyama, R. Mitsuyasu, C. Akao, H. Abu, II, N. Sato, T. Tanaka, T. Higashi, H. Arima, *Mol Pharm* **2015**, *12*, 3129-3136.

[9] (a) T. Asai, T. Tsuzuku, S. Takahashi, A. Okamoto, T. Dewa, M. Nango, K. Hyodo, H. Ishihara, H. Kikuchi, N. Oku, *Biochem Biophys Res Commun* **2014**, *444*, 599-604; (b) A. M. Chen, O. Taratula, D. Wei, H. I. Yen, T. Thomas, T. J. Thomas, T. Minko, H. He, *ACS Nano* **2010**, *4*, 3679-3688.

[10] (a) Y. Jiang, R. Tang, B. Duncan, Z. Jiang, B. Yan, R. Mout, V. M. Rotello, *Angew Chem* **2015**, *54*, 506-510; (b) P. Resnier, P. LeQuinio, N. Lautram, E. Andre, C. Gaillard, G. Bastiat, J. P. Benoit, C. Passirani, *Biotechnol J* **2014**, *9*, 1389-1401.

[11] (a) L. Gallego-Yerga, M. Lomazzi, V. Franceschi, F. Sansone, C. Ortiz Mellet, G. Donofrio, A. Casnati, J. M. Garcia Fernandez, *Org Biomol Chem* **2015**, *13*, 1708-1723; (b) J. L. Jimenez Blanco, F. Ortega-Caballero, L. Blanco-Fernandez, T. Carmona, G. Marcelo, M. Martinez-Negro, E. Aicart, E. Junquera, F. Mendicuti, C. Tros de Ilarduya, C. Ortiz Mellet, J. M. Garcia Fernandez, *Chem Commun* **2016**, *52*, 10117-10120; (c) Y. Chang, K. Yang, P. Wei, S. Huang, Y. Pei, W. Zhao, Z. Pei, *Angew Chem* **2014**, *53*, 13126-13130; (d) V. Bagnacani, V. Franceschi, M. Bassi, M. Lomazzi, G. Donofrio, F. Sansone, A. Casnati, R. Ungaro, *Nat Commun* **2013**, *4*, 1721; (e) V. Bagnacani, V. Franceschi, L. Fantuzzi, A. Casnati, G. Donofrio, F. Sansone, R. Ungaro, *Bioconjug Chem* **2012**, *23*, 993-1002; (f) V. Bagnacani, F. Sansone, G. Donofrio, L. Baldini, A. Casnati, R. Ungaro, *Org Lett* **2008**, *10*, 3953-3956.

[12] (a) C. McGregor, C. Perrin, M. Monck, P. Camilleri, A. J. Kirby, *J Am Chem Soc* **2001**, *123*, 6215-6220; (b) S. P. Jones, N. P. Gabrielson, C. H. Wong, H. F. Chow, D. W. Pack, P. Posocco, M. Fermeglia, S. Pricl, D. K. Smith, *Mol Pharm* **2011**, *8*, 416-429; (c) A. Kwok, G. A. Eggimann, J. L. Reymond, T. Darbre, F. Hollfelder, *ACS Nano* **2013**, *7*, 4668-4682.

[13] J. L. Reymond, T. Darbre, *Org Biomol Chem* **2012**, *10*, 1483-1492.

[14] A. Kwok, S. L. Hart, *Nanomedicine* **2011**, *7*, 210-219.

[15] Y. Fedorov, A. King, E. Anderson, J. Karpilow, D. Ilsley, W. Marshall, A. Khvorova, *Nat Meth* **2005**, *2*, 241.

[16] A. Kwok, D. McCarthy, S. L. Hart, A. D. Tagalakis, *Chemical Biology & Drug Design* **2015**, *87*, 747-763. .

[17] M. X. Tang, C. T. Redemann, F. C. Szoka, Jr., *Bioconjug Chem* **1996**, *7*, 703-714.

[18] O. M. Merkel, M. A. Mintzer, D. Librizzi, O. Samsonova, T. Dicke, B. Sproat, H. Garn, P. J. Barth, E. E. Simanek, T. Kissel, *Mol Pharm* **2010**, *7*, 969-983.

[19] (a) T. W. Geisbert, A. C. Lee, M. Robbins, J. B. Geisbert, A. N. Honko, V. Sood, J. C. Johnson, S. de Jong, I. Tavakoli, A. Judge, L. E. Hensley, I. Maclachlan, *Lancet* **2010**, *375*, 1896-1905; (b) E. P. Thi, C. E. Mire, A. C. Lee, J. B. Geisbert, J. Z. Zhou, K. N. Agans, N. M. Snead, D. J. Deer, T. R. Barnard, K. A. Fenton, I. MacLachlan, T. W. Geisbert, *Nature* **2015**, *521*, 362-365.

[20] N. Maillard, A. Clouet, T. Darbre, J. L. Reymond, *Nat Protoc* **2009**, *4*, 132-142.

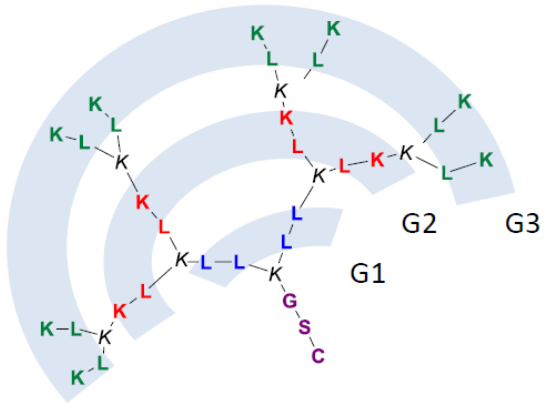

Figure 1. Structure of peptide dendrimer G2,3-KL as an example of the collection tested in this work. The shorthand G2,3-KL represents that this PD has an LL diad in the first generation (G1) and a KL diad in the second (G2) and third (G3) generations. The branching lysines are shown in italics. The cysteine residue is the C-terminus (amidated) and the eight lysines in the G3 are the N-termini with free amino groups. Full formulae are shown in Table 1 and complete chemical structures of all compounds tested are shown in the SI.

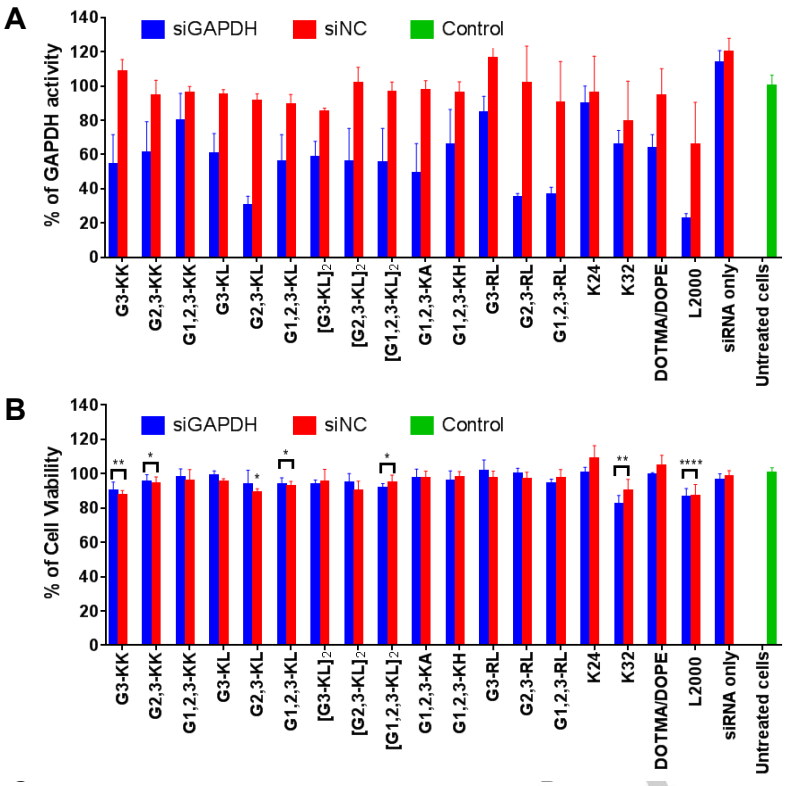

Figure 2. [A] Transfection by physically associated lipid-dendrimer-siRNA complexes. [B] Cell viability following transfection by lipid-dendrimer-siRNA complexes. HeLa cells were transfected with siGAPDH (siRNA targeting GAPDH) and siNC (non-active siRNA control) using dendrimers (N/P ratio = 10:1) and DOTMA/DOPE (w/w ratio = 2:1). Error bars refer to the mean ± SD carried out in sextuplicate. See the SI for detailed experimental protocols and for statistical analysis of the significance of the differences in gene silencing observed.

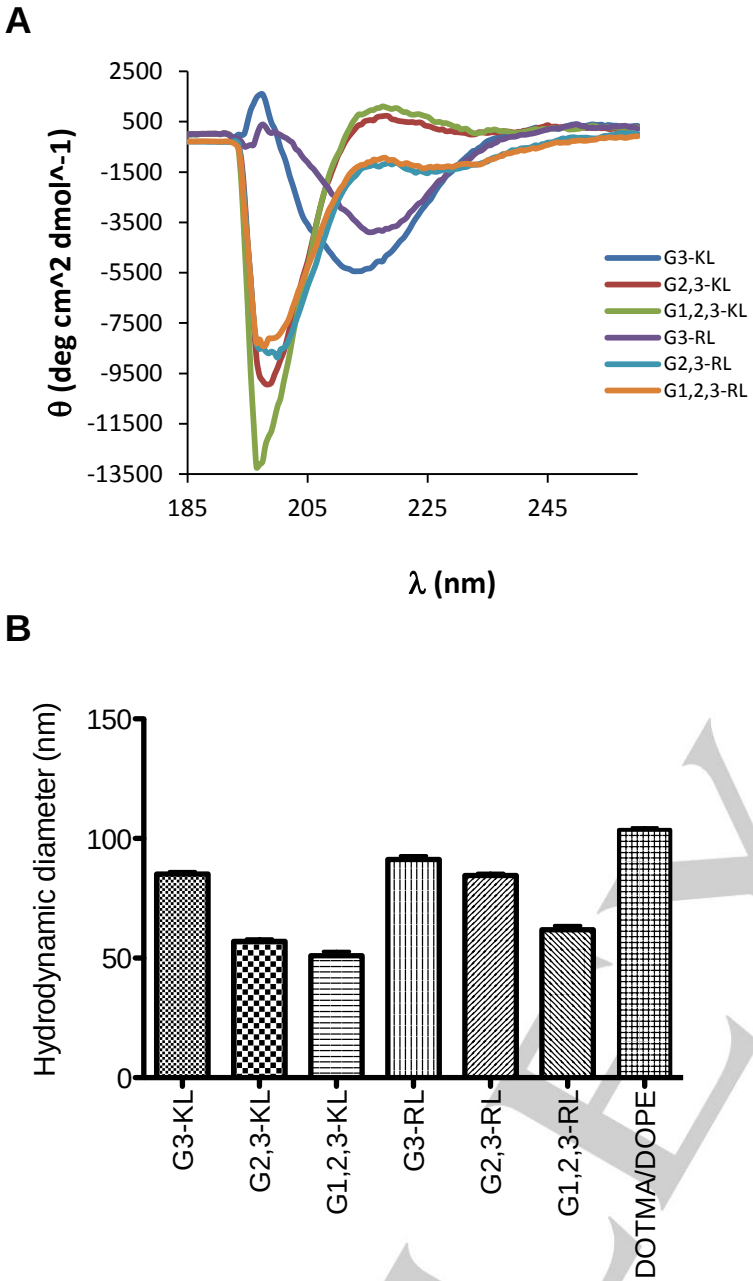

Figure 3. Biophysical characterisation of PDs. **(A)** CD spectra of 0.1 mg/mL peptide dendrimers in phosphate buffer (pH 7.4) at room temperature. (B) Hydrodynamic diameters of the lipid-dendrimer-siRNA complexes. siRNA (10 µg) was added to the dendrimers at an N/P ratio of 10:1 in the supplement with DOTMA/DOPE (w/w=2:1) at room temperature. The DOTMA/DOPE and siRNA complexes were used as a control. Following a 30 minute incubation, the complexes were transferred to a low-volume transparent cuvette for the hydrodynamic diameters measurement. The hydrodynamic diameter was recorded and analysed by Dynamic Light Scattering. n=3-7 for each formulation and SD is shown. All the data shown have a PDI lower than 0.5.

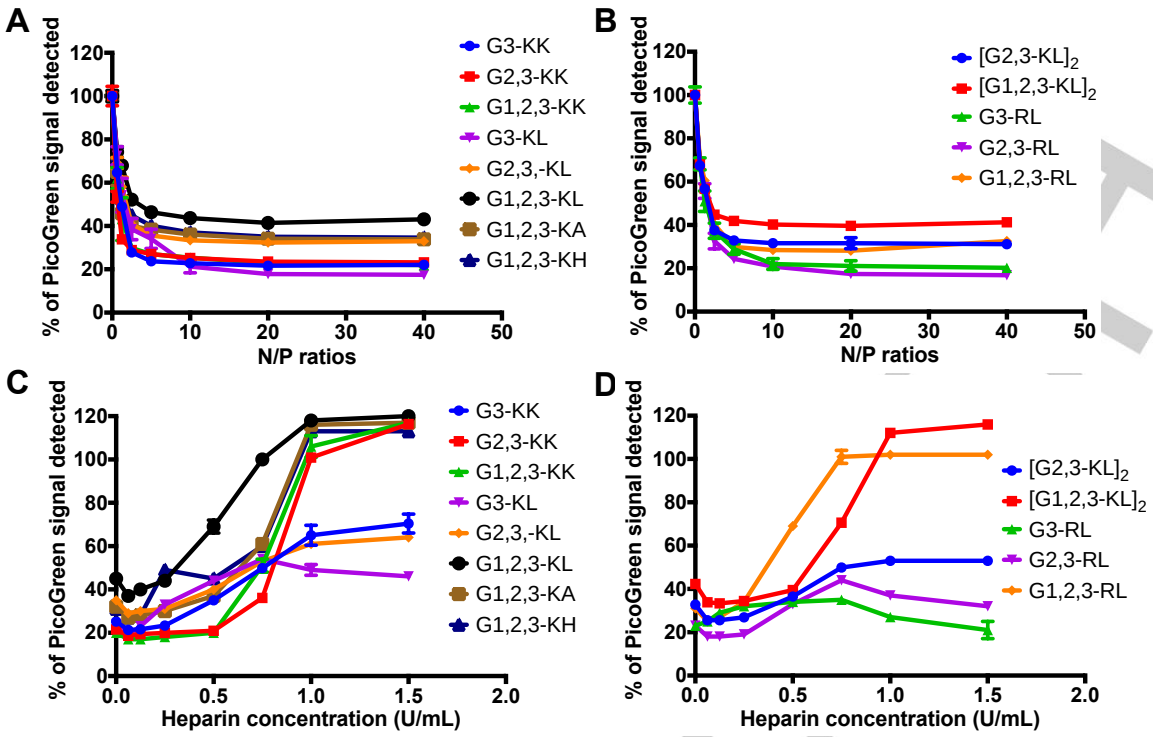

Figure 4. [A] and [B] Binding data of lipid-dendrimer-siRNA complexes. PicoGreen-labelled siGAPDH was used to form complexes with dendrimers at different N/P ratios, in the presence of DOTMA/DOPE (2:1 to siRNA w/w). The PicoGreen signal detected from the complexes was normalized by the siRNA alone control to yield the percentage of PicoGreen signal detected. [C] and [D] siRNA unloading data of lipid-dendrimer-siRNA complexes. Complexes were formed as described in [A], then, the complexes were challenged with heparin. Error bars refer to the mean ± SD for experiments carried out in triplicates.

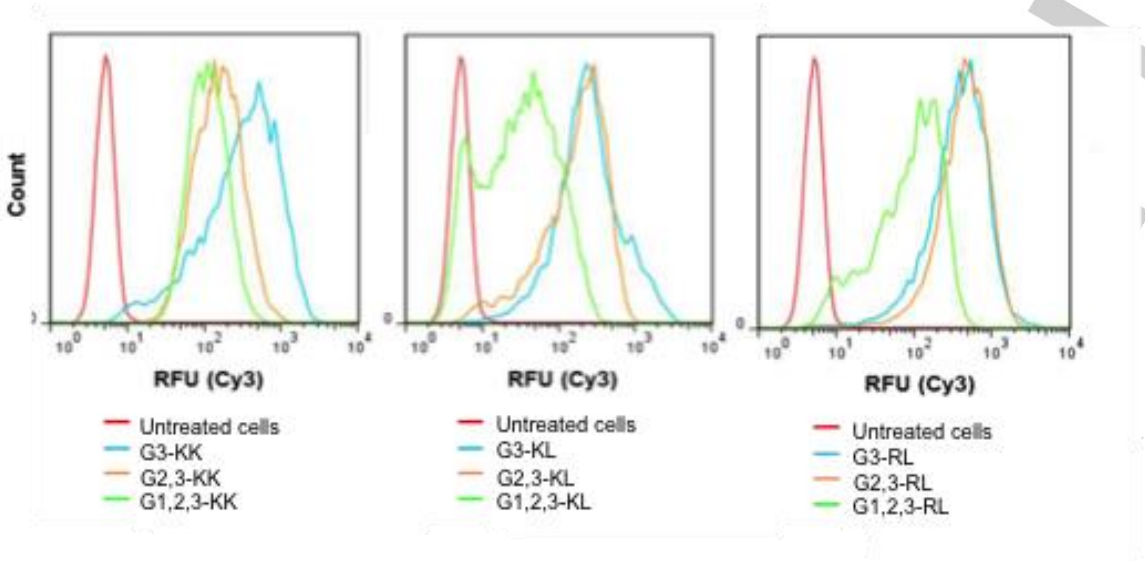

Figure 5. Cellular uptake of lipid-dendrimer-siRNA complexes. HeLa cells were transfected with complexes of Cy3-labelled siGAPDH (50 pmol, 25 µL) with dendrimers (N/P = 10) and DOTMA/DOPE (2:1 to siRNA, w/w) for 4 hours. Transfected cells were washed (3 times) with heparin solution (2 mg/mL) to remove surface-bound siRNA. The cells were then treated with trypsin and the internalization of the siRNA complexes was measured by flow cytometry (RFU: relative fluorescence units). See Fig. S4, SI for the controls.

Table 1. Peptide dendrimers (PDs) studied in this work. PDs are identified by the generation modified (G) and the substitution pattern in this shell (e.g. G3-KK has the motif KK in the third shell and the motif LL in the first and second shell; G1,2,3-KK has a KK motif in *all* three shells. See the SI for full chemical structures.

Transfectant	Sequence[a]	Total charge (+)[b]	Side chains		GAPDH Activity [%][d]
			Charged (+)[c]	Hydro-phobic	
G3-KK	$(KK)_8(\mathit{K}LL)_4(\mathit{K}LL)_2\mathit{K}GSC$	24	16	-	54 ± 17
G2,3-KK	$(KK)_8(\mathit{K}KK)_4(\mathit{K}LL)_2\mathit{K}GSC$	32	24	-	61 ±18
G1,2,3-KK	$(KK)_8(\mathit{K}KK)_4(\mathit{K}KK)_2\mathit{K}GSC$	36	28	-	80 ± 16
G3-KL	$(KL)_8(\mathit{K}LL)_4(\mathit{K}LL)_2\mathit{K}GSC$	16	8	20	60 ± 12
G2,3-KL	$(KL)_8(\mathit{K}KL)_4(\mathit{K}LL)_2\mathit{K}GSC$	20	12	16	30 ± 5
G1,2,3-KL	$(KL)_8(\mathit{K}KL)_4(\mathit{K}KL)_2\mathit{K}GSC$	22	14	14	56 ± 16
G3-RL	$(RL)_8(\mathit{K}LL)_4(\mathit{K}LL)_2\mathit{K}GSC$	16	8	20	84 ± 10
G2,3-RL	$(RL)_8(\mathit{K}RL)_4(\mathit{K}LL)_2\mathit{K}GSC$	20	12	16	35 ± 3
G1,2,3-RL	$(RL)_8(\mathit{K}RL)_4(\mathit{K}RL)_2\mathit{K}GSC$	22	14	14	36 ± 5
G1,2,3-KA	$(KA)_8(\mathit{K}KA)_4(\mathit{K}KA)_2\mathit{K}GSC$	22	14	14	49 ± 18
G1,2,3-KH	$(KH)_8(\mathit{K}KH)_4(\mathit{K}KH)_2\mathit{K}GSC$	>22[c]	14	>14[c]	66 ± 21
[G3-KL]$_2$	$[(KL)_8(\mathit{K}LL)_4(\mathit{K}LL)_2\mathit{K}GSC]_2$	32	16	40	55 ± 10
[G2,3-KL]$_2$	$[(KL)_8(\mathit{K}KL)_4(\mathit{K}LL)_2\mathit{K}GSC]_2$	40	24	32	58 ± 10
[G1,2,3-KL]$_2$	$[(KL)_8(\mathit{K}KL)_4(\mathit{K}KL)_2\mathit{K}GSC]_2$	44	28	28	55 ± 20
Linear **K24**	K_{24}	24	-	-	n.d
Linear **K32**	K_{32}	32	-	-	n.d
DOTMA/DOPE	n.a	n.a	-	-	68 ± 9
L2000	n.a	n.a	-	-	22 ± 3 [e]

[a] One letter codes for amino acids. Branching lysines in italics. All peptides are carboxamide ($CONH_2$) at the C-terminus. [b] Number of cationic amino acid residues (Lys or Arg) and free N-termini. [c] R or K. [d] His residues are partially protonated at pH 7. [d] GAPDH activity (%) is defined by the remaining GAPDH activity following transfection normalised against the GAPDH activity of untreated cells (set as 100%). [e] siRNA transfection with L2000 leads to the knock-down of more than one gene. This non-specific gene knockdown is significant and amounts to 35%. By contrast, all PDs only affect the targeted GAPDH.

The mean ± SD is for experiments carried out in sextuplicate; n.d: not detectable above the siNC or untreated cells (< exp. error); n.a: not applicable.

Table 2. Hydrodynamic radii of the peptide dendrimers.

Compound	Solvent	pH	radius[a]
G3-KL	D_2O	≈7.4	3.71±0.18
G2,3-KL	D_2O	≈7.4	2.22±0.02
G1,2,3-KL	D_2O	≈7.4	2.3±0.04
G3-RL	D_2O	≈7.4	4.15±0.23
G2,3-RL	D_2O	≈7.4	2.27±0.03
G1,2,3-RL	D_2O	≈7.4	2.52±0.04

[a] The hydrodynamic radii $\mathbf{R_h}$ were calculated from diffusion coefficients D, which are the median values from an intensity fit analysis of different ^1H signals, were calculated from the Stokes-Einstein equation $R_h = k*T/6\pi*\eta*D$, with Boltzmann constant $k = 1.380*10^{-23}$ $J*K^{-1}$, temperature T = 303 K and viscosity h = 1.089 mPa*s for D_2O.

Entry for the Table of Contents

COMMUNICATION

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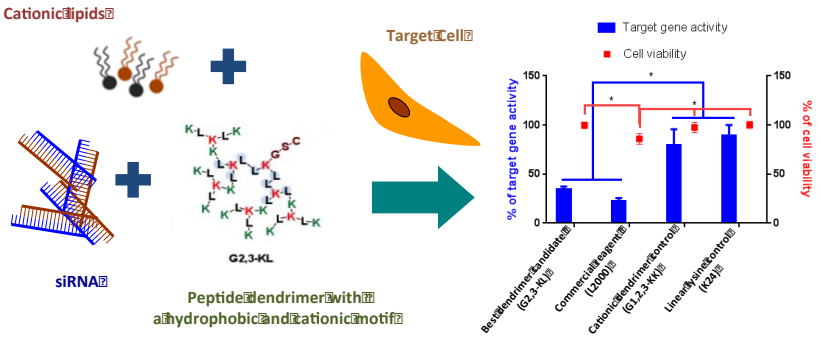
Page XXX – Page XXX

Efficient Transfection of siRNA by Peptide Dendrimer-Lipid Conjugates

Peptide dendrimers can transfer siRNA into living cells and achieve gene knock-down with efficiency comparable to commercial reagents, avoiding toxicity and yielding structure-function relationships.



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Supporting Info (with changes marked in red)