Polycationic phosphorus dendrimers: synthesis, characterization, study of cytotoxicity, complexation of DNA, and transfection experiments†‡

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Four series of phosphorus dendrimers (generations 1 and 4) having various types of amine terminal groups (pyrrolidine, morpholine, methyl piperazine, or phenyl piperazine) are synthesized. After protonation, the fourth generations of three of them are found water-soluble, and used for several biological experiments. First, the cytotoxicity of these polycationic dendrimers towards three cell strains (one healthy: HUVEC, and two cancerous: HEK 293 and HeLa) is assayed and found low. Second, their ability to interact with DNA is tested by electrophoresis: the dendrimer terminated by pyrrolidinium groups is found efficient to form dendriplexes. Finally, the polycationic dendrimers are used as transfection agents to deliver single- and double-stranded DNA into the three above-mentioned cell strains. Here also the dendrimer having pyrrolidinium groups is found the most efficient.

Introduction

The use of dendrimers for biological experiments is a flourishing area of research,[1] due to their precisely defined structure, their multiple functional groups, and their high tunability allowing innumerable modifications of both their periphery and their internal domain. In particular, dendrimers appear as an appealing alternative to other delivery systems such as polymers and liposomes. Interaction of dendrimers with DNA[2] has been recognised very early as suitable for transfection experiments.[3] Several types of dendritic structures, mostly based on poly(amidoamine) PAMAM dendrimers,[4] but also on poly(propyleneimine) PPI,[5] poly(lysine),[6] carbosilane,[7] and phosphorus[8] dendrimers have been used successfully for such experiments. However, none of the already proposed systems is able to compete with the efficiency of viral carriers, thus the design of more efficient candidates by modifying the structure of the dendrimers is still a quest.

We have already shown that the backbone of the phosphorus dendrimers we synthesize[9] is biocompatible, and that they are active as anti-prion agents,[10] anti-HIV agents,[11] able to stimulate immune blood cells[12] and to multiply human natural killer (NK) cells,[13] depending on the type of terminal groups they bear. We have already shown also that the transfection efficiency of the phosphorus dendrimers having multiple ammonium terminal groups depends on the reversibility of protonation: tertiary ammonium groups are less toxic and more efficient than quaternary ammonium groups, presumably due to the adaptability of tertiary ammonium groups to pH (amine/ammonium). In addition, we have shown that the generation of the dendrimers has an important influence on the transfection efficiency, with the fourth generation being the best compromise between the transfection efficiency and the time needed to synthesize the dendrimer. The sole phosphorus dendrimer having ammonium terminal group that we have tested up to now as transfecting agent is terminated by *N*,*N*-diethylethylenediamine.[8]

In this paper, we report the synthesis of several new, first and fourth generations of phosphorus dendrimers ended by derivatives of pyrrolidine, morpholine, methyl- and phenylpiperazine. The biological behaviour of the dendrimers **2–4** of fourth generation is studied first to check their cytotoxicity towards three cells strains, then their ability to complex double-stranded oligonucleotides, and finally their transfection efficiency is assayed.

Results and discussion

Syntheses

The first generation dendrimer **1-G$_1$**,[14] built from a cyclotriphosphazene core possesses 12 chloride residues as terminal groups; it is used in the presence of caesium carbonate as base to test the reactions with various diamino derivatives: 1-(2-aminoethyl)pyrrolidine (**2**), 4-(2-aminoethyl)morpholine (**3**), 1-methylpiperazine (**4**), and 1-phenylpiperazine (**5**) (Scheme 1). We have previously shown that primary amines react easily with P(S)Cl$_2$ terminal groups of dendrimers to afford disubstituted derivatives (P(S)(NHR)$_2$) but also that secondary amines afford only monosubstituted derivatives

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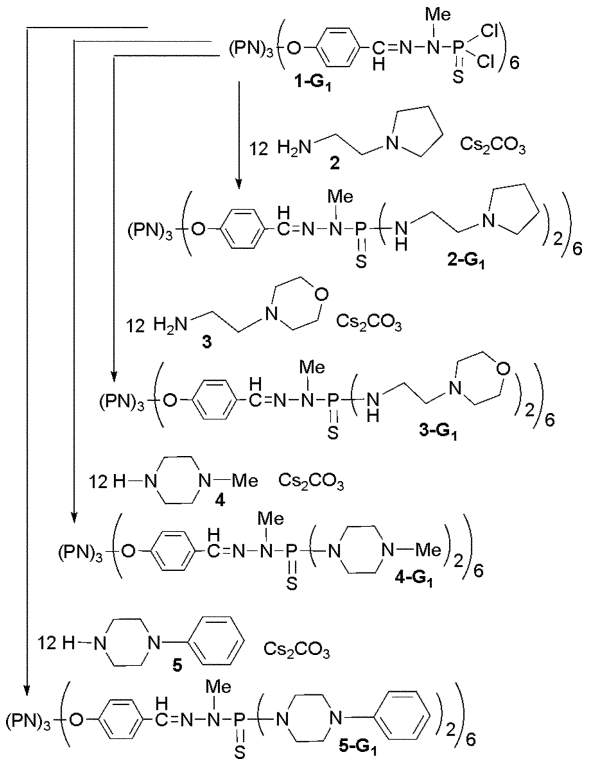
† Dedicated to Prof. Jean-Pierre Sauvage, on the occasion of his 65th birthday.

‡ Electronic supplementary information (ESI) available: Copy of ^{31}P NMR spectra for dendrimers **2-G$_n$**, **3-G$_n$**, **4-G$_n$**, **5-G$_n$** (n = 1 and 4), **2′-G$_4$**; transfection efficiency of GFP-coding plasmid in HUVEC and HeLa cells. See DOI: 10.1039/b815259d



Scheme 1 Synthesis of first generation dendrimers having tertiary amines as terminal groups.

($P(S)ClNR_2$), even when using a large excess of amine.[15] Thus, we expected no problem for the reaction with amines **2** and **3**, using Cs_2CO_3 as base. Indeed, monitoring the reactions by ^{31}P NMR displays in all cases the disappearance of the singlet at 65.9 ppm corresponding to $P(S)Cl_2$ on behalf of an intermediate signal at $\delta(^{31}P)$ = *ca.* 78 ppm (monosubstitution). This signal rapidly disappears on behalf of a signal corresponding to $P(S)(NHR)_2$ terminal groups at $\delta(^{31}P)$ = *ca.* 72 ppm for **2-G$_1$** and **3-G$_1$**, showing the completion of the substitutions.

In the case of compounds **4** and **5**, we observed the monosubstitution as expected ($\delta(^{31}P)$ = 80 ppm), but to our surprise the reaction continued to afford the disubstitution, characterized by a signal at *ca.* 76.5 ppm for **4-G$_1$** and **5-G$_1$** after one night at room temperature (Scheme 1). We believe that these cyclic secondary amines induce less steric crowding around phosphorus than the dialkylamines we used previously;[15] that might be the reason for this unexpected result.

All these first generation dendrimers are obtained quantitatively as shown by ^{31}P NMR, and isolated in relatively good yields after work-up. These dendrimers are also characterized by ^{1}H and ^{13}C NMR. In all cases, the CH_2 groups linked to the nitrogen that underwent the reaction appear as a doublet in the ^{13}C NMR spectra, due to the $^{2}J_{CP}$ coupling with phosphorus. These neutral species are soluble in organic solvents, but not in water. The protonated derivatives are obtained by adding HCl in ether to a solution of the dendrimer in THF. The amount of HCl is chosen to protonate 90% of the terminal groups; the aim is to induce the solubility in water, then an acidic–basic equilibrium should occur in the physiological media. The protonated dendrimers precipitate in THF and are recovered by filtration, then they are tentatively dissolved in water. The protonated dendrimers **2′-G$_1$**, **3′-G$_1$** and **4′-G$_1$** (obtained from **2-G$_1$**, **3-G$_1$** and **4-G$_1$**, respectively) are soluble in water, but not **5′-G$_1$**, probably because few terminal nitrogens are protonated in this case. The protonated dendrimers are only characterized by ^{31}P NMR, in order to ascertain the integrity of the internal skeleton: the ^{31}P NMR chemical shifts are analogous before and after protonation of the terminal amines.

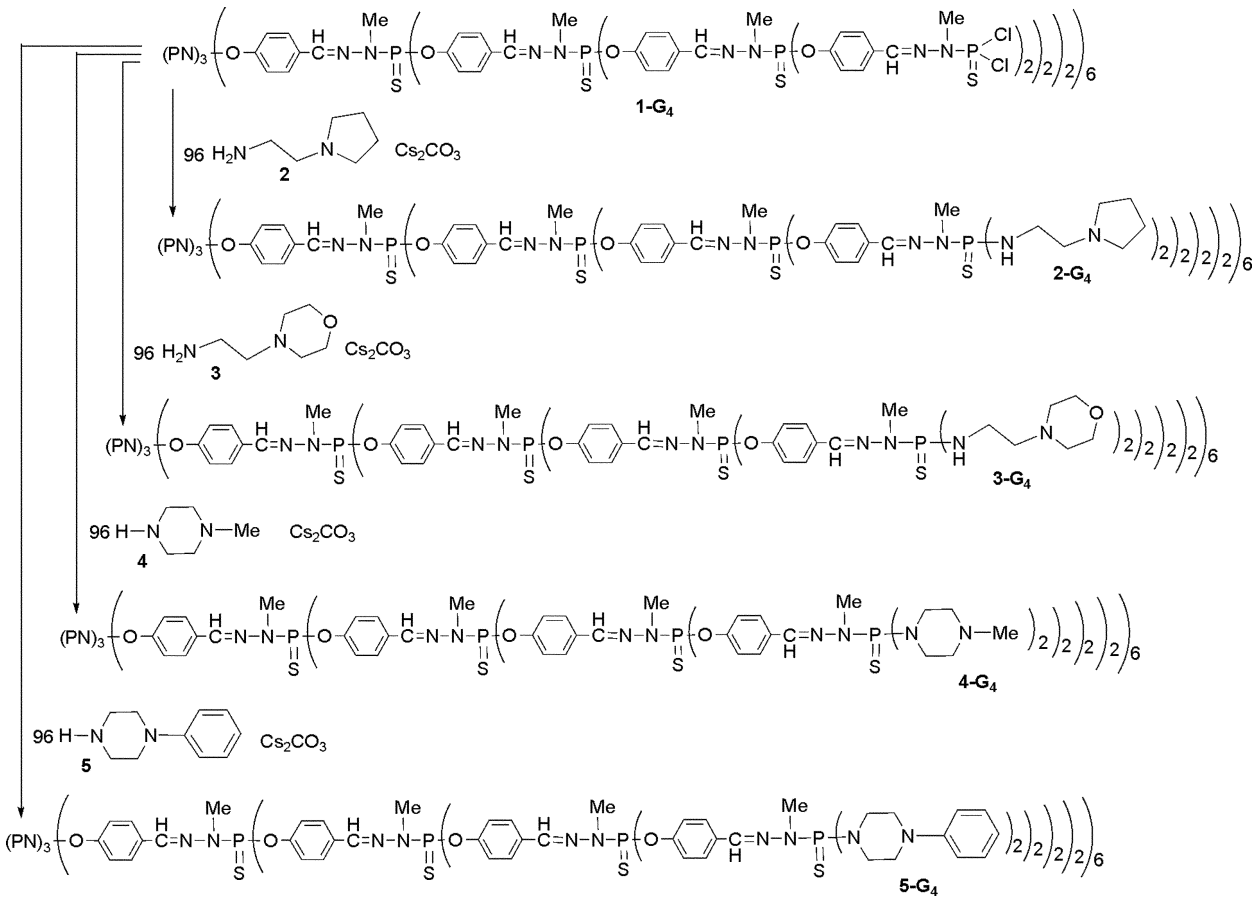
Having in hand the method to functionalize the first generation, we applied the same method to the fourth generation **1-G$_4$** possessing 96 terminal chloride residues. In this case also the reactions with amines **2–5** are completed after one night at room temperature, to afford dendrimers **2-G$_4$**, **3-G$_4$**, **4-G$_4$** and **5-G$_4$** (Scheme 2). In all cases we observe by ^{31}P NMR an intermediate signal corresponding to the monosubstitution on each terminal group, which disappears on behalf of the signal corresponding to the full substitution, as for the first generations. All these dendrimers are also isolated in good yield after work-up. As for the first generation, water solubility is obtained after protonation in the case of **2′-G$_4$**, **3′-G$_4$** and **4′-G$_4$** but not for **5′-G$_4$**.

Biological assays

In view of the previous experiments with the series **6′-G$_n$** (n = 1–5) (Fig. 1) in which the first generation was practically non-active,[8] we decided to study the biological properties of the fourth generations only. The first biological assays were carried out in order to determine the cytotoxicity of dendrimers **2′-G$_4$**, **3′-G$_4$** and **4′-G$_4$** towards three human cell strains: one healthy strain (HUVEC: human umbilical vein endothelial cell) and two cancerous lines (HeLa: human epitheloid cervical carcinoma; HEK 293: human transformed primary embryonal kidney).

The amount of cells that survived after the experiments is determined by MTT assay. This test consists in the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) by the succinate dehydrogenase intervening in the respiratory mitochondrial chain of viable cells. In this reaction, the water-soluble yellow MTT is converted to water-insoluble purple formazan, which is later dissolved in organic solvents and dosed by spectrophotometry.[16] The percentage of living cells is deduced from the corrected formazan absorbance.

Solutions of dendrimers are first prepared in a PBS buffer (phosphate/saline buffer) free of Mg^{2+} and Ca^{2+}, pH 7.4; only dendrimer **2′-G$_4$** is soluble in these conditions, whereas dendrimers **3′-G$_4$** and **4′-G$_4$** precipitate (dendrimer **5′-G$_4$** was not tested). Addition of a biocompatible solvent (DMSO, acetonitrile) does not improve the solubility. This shows that the acidic–basic equilibrium of the amino terminal groups is shifted towards the neutral form at physiological pH, inducing the precipitation of the dendrimer. Better solubility of test dendrimers is observed in an OPTI-MEM 1 cell culture medium, used routinely for cell transfection. Solutions up to 50 μg mL^{-1} are stable in these conditions for at least for an hour and no precipitates are observed. Thus, in a next attempt, the OPTI-MEM 1 solutions of dendrimers are used for transfection of HUVEC, HeLa and HEK 293 cells and cytotoxicity tests. The quantity of dendrimer added to cells



Scheme 2 Synthesis of fourth generation dendrimers having tertiary amines as terminal groups.

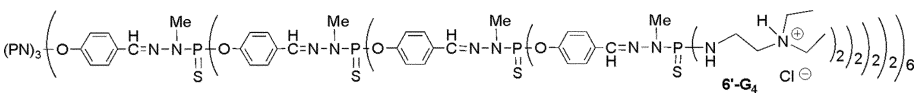


Fig. 1 Chemical structure of the previously used phosphorus dendrimer as transfection agent, **6′-G$_4$**.

culture allows to reach a final theoretical concentration of 0.5, 1, 2, 5, 10, 20, or 50 μg mL^{-1} in each well for a final volume of 200 μL. Observation of the cells by phase contrast microscope shows a partial precipitation of dendrimers in all cases except for **2′-G$_4$** in the culture of HeLa cells. After 48 or 72 h, HEK 293 and HeLa cells in the presence of the dendrimer display a behaviour different from those cultured without dendrimers, forming packs instead of single layers. After 24, 48 and 72 h, a solution of MTT is added and allowed to interact with the cells for 2 h. An intense purple coloration is observed in all cases. The cells are then lysed (sodium dodecyl sulfate and DMF) and after 24 h, all the formazan has diffused in the solution. The results of the MTT assays are given in Fig. 2; each value is the result of the measurement of at least four concordant values. No significant cytotoxicity is detected for the dendrimers **2′-G$_4$** and **3′-G$_4$**, even if the HUVEC cells (non-cancerous strain) are more sensitive to the presence of the dendrimers than the cancerous cells. On the other hand, dendrimer **4′-G$_4$** having methylpiperazine extremities is the most toxic against HUVEC cells and seems to increase the percentage of cancerous cells (HEK 293 and HeLa). Consequently, it will not be the best candidate for transfection experiments.

The second biological test that we have carried out consists in measuring the strength of the association between dendrimers and DNA. For this purpose, a ^{32}P labelled 20-mer double stranded oligodeoxyribonucleotide (0.025 nmol) was incubated/complexed with different concentrations of the three dendrimers, **2′-G$_4$**, **3′-G$_4$** and **4′-G$_4$**. The result of the possible association between the dendrimers and the oligonucleotides, often called ''dendriplexes''[17] was checked by electrophoresis. This technique allows to separate species on a gel support, depending on their charge and molecular mass: free DNA (negatively charged) should migrate towards the anode faster than DNA complexed with positively charged dendrimers, since most charges should be neutralized in this high molecular mass complex. This assumption is true if dendriplexes exhibit an ability to migrate in the gel. Fig. 3 displays the results with increasing quantities of dendrimers (from 0.001 to 2.24 nmol for **2′-G$_4$**, from 0.035 to 1.12 nmol for **3′-G$_4$**, and from 0.035 to 2.24 nmol for **4′-G$_4$**). Only dendrimer **2′-G$_4$** is able to interact with DNA, as shown by the disappearance of the black band in the middle of the gel. Surprisingly, there is no modification of the distance of migration, depending on the quantity of dendrimer: either

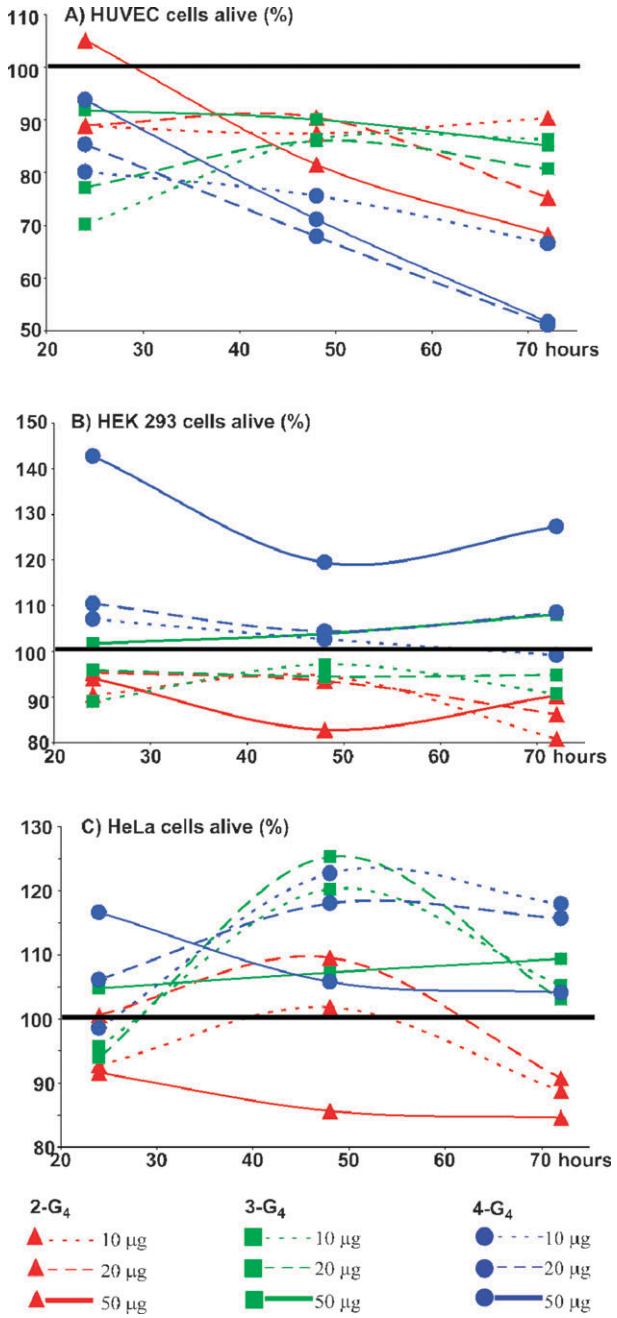


Fig. 2 Cytotoxicity measured by MTT assays, with various concentrations of dendrimers **2′-G$_4$**, **3′-G$_4$** and **4′-G$_4$**. (A) for HUVEC cells; (B) for HEK 293 cells; (C) for HeLa cells. The black line indicates 100% cell viability.

the oligonucleotide migrates as if it is free, or it remains at the well when the quantity of dendrimers is adequate to neutralize the charges. These results may suggest that (i) **2′-G$_4$**/DNA dendriplexes are readily formed and (ii) as neutral species they do not exhibit electrophoretic mobility. So, the spots in the lower part of the gels represent a free fraction of DNA. The fraction of bound DNA either stays in the well as charge-free dendriplex (as in **2′-G$_4$** (Fig. 3, lanes d–g)) or is eluted out of the gel in a positively charged complex (as in lanes h–l of **2′-G$_4$**).

We could deduce from the electrophoretic experiments that dendrimer **2′-G$_4$** should be the best choice for transfection

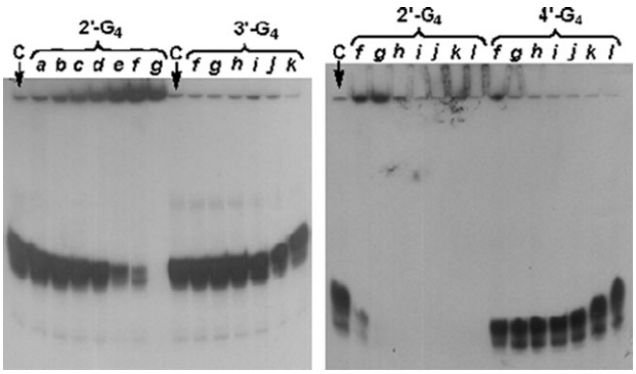


Fig. 3 Electrophoresis experiments for the association between dendrimers (**2′-G$_4$**, **3′-G$_4$** or **4′-G$_4$**) and a ^{32}P labelled 20-mer double stranded oligonucleotide. C→: control (no dendrimer); ***a*–*l***: various concentrations of dendrimers in nmol (and N : P ratio ammonium terminal groups of dendrimer to DNA phosphates); ***a***: 0.001 (1 : 10); ***b***: 0.002 (1 : 5); ***c***: 0.0041 (1 : 2.5); ***d***: 0.0082 (1 : 1.25); ***e***: 0.017 (1 : 0.62); ***f***: 0.035 (1 : 0.31); ***g***: 0.070 (1 : 0.15); ***h***: 0.14 (1 : 0.075); ***i***: 0.28 (1 : 0.037); ***j***: 0.56 (1 : 0.018); ***k***: 1.12 (1 : 0.009); ***l***: 2.24 (1 : 0.0045).

experiments. Such experiments are performed with two concentrations (10 and 20 μg mL^{-1}) of dendrimers **2′-G$_4$**, **3′-G$_4$**, **4′-G$_4$**, as well as of **6′-G$_4$** and lipofectin (1 μL/1 μg DNA) (a standard agent for transfection)[18] which are used to deliver plasmid DNA (pHygEGFP)[8b] to HUVEC, HEK 293 and HeLa cells, using two types of media: either a classical cell culture medium (DMEM, RPMI 1640), or the OPTI-MEM 1 medium. The efficiency of transfection is deduced from the fluorescence of the green fluorescent protein (GFP) expressed in cells from lipofectin- or dendrimer-delivered plasmid DNA. A blank experiment is carried out in all cases (without transfection agent, but with the GFP-coding plasmid) and the fluorescence measured for the blank experiments is subtracted from the fluorescence measured in the case of transfection with dendrimer or lipofectin. Each value is the mean value of at least four concordant values. Fig. 4 represents typical transfection experiment of HEK 293 cells with lipofectine and tested dendrimers.

In this case dendrimer **2′-G$_4$** is the most active in DMEM medium with 10% FBS and antibiotics, whereas lipofectin and dendrimer **6′-G$_4$** are the most active in OPTI-MEM 1. Similar results are obtained for HUVEC and HeLa cells, where again the most efficient transfection agent is **2′-G$_4$** in cell culture medium, whereas lipofectin exhibits higher transfection efficiency in OPTI-MEM 1 medium, even if the difference in efficiency is less pronounced in these cases (see ESI‡). Thus, these experiments confirm what was already deduced from the cytotoxicity and electrophoresis experiments: among dendrimers **2′-G$_4$**, **3′-G$_4$** and **4′-G$_4$**, only **2′-G$_4$** has an interesting potential as transfection agent. In consequence, for the forthcoming experiments, only **2′-G$_4$** might be used and compared with dendrimer **6′-G$_4$** and lipofectin. In these experiments the cellular up-take of fluorescently labeled single stranded DNA (ODN-FITC, 35-nt oligodeoxyribonucleotide of a DNAzyme sequence, complementary to the mRNA of the β3 subunit of integrin, 3′- and 5′-terminated with 2′-OMe units and 5′-conjugated with fluorescein residue) into three previously mentioned cells strains (HUVEC, HEK 293 and HeLa) was

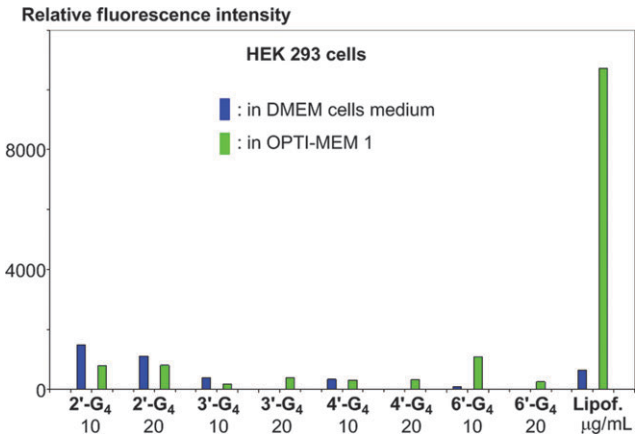


Fig. 4 Transfection efficiency of GFP-coding plasmid in HEK 293 cells, using various dendrimers with a concentration of 10 and 20 μg mL^{-1}, and lipofectin, measured by the relative intensity of fluorescence.

evaluated. The high levels of fluorescence values obtained for cells transfected in the presence of test dendrimers, in comparison with the data obtained for cells transfected in the presence of lipofectin (data not shown) were suspicious, as we might expect that cellular uptake of ssDNA would be similar to previously obtained results of a cellular up-take of the plasmid dsDNA. Careful microscopic analysis of HEK 293 cells after transfection in the presence of **2′-G_4** has shown insoluble fluorescent aggregates in the OPTI-MEM 1 medium and only very weak, if any, fluorescence of the cells (Fig. 5, upper pictures). In contrary, the microscopic picture of HEK 293 cells transfected with ODN-FITC in the presence of lipofectin shows more efficient cellular up-take of fluorescently labeled oligonucleotide into the cells (Fig. 5, lower pictures). Thus, from these experiments we can deduce that the newly synthesized dendrimers **2′-G_4** form tight aggregates with single stranded DNA conjugated with fluorescein. These aggregates do not exhibit potential for cellular up-take (by phagocytosis) in contrast to soluble dendriplexes of plasmid DNA with test dendrimers which to some extent introduce nucleic acids (cellular up-take by endocytosis).

Conclusions

We have synthesized four new series of polycationic dendrimers (generations 1 and 4) and found that three of them are soluble in water. Cytotoxicity measurements towards three cell strains (one healthy, two cancerous) using the fourth generation of these three dendrimers shows that dendrimers **2′-G_4** and **3′-G_4** (pyrrolidine and morpholine derivatives) are practically non-cytotoxic, whereas dendrimer **4′-G_4** (methylpiperazine derivative) is the most cytotoxic with healthy cells and has a tendency to increase the quantity of cancerous cells. The association between these dendrimers and a double stranded DNA affords a dendriplex only in the case of dendrimer **2′-G_4**, as shown by electrophoresis. Two types of transfection experiments carried out with either dsDNA (GFP-coding plasmids) or an ODN labelled by FITC and in two different cells media demonstrated that dendrimer **2′-G_4** is the most efficient and exhibits in all cases some potential for facilitation of cellular up-take of nucleic acids. More studies are needed to fully characterize this dendrimer as transfection agent, but it is as

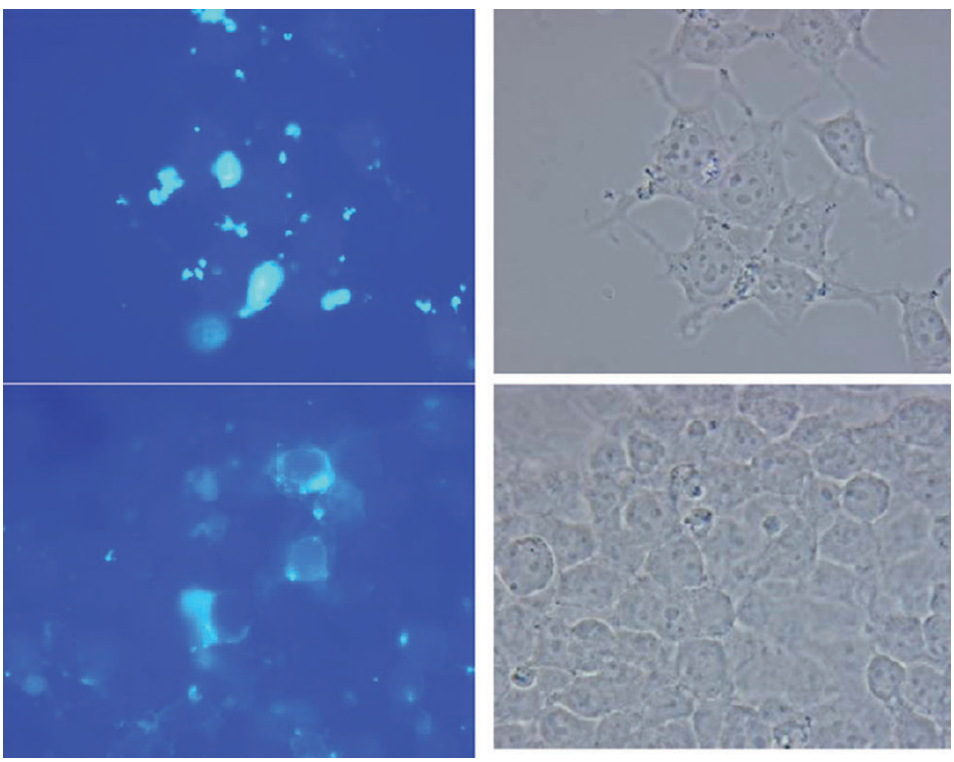


Fig. 5 Microscopic pictures (left: fluorescence microscope; right: phase contrast microscope) of HEK 293 cells transfected with ODN-FITC/**2′-G_4** (up) and control cells transfected with ODN-FITC and lipofectin (down) in the presence of OPTI-MEM 1 cell medium.

efficient as our previous most efficient transfecting agent (**6′-G$_4$**), at least in OPTI-MEM 1 medium, and more efficient in cell growing medium (DMEM for HEK 293 and RPMI 1640 medium for HeLa and HUVEC cells). One can remark that **2′-G$_4$** and **6′-G$_4$** bear as terminal groups ammonium groups known to have the highest pK_a values in the series of compounds used here.[19] Thus, subtle changes in the periphery of dendrimers are able to totally modify the properties of dendrimers, and this is particularly sensitive and important when carrying out biological experiments.

Experimental

Chemistry

General. All manipulations were carried out with standard high vacuum and dry-argon techniques. The solvents were dried and distilled prior to use (THF and diethyl ether over sodium/benzophenone, pentane over phosphorus pentoxide). Conventional ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with Bruker AC 200, AC 250, DPX 300 or AMX 400 spectrometers. References for NMR chemical shifts are 85% H_3PO_4 in water for ^{31}P NMR, $SiMe_4$ for ^{1}H and ^{13}C NMR. The attribution of ^{13}C NMR signals has been done using J_{mod}, two-dimensional HMBC, and HMQC, Broad-band or CW ^{31}P decoupling experiments when necessary. The numbering used for NMR assignments is shown in Fig. 6. Dendrimers **1-G$_1$** and **1-G$_4$** were synthesized as previously published,[14] as well as dendrimer **6′-G$_4$**.[8a]

General method of synthesis of dendrimers. 2.1 equivalents of amine (**2**, **3**, **4** or **5**) per Cl were added to a solution of 0.5 g of dendrimer **1-G$_n$** (n = 1 or 4) in THF (50 mL) at 0 °C in the presence of caesium carbonate (0.5 g). The resulting mixture was stirred overnight at room temperature, then filtered and evaporated to dryness. The residue was added to an aqueous solution of 50 mL of sodium hydroxide (0.1 mol/L), which was then extracted three times with 100 mL of ethyl acetate. The organic phases were gathered, dried over sodium sulfate, filtered, and evaporated to dryness. The resulting oil was precipitated into 50 mL of a mixture of ether–pentane (1 : 4). The resulting white powder (dendrimers **2-G$_n$**, **3-G$_n$**, **4-G$_n$** or **5-G$_n$** (n = 1 or 4)) was recovered by filtration and dried under vacuum.

The protonated dendrimers (**2′-G$_n$**, **3′-G$_n$**, **4′-G$_n$**, **5′-G$_n$**; n = 1 or 4) were obtained by adding a solution of HCl in ether (1.0 M) to a solution of dendrimer in THF. The amount of HCl was calculated to induce theoretically the protonation of 90% of the terminal nitrogen. The protonated dendrimers precipitated in THF and were simply recovered by filtration. These protonated dendrimers were only characterized by ^{31}P NMR, in order to ascertain the integrity of the structure.

Dendrimer 2-G$_1$. This compound was isolated as a white powder in 70% yield. ^{31}P{^{1}H} NMR ($CDCl_3$): δ 11.7 (P_0), 72.0 (P_1); ^{1}H NMR ($CDCl_3$): δ 1.71 (br s, 48H, C_dH_2), 2.50–2.60 (m, 72H, C_bH_2, C_cH_2), 2.98–3.09 (m, 24H, C_aH_2), 3.14 (d, $^3J_{HP}$ = 9.4 Hz, 18H, CH_3NP), 4.10 (m, 12H, NH), 6.91 (d, $^3J_{HH}$ = 8.4 Hz, 12H, C_0^2H), 7.45 (d, $^3J_{HH}$ = 8.4 Hz, 12H, C_0^3H), 7.48 (s, 6H, CH=N); ^{13}C{^{1}H} NMR ($CDCl_3$): δ 23.5 (C_d), 30.8 (d, $^2J_{CP}$ = 11.4 Hz, CH_3NP_1), 39.7 (C_b), 53.8 (C_c), 56.2 (d, $^2J_{CP}$ = 8.0 Hz, C_a), 121.1 (C_0^2), 127.5 (C_0^3), 132.9 (C_0^4), 135.8 (d, $^2J_{CP}$ = 12.8 Hz, CH=NNP$_1$), 150.7 (br s, C_0^1). Anal. Calc. for $C_{120}H_{204}N_{39}O_6P_9S_6$ (2760): C, 52.22; H, 7.45; N, 19.79. Found: C, 52.48; H, 7.54; N, 19.61%.

2′-G$_1$. ^{31}P{^{1}H} NMR (D_2O): δ 12.7 (P_0), 73.3 (P_1).

Dendrimer 2-G$_4$. This compound was isolated as a white powder in 73% yield. ^{31}P{^{1}H} NMR ($CDCl_3$): δ 11.7 (P_0), 66.0 (P_1), 66.1 (P_2), 66.2 (P_3), 71.8 (P_4); ^{1}H NMR ($CDCl_3$): δ 1.68 (br s, 384H, C_dH_2), 2.51–2.60 (m, 576H, C_bH_2, C_cH_2), 3.08–3.12 (m, 192H, C_aH_2), 3.10 (br d, $^3J_{HP}$ = 9.3 Hz, 144H, CH_3NP_4), 3.26 (br d, $^3J_{HP}$ = 9.9 Hz, 126H, $CH_3NP_{1,2,3}$), 4.17 (br s, 96H, NH), 7.11–7.15 (m, 180H, C_n^2H), 7.41–7.65 (m, 270H, C_n^3H, CH=N); ^{13}C{^{1}H} NMR ($CDCl_3$): δ 23.4 (C_d), 30.7 (d, $^2J_{CP}$ = 12.6 Hz, CH_3NP_4), 33.1 (br d, $^2J_{CP}$ = 12.6 Hz, $CH_3NP_{1,2,3}$), 39.5 (C_b), 53.8 (C_c), 56.2 (d, $^2J_{CP}$ = 8.0 Hz, C_a), 121.6 (br s, C_0^2, C_1^2, C_2^2, C_3^2), 127.6 (C_3^3), 128.3 (br s, C_0^3, C_1^3, C_2^3), 132.2 (C_0^4, C_1^4), 132.3 (C_2^4), 133.2 (C_3^4), 135.6 (d, $^2J_{CP}$ = 10.9 Hz, CH=NNP$_4$), 139.1 (br m, CH=N), 150.5 (d, $^2J_{CP}$ = 7.1 Hz, C_3^1), 151.2 (m, C_0^1, C_1^1, C_2^1). Anal. Calc. for $C_{1296}H_{1968}N_{375}O_{90}P_{93}S_{90}$ (30009): C, 51.87; H, 6.61; N, 17.50. Found: C, 52.08; H, 6.75; N, 17.32%.

2′-G$_4$. ^{31}P{^{1}H} NMR (D_2O): δ 12.7 (P_0), 66.8 (br s, P_1, P_2, P_3), 73.2 (P_4).

Dendrimer 3-G$_1$. This compound was isolated as a white powder in 74% yield. ^{31}P{^{1}H} NMR ($CDCl_3$): δ 11.5 (P_0), 72.0 (P_1); ^{1}H NMR ($CDCl_3$): δ 2.48 (br s, 72H, C_bH, C_cH), 2.95–3.14 (m, 24H, C_aH), 3.16 (d, $^3J_{CP}$ = 9.6 Hz, 18H, CH_3NP), 3.69 (br s, 48H, C_dH), 4.04 (br s, 12H, NH), 6.97 (d, $^3J_{HH}$ = 8.4 Hz, 12H, C_0^2H), 7.50 (d, $^3J_{HH}$ = 8.4 Hz, 12H, C_0^3H), 7.52 (s, 6H, CH=N); ^{13}C{^{1}H} NMR ($CDCl_3$): δ 30.8 (d, $^2J_{CP}$ = 8.3 Hz, CH_3NP_1), 37.3 (C_b), 53.3 (C_c), 58.6 (d, $^2J_{CP}$ = 8.0 Hz, C_a), 66.8 (C_d), 121.1 (C_0^2), 127.4 (C_0^3), 132.7 (C_0^4), 136.1 (d, $^2J_{CP}$ = 12.2 Hz, CH=NNP$_1$), 150.6 (br s C_0^1). Anal. Calc. for $C_{120}H_{204}N_{39}O_{18}P_9S_6$ (2952): C, 48.82; H, 6.96; N, 18.50. Found: C, 48.95; H, 7.03; N, 18.41%.

3′-G$_1$. ^{31}P{^{1}H} NMR (D_2O): δ 12.6 (P_0), 73.3 (P_1).

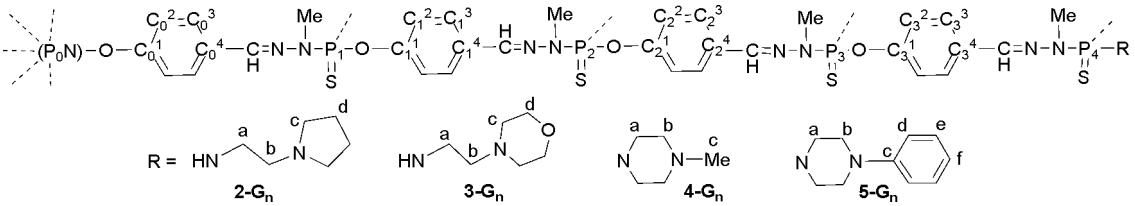


Fig. 6 Numbering used for NMR assignments.

Dendrimer 3-G$_4$. This compound was isolated as a white powder in 75% yield. $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ 11.6 (P_0), 66.1 (P_1), 66.2 (P_2), 66.3 (P_3), 71.8 (P_4); ^1H NMR ($CDCl_3$): δ 2.37 (br s, 576H, C_bH, C_cH), 2.95–3.10 (m, 192H, C_aH), 3.10 (d, $^3J_{HP}$ = 9.2 Hz, 144H, CH_3NP_4), 3.28 (d, $^3J_{HP}$ = 9.6 Hz, 126H, $CH_3NP_{1,2,3}$), 3.60 (br s, 384H, C_dH), 3.89 (br s, 96H, NH), 7.10–7.20 (m, 180H, $C_n{}^2H$), 7.42–7.66 (m, 270H, $C_n{}^3H$, CH=N); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 30.8 (d, $^2J_{CP}$ = 10.9 Hz, CH_3NP_4), 33.0 (d, $^2J_{CP}$ = 12.5 Hz, $CH_3NP_{1,2,3}$), 37.3 (C_b), 53.3 (C_c), 58.6 (d, $^2J_{CP}$ = 5.9 Hz, C_a), 66.8 (C_d), 121.7 ($C_0{}^2$, $C_1{}^2$, $C_2{}^2$, $C_3{}^2$), 127.5 ($C_3{}^3$), 128.3 (br s, $C_0{}^3$, $C_1{}^3$, $C_2{}^3$), 132.3 (br s, $C_0{}^4$, $C_1{}^4$, $C_2{}^4$), 132.9 ($C_3{}^4$), 135.7 (d, $^2J_{CP}$ = 12.2 Hz, CH=NNP_4), 139.1 (br s, CH=N), 150.6 (d, $^2J_{CP}$ = 7.1 Hz, $C_3{}^1$), 151.3 (br d, $^2J_{CP}$ = 6.8 Hz, $C_0{}^1$, $C_1{}^1$, $C_2{}^1$). Anal. Calc. for $C_{1296}H_{1968}N_{375}O_{186}P_{93}S_{90}$ (31545): C, 49.35; H, 6.29; N, 16.65. Found: C, 49.55; H, 6.37; N, 16.52%.

3′-G$_4$. $^{31}P\{^1H\}$ NMR (D_2O): δ 12.6 (P_0), 66.9 (br s, P_1, P_2, P_3), 73.1 (P_4).

Dendrimer 4-G$_1$. This compound was isolated as a white powder in 75% yield. $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ 11.5 (P_0), 76.7 (P_1); ^1H NMR ($CDCl_3$): δ 2.18 (s, 36H, C_cH_3), 2.29 (br s, 48H, C_bH_2), 3.03–3.17 (m, 48H, C_aH_2), 3.22 (d, $^3J_{HP}$ = 8.8 Hz, 18H, CH_3NP), 6.97 (d, $^3J_{HH}$ = 8.5 Hz, 12H, $C_0{}^2H$), 7.38 (s, 6H, CH=N), 7.40 (d, $^3J_{HH}$ = 8.5 Hz, 12H, $C_0{}^3H$); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 33.2 (d, $^2J_{CP}$ = 10.9 Hz, CH_3NP), 45.4 (C_c), 46.2 (C_b), 55.2 (d, $^2J_{CP}$ = 6.9 Hz, C_a), 121.2 ($C_0{}^2$), 127.4 ($C_0{}^3$), 132.9 ($C_0{}^4$), 135.6 (d, $^2J_{CP}$ = 12.5 Hz, CH=NNP_1), 150.6 (d, $^2J_{CP}$ = 4.2 Hz, $C_0{}^1$). Anal. Calc. for $C_{108}H_{180}N_{39}O_6P_9S_6$ (2592): C, 50.05; H, 7.00; N, 21.08. Found: C, 50.18; H, 7.08; N, 20.96%.

4′-G$_1$. $^{31}P\{^1H\}$ NMR (D_2O): δ 12.6 (P_0), 78.1 (P_1).

Dendrimer 4-G$_1$. This compound was isolated as a white powder in 78% yield. $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ 11.6 (P_0), 65.9–66.1 (P_1, P_2, P_3), 76.3 (P_4); ^1H NMR ($CDCl_3$): δ 2.22 (br s, 288H, C_cH_3), 2.33 (br s, 384H, C_bH_2), 2.95–3.35 (m, 654H, C_aH_2, CH_3NP), 6.78–6.97 (m, 84H, CH_{Ar}), 7.18 (d, $^3J_{HH}$ = 8.3 Hz, 96H, $C_3{}^2H$), 7.41 (s, 48H, $C_3{}^4CH$=N), 7.51 (d, $^3J_{HH}$ = 8.3 Hz, 96H, $C_3{}^3H$), 7.66 (m, 126H, CH_{Ar}, CH=N); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 33.1 (m, CH_3NP), 44.6 (C_c), 46.2 (C_b), 55.2 (d, $^2J_{CP}$ = 6.9 Hz, C_a), 121.7 ($C_0{}^2$, $C_1{}^2$, $C_2{}^2$, $C_3{}^2$), 127.5 ($C_3{}^3$), 128.4 (br s, $C_0{}^3$, $C_1{}^3$, $C_2{}^3$, $C_3{}^3$), 132.4 ($C_0{}^4$, $C_1{}^4$, $C_2{}^4$), 133.2 ($C_3{}^4$), 135.7 (d, $^2J_{CP}$ = 11.9 Hz, CH=NNP_4), 139.1 (br s, CH=N), 150.6 (d, $^2J_{CP}$ = 7.2 Hz, $C_3{}^1$), 151.2 (d, $^2J_{CP}$ = 6.9 Hz, $C_0{}^1$, $C_1{}^1$, $C_2{}^1$). Anal. Calc. for $C_{1200}H_{1776}N_{375}O_{90}P_{93}S_{90}$ (28662): C, 50.29; H, 6.25; N, 18.33. Found: C, 50.40; H, 6.31; N, 18.21%.

4′-G$_4$. $^{31}P\{^1H\}$ NMR (D_2O): δ 12.6 (P_0), 67.0 (br s, P_1, P_2, P_3), 77.7 (P_4).

Dendrimer 5-G$_1$. This compound was isolated as a white powder in 77% yield. $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ 11.5 (P_0), 76.5 (P_1); ^1H NMR ($CDCl_3$): δ 3.08 (br s, 48H, C_bH_2), 3.26 (d, $^3J_{HP}$ = 9.2 Hz, 18H, CH_3NP), 3.35 (m, 48H, C_aH_2), 6.83 (d, $^3J_{HH}$ = 8.5 Hz, 24H, C_dH), 6.99 (d, $^3J_{HH}$ = 8.4 Hz, 12H, $C_0{}^2H$), 7.23 (d, $^3J_{HH}$ = 8.5 Hz, 24H, C_eH), 7.26 (s, 12H, C_fH), 7.39 (s, 6H, CH=N), 7.41 (d, $^3J_{HH}$ = 8.4 Hz, 12H, $C_0{}^3H$); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 33.2 (d, $^2J_{CP}$ = 10.6 Hz, CH_3NP), 45.5 (C_b), 49.7 (d, $^2J_{CP}$ = 7.2 Hz, C_a), 116.3 (C_d), 120.2 (C_f), 121.3 ($C_0{}^2$), 127.4 ($C_0{}^3$), 129.2 (C_e), 132.8 ($C_0{}^4$), 135.9 (d, $^2J_{CP}$ = 11.6 Hz, CH=N), 150.7 (br s, $C_0{}^1$), 151.2 (C_c). Anal. Calc. for $C_{168}H_{204}N_{39}O_6P_9S_6$ (3337): C, 60.47; H, 6.16; N, 16.37. Found: C, 60.52; H, 6.21; N, 16.31%.

5′-G$_1$. $^{31}P\{^1H\}$ NMR (DMF): δ 11.5 (P_0), 72.5 (P_4).

Dendrimer 5-G$_4$. This compound was isolated as a white powder in 80% yield. $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ 11.7 (P_0), 65.8 (P_2), 65.9 (P_3), 66.1 (P_1), 76.3 (P_4); ^1H NMR ($CDCl_3$): δ 3.09 (br s, 384H, C_bH_2), 3.31 (br s, 1038H, CH_3NP, C_aH_2), 6.75–6.95 (m, 288H, CH_{Ar}), 7.16–7.25 (m, 372H, CH_{Ar}), 7.42–7.61 (m, 270H, CH=N, CH_{Ar}); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ 33.1 (m, CH_3NP), 45.5 (C_b), 49.5 (br s, C_a), 116.3 (C_d), 120.1 (C_f), 121.8 ($C_0{}^2$, $C_1{}^2$, $C_2{}^2$, $C_3{}^2$), 127.6 ($C_3{}^3$), 128.4 (br s, $C_0{}^3$, $C_1{}^3$, $C_2{}^3$, $C_3{}^3$), 129.2 (C_e), 132.1 ($C_0{}^4$, $C_1{}^4$), 132.4 ($C_2{}^4$), 133.2 ($C_3{}^4$), 136.1 (d, $^2J_{CP}$ = 11.2 Hz, CH=NNP_4), 139.0 (br s, CH=N), 150.7 (d, $^2J_{CP}$ = 11.2 Hz, $C_0{}^1$, $C_1{}^1$, $C_2{}^1$, $C_3{}^1$), 151.2 (C_c). Anal. Calc. for $C_{1680}H_{1968}N_{375}O_{90}P_{93}S_{90}$ (34621): C, 58.28; H, 5.73; N, 15.17. Found: C, 58.40; H, 7.81; N, 15.08%.

5′-G$_4$. $^{31}P\{^1H\}$ NMR (DMF): δ 11.7 (P_0), 62.0 (br s, P_1, P_2, P_3), 72.4 (P_4).

Biology

Cell cultures. Human epithelial cervical carcinoma cell line (HeLa) was maintained in RPMI 1640 medium (Gibco BRL, Paisley, UK) supplemented with 10% thermally inactivated foetal bovine serum (FBS, Gibco BRL, Paisley, UK), 100 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin. Human transformed primary embryonal kidney cell line (HEK 293) was cultured in Dulbecco's modification of Eagle's medium (DMEM, Sigma, St. Louis, MO), supplemented with 10% FBS and antibiotics as above. Glass wells used for experiments with HEK 293 cells were coated with poly-L-lysine. Thus, they were covered for 5 min with 0.01% aqueous solution of poly-L-lysine (Sigma, St. Louis, MO), washed with sterile water and dried for 2 h on air under sterile conditions. Human umbilical vein endothelial cells (HUVEC) were isolated from freshly collected umbilical cords as previously described,[20] and cultured in plastic dishes coated with gelatin, in RPMI 1640 medium supplemented with 20% FBS, 90 U mL^{-1} heparin, 150 μg mL^{-1} ECGF (Endothelial Cell Growth Factor, Roche Diagnostics, Mannheim, Germany) and antibiotics (as above). All cell lines were grown in monolayer, at 37 °C in an atmosphere of 5% CO_2.

Nucleic acid models

5′-Radioactive labeled ds oligodeoxyribonucleotide. ODNs of following sequences: sense 5′-TCTTCAAGAATTC-AGGACTA-3′ and antisense 5′-TAGTCCTGAATTCTTG-AAGA-3′ (substrate sequence for EcoRI taken from pUC plasmid) were synthesized in house. The ODN of the sense strand (0.1 OD, 0.49 nmol) was radioactively 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase according to the previously described procedure.[21] Molar equivalents of oligomers were annealed in water by heating for 2 min at 95 °C, and cooling to room temperature.

The plasmid. The pHygEGFP Plasmid (BD Biosciences Clontech) expressing a fusion of the hygromycin resistance gene with enhanced green fluorescent protein (EGFP) under control of the human CMV promoter was used.

Oligonucleotide–fluorescein conjugate. Oligodeoxyribonucleotide used for transfection experiments was the 35-nt ODN of the following sequence 5′-GAG TCC CAT AGG CTA GCT ACA ACG AAA GAC TTG AG-3′. This ODN was designed as DNAzyme complementary to mRNA of β3 integrin subunit.[22] The oligonucleotide was synthesized in -house according to previously described procedure.[8b]

Cytotoxicity studies. The cytotoxicity of dendrimers was studied for HeLa, HEK 293 and HUVEC cell strains using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO] assay (activity of the mitochondrial respiratory chain).[23] Cells were trypsinized and diluted with appropriate cell culture medium to a density of 5000 cells/200 μL for HeLa and HEK 293, and 7000 cells/200 μL for HUVEC cells. Cultured media were supplemented with FBS to a concentration of 10 or 20%. Cell suspensions prepared as above were added to the 96-well plates (200 μL per well), coated with poly-L-lysine or gelatin, for HEK 293 and HUVEC cells, respectively. After 24 h of cell cultivation in wells, dendrimers were added to a final concentration 0.5, 1.0, 2.5, 5, 10, 20 or 50 μg per mL of cell culture. Lipofectin was used at the final concentration of 10 or 20 μg mL^{-1}. Transfected cells were incubated for 24, 48 and 72 h. As a control, cultured cells were grown in the absence of dendrimers or lipofectin. After incubation with dendrimers or lipofectin, 25 μL of MTT solution (5 mg mL^{-1}) in PBS buffer (155 mM NaCl, 4.2 mM KH_2PO_4/Na_2HPO_4, pH 7.4) was added to each well and incubated for 2 h at 37 °C with continuous observation of cells under a phase-contrast microscope. Finally, 95 μL of lysis buffer (20% SDS, 50% aqueous dimethylformamide, pH 4.5) was added into each well and incubated at 37 °C for an additional 24 h. Absorbance of a given sample was measured at 570 nm, with the reference wavelength 630 nm (Microplate Reader 450, BioRad). The percentage of living cells (PLC) is obtained by PLC = (AS − AM)/(AC − AM) × 100%, where AS corresponds to the sample treated by the chemical agent to be tested (the dendrimer), AM is the absorbance due to the diffusion media, and AC is the absorbance of a sample reference in the absence of the chemical agent to be tested. Data points represent means of at least 8 repeats.

Electrophoresis of dendriplexes. Samples of dendrimers in the following concentrations: **2′-G_4**: 0.001–2.24 nmol, **3′-G_4**: 0.035–1.12 nmol, and **4′-G_4**: 0.035–2.24 nmol were incubated with ^{32}P-labelled 20-mer double stranded oligodeoxyribonucleotide (0.025 nmol portions) for 0.5 h at 37 °C and then loaded in formamide/dye solution (15 μL total volume) onto the native 20% polyacrylamide gel. Electrophoresis was performed in a TBE buffer (0.9 M Tris pH 8.3, 0.85 M H_3BO_3, 0.025 M EDTA) for 2 h at 1000 V. The resulting gel was visualized on a X-ray sensitive film.

Transfection of cells with dendrimer-complexed plasmid DNA. HeLa, HEK 293 and HUVEC cells, grown in appropriate cell culture medium (as above) were transferred to the 96-well plates in 50 μL aliquots (10.000 and 15.000 cells per well, respectively) 24 hours before the transfection. The experiments were performed in either DMEM medium for HEK 293 cells, RPMI 1640 medium supplemented with 10% FBS for HeLa and HUVEC cells, or in OPTI-MEM 1 medium. The transfection mixtures were prepared of dendrimers **2′-G_4**, **3′-G_4**, **4′-G_4**, as well as of **6′-G_4** (10 and 20 μg mL^{-1}) or lipofectin (1 μL/1 μg DNA) and pHygEGFP plasmid DNA (1 μg mL^{-1}) in appropriate media, pre-incubated for 30 min at room temperature. The transfection mixtures containing lipofectin instead of dendrimers in the same media served as positive controls, whereas the mixtures containing dendrimers (10 or 20 μg mL^{-1}) in cell medium without plasmid served as negative controls to correct the influence of dendrimers on fluorescence read-out. Other negative controls were wells containing cells suspended in either of above media (50 μL) into which the solution of plasmid alone was added (1 μg mL^{-1}). Each transfection experiment was repeated in four separate wells.

The plates with transfection mixtures were incubated for 6 h at 37 °C in 5% CO_2 atmosphere. Then, the supernatant was removed and the cells were post-incubated for 24 h under the same conditions with 50 μL of appropriate fresh cell culture medium. Before the fluorescence reading, the cells were washed three times with PBS (phosphate buffered saline, pH 7.4) without Ca^{2+} and Mg^{2+} and lysed with NP 40 buffer (150 mM NaCl, 1% IGEPAL CA-630, 1 mM PMSF, 50 mM Tris-HCl, pH 8.0). The intensity of fluorescence was measured with a Synergy HT plate reader (Bio-Tek Instruments, INC. Winooski, Vermont) with filter of excitation for wavelength of 485/20 nm and emission of 528/20 nm.

Transfection of cells with ODN-FITC. Transfection experiments were performed analogously to the above presented procedure in 96-well plate. The ODN-FITC was used in 0.5 μM concentration, while dendrimers **2′-G_4** and **6′-G_4** in 15 μg mL^{-1} concentration. Cells treated with lipofectin (1 μL/1 μg DNA) and ODN-FITC (0.5 μM) were used as controls. The plates with transfection mixtures were incubated for 12 h at 37 °C in 5% CO_2 atmosphere. The following post-incubation was carried out for the next 24 h under the same conditions and after work-up as above (cells washing and lysis) the fluorescence intensity was measured with a Synergy HT plate reader in conditions described previously.

Microscopic analysis of cells transfected with ODN-FITC. For microscopic analysis HeLa and HEK 293 cells were plated at a density of 25 000/250 μL on Thermanox Coverslips in 8-well cell culture chamber slides (Nunc, Roskilde, Denmark) coated with fibronectin (5 μg mL^{-1}). The cells were grown to 70–80% of confluence. Before performing the assays, cells were washed three times with pre-warmed PBS buffer and 400 μL of appropriate medium was added. Then, 100 μL of the solution containing fluorescein-labeled oligonucleotide complexed (final concentration 0.5 μM) with dendrimer **2′-G_4** (15 μg mL^{-1}) or lipofectin (1 μL/1 μg DNA) in corresponding

medium was added to the cell culture (total volume 500 μL) and incubation was continued for 12 h at 37 °C in 5% CO_2 atmosphere. After incubation, cells were washed three times with PBS buffer, fixed in a freshly prepared 3.8% paraformaldehyde solution, and again washed three times with PBS buffer. The slides were mounted in glycerol/PBS mixture (9 : 1, v/v) containing 2.5% DABCO (Sigma, St. Louis, MO). Analyses and micrographs were performed under epifluorescence, with a phase-contrast Optiphot-2 microscope (Nikon), using B-2A filter (Ex 450–490 nm) and UV-2A filter (Ex 330–380 nm). The DXM 1200 camera equipped with Lucia G 4.61 program was employed for documentation of the oligonucleotide uptake process.

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