Preparation of Water-Soluble Cationic Phosphorus-Containing Dendrimers as DNA Transfecting Agents

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Abstract: A new class of phosphorus-containing dendrimers (abbreviated as P-dendrimer) with protonated or methylated terminal tertiary amines has been prepared and characterized. Five different generations of protonated dendrimers (**2-[G_1]** to **2-[G_5]**) and the corresponding methylated series (**5-[G_1]** to **5-[G_5]**) have been examined as transfecting agents of the luciferase gene within 3T3 cells. The capability of these dendrimers to transfect cells appears to depend on the size of the dendrimers (the third, fourth, and fifth generation being the most efficient ones) and the chemical nature of the terminal tertiary amines (the protonated forms being more efficient than the methylated ones). The most efficient representatives of this series of P-dendrimers have a transfection activity level comparable with linear PEI with only five equivalents of tertiary amine per nucleotide. Unexpectedly and fortunately, these dendrimers have a better transfection efficiency in the presence of serum than without.

Keywords: cationic dendrimer · DNA · phosphorus · transfection

Introduction

Within a few decades, the high level of accumulated knowledge on DNA at the molecular level allowed the genetic engineering of microorganisms, plants, and animals. Among the different possibilities offered by this new field of molecular biology, it is now possible to create new therapeutic approaches based on the introduction of genes to cells that have chromosomal defects to cure the related diseases. However, the development of gene therapy in medicine is highly dependent on the transfer efficiency of genes within the desired type of cells.[1, 2]

Viral-based gene delivery is currently one of the most efficient methods to introduce exogeneous genes within cells.[3, 4] However, immunogenic or potential oncogenic effects are the two main limits for this transfection method. To overcome these problems and to diversify the different modes of gene delivery, alternative methods have been developed that involve cationic lipid preparations,[5–8] DNA encapsulation in liposomes,[9–11] and polyethyleneimine[12] and more recently polyamine dendrimers.[13, 14] The efficiency of these last transfecting agents is dependent on chemical modification of the initial dendrimer structure by a thermal treatment; this leads to a heterodisperse population of compounds.[15] Consequently, the transfection activity of monodispersed dendrimers should be evaluated. We decided to investigate the possibility of using a new series of cationic dendrimers, related to the intensive development of the dendrimer chemistry in one of our groups, by using phosphorus-containing synthons, which leads to diversified and controlled polymeric structures.[16, 17] These macromolecules built up to the highest generation known till now (generation 12) are of great interest, since they can be easily functionalized not only on the surface but also selectively in the internal layers. Moreover they are thermally stable up to 250 °C and are not water sensitive in a large range of pH (from 3 to 12). Lastly flexibility of the branches and porosity of these phosphorus-containing dendrimers (P-dendrimers) have been evidenced (see refs. [18, 19] for recent review articles on dendrimers, and also refs. [20–22]). Consequently these dendrimers appear to be excellent candidates as transfecting agents after grafting positively charged functions at their periphery.

Here we report the preparation of new cationic P-dendrimers with protonated or methylated terminal tertiary amines and the results when using them as transfecting agents of the luciferase gene within 3T3 cells.

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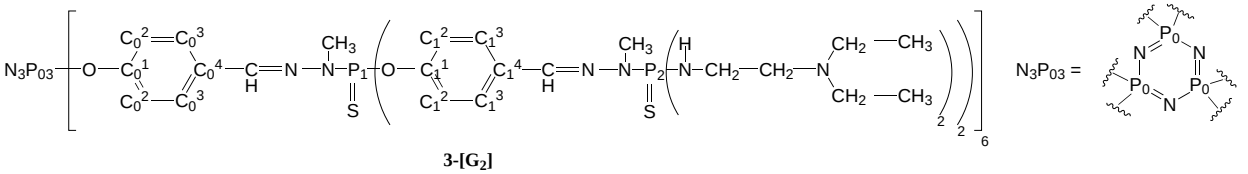


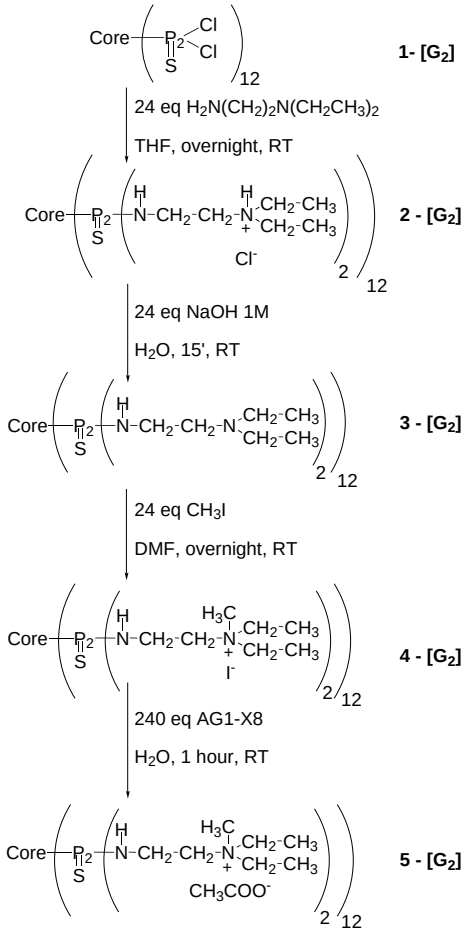
3-[G$_2$]

Scheme 1. Numbering of carbons atoms used in the description of NMR spectra of the dendrimers **3-[G$_n$]**, case of **3-[G$_2$]**. The structure of the cyclophosphazene **N$_3$P$_{03}$** unit used as core of these phosphorus-containing dendrimers is also depicted.

Results and Discussion

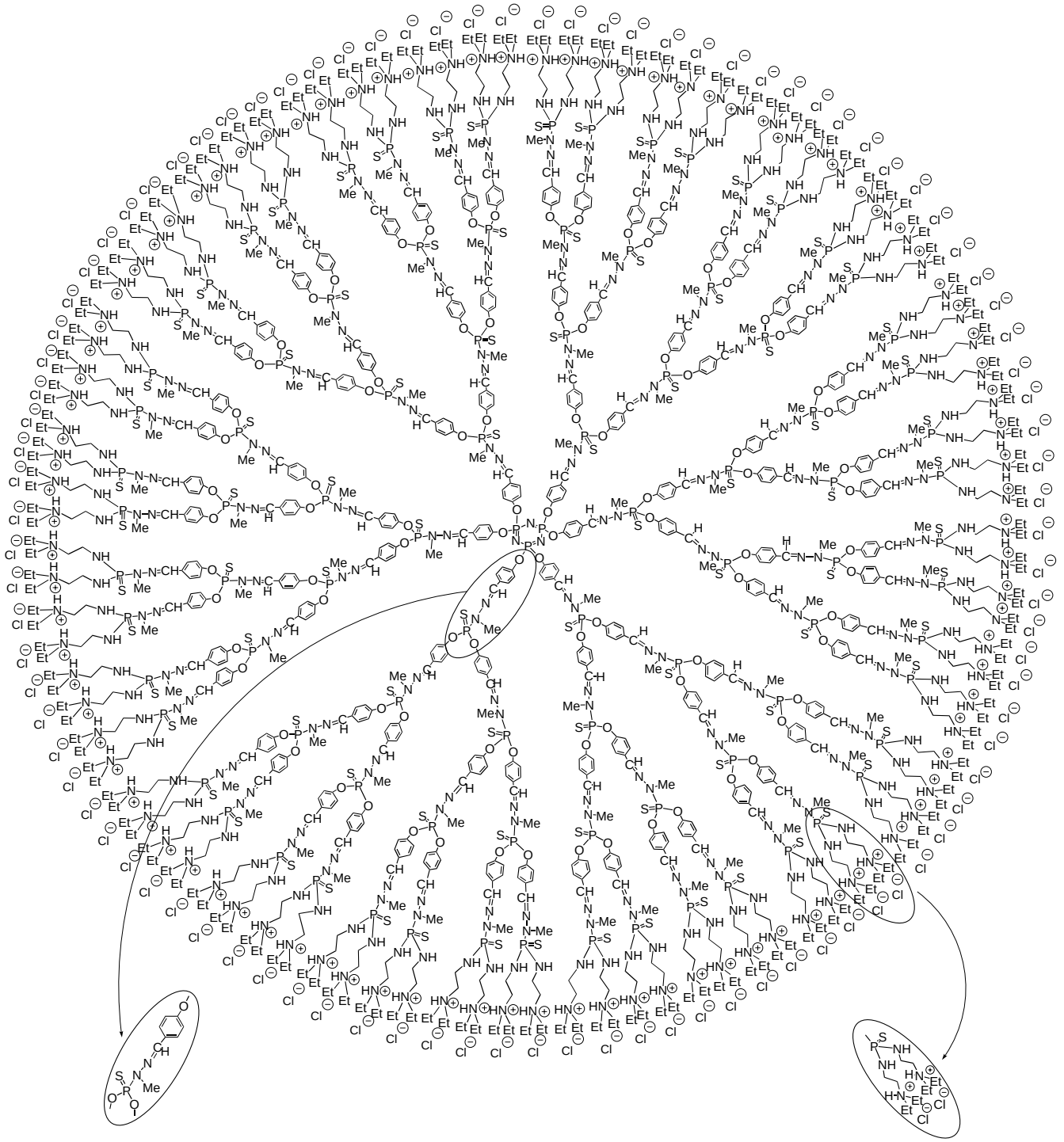
These P-dendrimers were obtained by controlled growth of the dendrimeric structure with successive layers of $H_2N{-}N(Me){-}P(S)Cl_2$ motifs. The central building block is a hexachlorocyclotriphosphazene unit modified with the triethylammonium salt of 4-hydroxybenzaldehyde (this starting material $N_3P_3(OC_6H_4CHO)_6$ is depicted in Scheme 1 and noted as core in Scheme 2). Then this core, with six terminal aldehyde functions is treated with dichlorophosphonomethylhydrazide $[H_2N{-}N(Me){-}P(S)Cl_2]$ to produce a dendron with terminal dichlorothiophosphine units; this dendron can be treated again with the sodium salt of hydroxybenzaldehyde to produce by iteration each generation of dendrimers. The first, second, third, fourth, and fifth generations have 6, 12, 24, 48, and 96 terminal $P(S)Cl_2$ units, respectively, which can be treated with *N*,*N*-diethylethylenediamine to produce cationic dendrimers after protonation (**2-[G$_1$]** to **2-[G$_5$]** dendrimers, see Scheme 3 for a representation of the structure of **2-[G$_4$]**) or methylation (**5-[G$_1$]** to **5-[G$_5$]** dendrimers). Consequently, the cationic dendrimers have 12, 24, 48, 96, or 192 peripheral positive charges for the first, second, third, fourth and fifth generations, respectively. The methylated forms (**5-[G$_1$]** to **5-[G$_5$]**) were prepared from the corresponding neutral terminal amines (**3-[G$_1$]** to **3-[G$_5$]**) by methylation with methyl iodide followed by exchange of the iodide counteranions for acetate ions with an exchange resin. The purity and the integrity of the dendrimers were monitored by ^{1}H, ^{13}C, and ^{31}P NMR. All these dendrimers have a high degree of purity. Only 8 to 10% of the terminal branches of the methylated dendrimers (**5-[G$_1$]** to **5-[G$_5$]**) exhibit a deficiency of methyl groups. This modification was observed during the exchange step to obtain the methylated dendrimers with acetate counterions. We checked that all these cationic P-dendrimers are stable in aqueous solution for several months.

Although cationic liposomes are the most commonly used non-viral vectors in transfection of eucaryotic cells, increasing interest for cationic polymers has arisen in recent years. In contrast with the first generation of polymers based on polylysine,[23, 24] PAMAM dendrimers[13, 25] and polyethyleneimine[12] have been shown to be very efficient in transfecting nucleic acids into cells. We studied the capacity of these new polycationic polymers, based on P-dendrimers, to mediate the



Scheme 2. Illustration of the preparation of the acetate form of **5-[G$_2$]** (second generation dendrimer) from the chlorohydrate form **2-[G$_2$]** in three steps via the deprotonated dendrimer **3-[G$_2$]** and the methylated form **4-[G$_2$]** with iodide as counteranions.

Abstract in French: *Cet article décrit la préparation et la caractérisation d'une nouvelle classe de dendrimères phosphorés ayant des amines tertiaires protonées ou méthylées comme terminaisons. Cinq générations de dendrimères protonés (**2-[G$_1$]** à **2-[G$_5$]**) et les cinq générations méthylées correspondantes (**5-[G$_1$]** à **5-[G$_5$]**) ont été testées comme agents de transfection du gène de la luciférase dans des cellules 3T3. La capacité de ces dendrimères à transfecter les cellules dépend de la taille des dendrimères (les troisièmes, quatrièmes et cinquièmes générations sont les plus efficaces) et de la nature chimique des amines tertiaires terminales (les formes protonées étant plus efficaces que les méthylées). Les molécules les plus actives de cette série de dendrimères phosphorés atteignent un niveau de transfection comparable à celui du PEI avec seulement cinq équivalents d'amines tertiaires par nucléotide. Il faut noter que ces dendrimères ont une meilleure capacité à transfecter en présence qu'en l'absence de sérum.*



Scheme 3. Schematic representation of the polycationic dendrimer **2-[G$_4$]** (fourth generation), which is efficient in the transfection of the luciferase gene in 3T3 cells.

in vitro delivery of nucleic acids into mammalian cells. Two different series were studied: the **2-[G$_n$]** series with chlorohydrate terminii (with the possibility of a deprotonation process when the pH value is increased) and the **5-[G$_n$]** series with methylammonium terminii (which creates an irreversible positive charge at the periphery of the dendrimer). The obtained data are reported in Figures 1 and 2 (see later), respectively. Our results with these different cationic polymers always show an optimal efficiency at a charge ratio N/P between five and ten (N/P ratio = number of terminal nitrogen atoms of dendrimer per DNA phosphate). Therefore, we decided to compare the transfection efficiency of different generations of P-dendrimers with that of the linear PEI ExGen500 at five and ten equivalents.

At five equivalents of positive charge per DNA phosphate, all five tested chlorohydrate forms, **2-[G$_n$]** series, showed a significant expression of transgene. Light units increased from 10^5 to nearly 10^9 RLU mg^{-1} of protein (RLU stands for

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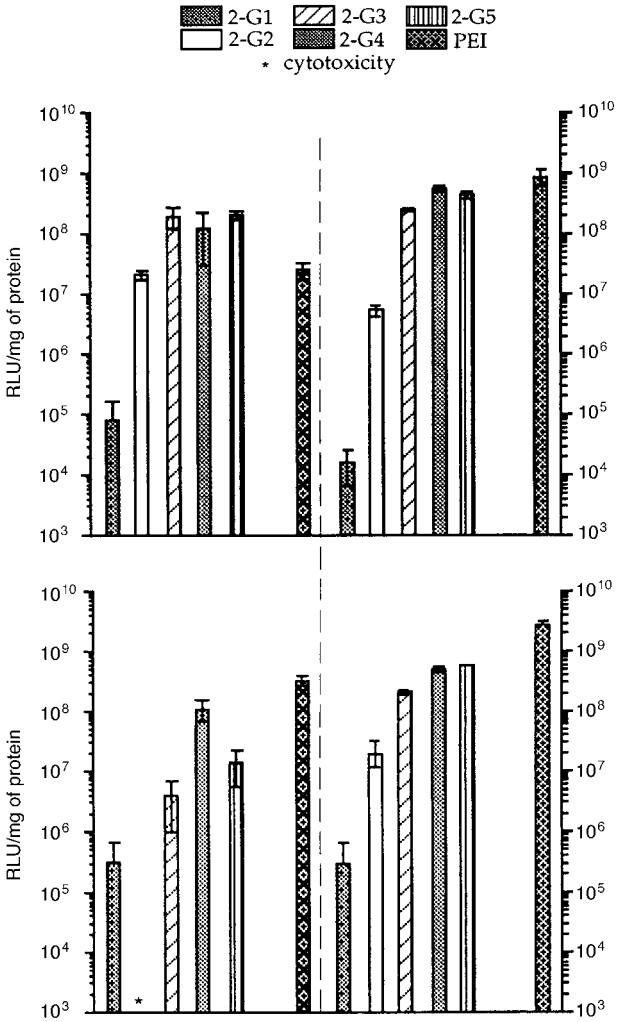

Figure 1. Chlorohydrate phosphorus-containing dendrimer-mediated gene transfer into eucaryotic cells. 3T3 cells were transfected with pCMV-luc plasmid (2 μg) complexed with the desired dendrimer in the absence (left side) or presence (right side) of 10% serum. Two N/P ratios 5 (top) and 10 equivalents (bottom) were studied in comparison with the linear PEI (ExGen500). Expression was stopped 24 hours after transfection and reporter gene activity was measured.

relative light units). However, the transfection efficiency increased with increasing generation number and reached a plateau for generations three to five (values of light units ranging from 10^8 to 10^9, see Figure 1 top). We consider **2-[G_4]** as a suitable candidate for further investigations of the transfection capacity of these new cationic P-dendrimers (see Scheme 3 for structure). Increasing the N/P ratio to ten enhanced the toxicity and therefore led generally to a lower transfection efficiency (Figure 1 bottom).

It should be noted that performing transfections in the presence of 10% of serum led to a lower cytotoxicity and thus to expression levels higher than those obtained in the absence of serum (compare the left side of Figure 1 for data obtained without serum with the right side for data with 10% of serum). Without any further optimization, the most efficient dendrimers (from the third to the fifth generation) were found to be as efficient as linear PEI used at its optimal conditions.

In contrast, the methylated forms **5-[G_n]** were found to be rather toxic and relatively inefficient in transfecting nucleic acids into eukaryotic cells, both with or without serum (Figure 2). This phenomenon might be due to a high, stable positive-charge density, which may disrupt the cell membrane and therefore lead to cell death. The reduction of the charge density is not possible for this methylated series without degradation of the dendrimers, whereas the charge density of

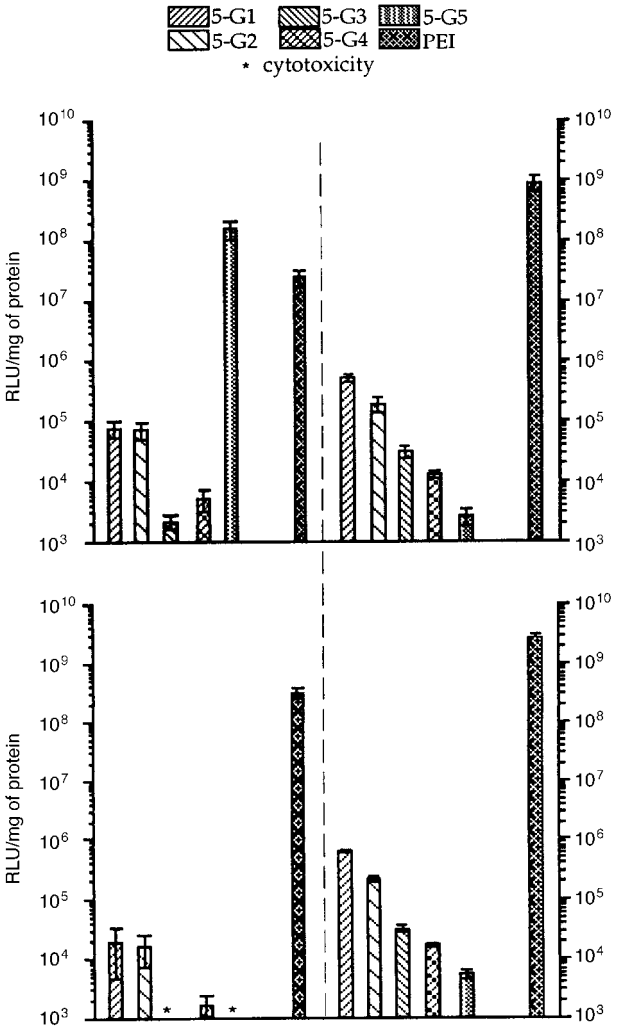

Figure 2. Methylated phosphorus-containing dendrimer mediated gene transfer into eucaryotic cells. 3T3 cells were transfected with pCMV-luc plasmid (2 μg) complexed with the desired dendrimer in the absence (left side) or presence (right side) of 10% serum. Two N/P ratio 5 (top) and 10 equivalents (bottom) were studied in comparison with the linear PEI (ExGen500). Expression was stopped 24 hours after transfection and reporter gene activity was measured.

chlorohydrate series **2-[G_n]** can be modulated by micro-environmental modification of the pH values when approaching the cell membrane. In addition the possibility of modulating the charge density of these chlorohydrate dendrimers might be a key factor for the release of the luciferase gene within endosomes. These dendrimers might act as proton reservoir within the cell compartments, their charge density being controlled by ATPase-driven proton pumps and by modifications of intracellular chloride concentration. The possibility of

reducing the cationic charge density of these P-dendrimers outside or inside cells should be favorable for in vivo experiments as previously mentioned.[26]

Conclusion

We found that P-dendrimers with terminal tertiary amines that can be protonated are suitable to mediate highly efficient transfection of the luciferase gene with cells in the presence of serum and with only five equivalents of amine per nucleotide. In addition, they are not toxic, but are stable in aqueous solutions and might be considered as a new class of transfecting agents. We are currently developing studies to evaluate the capacity of these new dendrimers as transfecting agents in vivo.

Experimental Section

Preparation of phosphorus-containing dendrimers

General methods and chemicals: All manipulations were carried out with standard high-vacuum or dry argon atmosphere techniques. ^{1}H, ^{31}P, ^{13}C NMR spectra were recorded on Bruker AC80, AC200, AC250 spectrometers. ^{31}P NMR chemical shifts are reported in ppm relative to 85% H_3PO_4. The numbering used for NMR is depicted in Scheme 1. Dendrimers **1-[G$_n$]** were synthesized according to published procedures.[16, 17] In the abbreviation **1-[G$_n$]** the number **1** stands for the dendrimers with P–Cl terminii, **2**, **3**, **4** or **5** for $-NH(Et)_2]^+(Cl^-)$, $-N(Et)_2$, $-NMe(Et)_2]^+(I^-)$ or $-NMe(Et)_2]^+(OAc^-)$ terminii, respectively, and ***n*** corresponds to the generation number. Methyl iodide and *N*,*N*-diethylethylenediamine were purchased from Aldrich and the strong anion exchange resin AG1-X8 from Bio-rad.

General procedure for the synthesis of protonated dendrimers 2-[G$_1$]–2-[G$_5$]: *N*,*N*-Diethylethylenediamine ($n=1$, 93 μL, 0.66 mmol; $n=2$, 71 μL, 0.5 mmol; $n=3$, 68 μL, 0.48 mmol; $n=4$, 61 μL, 0.43 mmol, $n=5$, 60 μL, 0.42 mmol) was added dropwise by syringe with strong stirring to a solution of dendrimer **1-[G$_n$]** (100 mg; $n=1$, 55 μmol; $n=2$, 21 μmol; $n=3$, 10 μmol; $n=4$, 4.5 μmol; $n=5$, 2.2 μmol) in distilled THF (15 mL). After stirring overnight at room temperature, the solvent was removed by filtration. The white powder was washed with distilled THF (2×20 mL) and evaporated to dryness. The protons produced during the coupling reaction were trapped by the terminal tertiary amine residues; consequently **2-[G$_n$]** dendrimers were obtained as chlorohydrate derivatives.

Dendrimer 2-[G$_1$]: Yield 80%; $^{31}P\{^1H\}$ NMR (CD_3OD): $\delta=7.9$ (P_0), 69.6 (P_1); ^1H NMR ([D$_6$]DMSO): $\delta=1.3$ (t, $^3J_{HH}=6.3$ Hz, 72H; CH_2CH_3), 3.0–3.5 (m, 114H; CH_3-N-P_1, CH_2), 5.7 (brm, 12H; N-H), 7.1 (d, $^3J_{HH}=8.4$ Hz, 12H; C_0^2-H), 7.9 (s, 6H; CH=N), 7.9 (d, $^3J_{HH}=8.4$ Hz, 12H; C_0^3-H), 10.8 (brs, 12H; $^+$N-H); $^{13}C\{^1H\}$ NMR (CD_3OD): $\delta=9.7$ (s, CH_2CH_3), 33.3 (d, $^2J_{CP1}=10.3$ Hz, CH_3-N-P_1), 37.9 (s, CH_2-N-P_1), 49.5 (s, CH_2CH_3), 53.9 (d, $^3J_{CP1}=6.2$ Hz, CH_2-CH_2-N-P_1), 122.6 (s, C_0^2), 129.8 (s, C_0^3), 135.0 (s, C_0^4), 139.3 (d, $^3J_{CP1}=11.6$ Hz, CH=N), 152.4 (d, $^2J_{CP0}=7.3$ Hz, C_0^1); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 284 nm (1.2×10^5).

Dendrimer 2-[G$_2$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): $\delta=8.5$ (P_0), 62.0 (P_1), 69.6 (P_2); ^1H NMR ([D$_6$]DMSO): $\delta=1.3$ (brs, 144H; CH_2CH_3), 3.0–3.6 (brm, 246H; CH_3-N-$P_{1,2}$, CH_2), 5.6 (brm, 24H; N-H), 7.0–7.4 (brm, 36H; C_0^2-H, C_1^2-H), 7.7–8.2 (m, 54H; CH=N, C_0^3-H, C_1^3-H), 10.7 (brs, 24H; $^+$N-H); $^{13}C\{^1H\}$ NMR (CD_3OD): $\delta=9.6$ (s, CH_2CH_3), 33.0 (d, $^2J_{CP2}=10.6$ Hz, CH_3-N-P_2), 34.2 (d, $^2J_{CP1}=11.8$ Hz, CH_3-N-P_1), 37.8 (s, CH_2-N-P_2), 49.2 (s, CH_2CH_3), 53.9 (d, $^3J_{CP2}=6.3$ Hz, CH_2-CH_2-N-P_2), 122.8 (s, C_0^2), 123.0 (d, $^3J_{CP1}=3.0$ Hz, C_1^2), 129.7 (s, C_1^3), 130.0 (s, C_0^3), 134.3 (s, C_0^4), 135.0 (s, C_1^4), 139.1 (d, $^3J_{CP2}=12.5$ Hz, CH=N), 141.3 (d, $^3J_{CP1}=15.4$ Hz, CH=N), 152.6 (d, $^2J_{CP1}=7.3$ Hz, C_1^1), 152.6 (s, C_0^1); UV/vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 284 nm (3.1×10^5).

Dendrimer 2-[G$_3$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): $\delta=8.6$ (P_0), 61.5 (P_1), 62.3 (P_2), 69.5 (P_3); ^1H NMR ([D$_6$]DMSO): $\delta=1.3$ (brs, 288H; CH_2CH_3), 3.0–3.5 (brm, 510H; CH_3-N-$P_{1,2,3}$, CH_2), 5.7 (brs, 48H; N-H), 7.0–7.5 (brm, 84H; C_0^2-H, C_1^2-H, C_2^2-H), 7.7–8.2 (brm, 126H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H), 10.8 (brs, 48H; $^+$N-H); $^{13}C\{^1H\}$ NMR (CD_3OD): $\delta=9.6$ (s, CH_2CH_3), 33.1 (d, $^2J_{CP3}=9.4$ Hz, CH_3-N-P_3), 34.2 (m, CH_3-N-$P_{1,2}$), 37.6 (s, CH_2-N-P_3), 49.2 (s, CH_2CH_3), 53.7 (d, $^3J_{CP3}=6.3$ Hz, CH_2-CH_2-N-P_3), 123.2 (brs, C_0^2, C_1^2, C_2^2), 129.6 (brs, C_0^3, C_1^3, C_2^3), 134.0 (s, C_0^4, C_1^4), 134.8 (s, C_2^4), 139.0 (brs, C_2^4-CH=N), 141.4 (brs, CH=N), 152.4 (d, $^2J_{CP2}=7.3$ Hz, C_2^1), 152.8 (brs, C_0^1, C_1^1); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 286 nm (7.3×10^5).

Dendrimer 2-[G$_4$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): $\delta=8.4$ (P_0), 62.0 ($P_{1,2,3}$), 69.4 (P_4); ^1H NMR ([D$_6$]DMSO): $\delta=1.3$ (brs, 576H; CH_2CH_3), 3.0–3.5 (m, 1038H; CH_3-N-$P_{1,2,3,4}$, CH_2), 5.6 (brs, 96H; N-H), 7.0–7.5 (brm, 180H; C_0^2-H, C_1^2-H, C_2^2-H, C_3^2-H), 7.7–8.2 (m, 270H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H, C_3^3-H), 10.8 (brs, 96H; $^+$N-H); $^{13}C\{^1H\}$ NMR (CD_3OD): $\delta=9.7$ (s, CH_2CH_3), 33.2 (d, $^2J_{CP4}=9.2$ Hz, CH_3-N-P_4), 34.3 (d, $^2J_{CP}=10.1$ Hz, CH_3-N-$P_{1,2,3}$), 37.7 (s, CH_2-N-P_4), 49.2 (s, CH_2CH_3), 53.8 (d, $^3J_{CP4}=5.5$ Hz, CH_2-CH_2-N-P_4), 123.1 (brs, C_0^2, C_1^2, C_2^2, C_3^2), 129.7 (brs, C_0^3, C_1^3, C_2^3, C_3^3), 134.2 (s, C_0^4, C_1^4, C_2^4), 134.9 (s, C_3^4), 139.2 (brs, C_3^4-CH=N), 141.5 (brs, CH=N), 152.5 (d, $^3J_{CP3}=7.4$ Hz, C_3^1), 153.0 (brs, C_0^1, C_1^1, C_2^1); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 288 nm (1.7×10^6).

Dendrimer 2-[G$_5$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): $\delta=62.0$ ($P_{1,2,3,4}$), 69.3 (P_5); ^1H NMR ([D$_6$]DMSO): $\delta=1.3$ (brs, 1152H; CH_2CH_3), 2.9–3.5 (brm, 2094H; CH_3-N-$P_{1,2,3,4,5}$, CH_2), 5.6 (brs, 192H; N-H), 7.0–7.5 (m, 372H; C_0^2-H, C_1^2-H, C_2^2-H, C_3^2-H, C_4^2-H), 7.7–8.2 (m, 558H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H, C_3^3-H, C_4^3-H), 10.8 (brs, 192H; $^+$N-H); $^{13}C\{^1H\}$ NMR (CD_3OD): $\delta=9.7$ (s, CH_2CH_3), 33.2 (brs, CH_3-N-P_5), 34.3 (brs, CH_3-N-$P_{1,2,3,4}$), 37.8 (s, CH_2-N-P_5), 49.2 (s, CH_2CH_3), 53.8 (s, CH_2-CH_2-N-P_5), 123.1 (brs, C_0^2, C_1^2, C_2^2, C_3^2, C_4^2), 129.7 (brs, C_0^3, C_1^3, C_2^3, C_3^3, C_4^3), 134.2 (s, C_0^4, C_1^4, C_2^4, C_3^4), 134.9 (s, C_4^4), 139.2 (brs, C_4^4-CH=N), 141.5 (brs, CH=N), 152.5 (s, C_4^1), 153.0 (brs, C_0^1, C_1^1, C_2^1 , C_3^1); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 286 nm (3.3×10^6).

General procedure for the synthesis of the neutral dendrimers 3-[G$_1$]–3-[G$_5$]: A solution of NaOH (1M; $n=1$, 372 μL, 0.37 mmol; $n=2$, 312 μL, 0.31 mmol; $n=3$, 288 μL, 0.28 mmol; $n=4$, 288 μL, 0.28 mmol; $n=5$, 288 μL, 0.28 mmol) was added dropwised to a stirred solution of dendrimer **2-[G$_n$]** (100 mg; $n=1$, 31 μmol; $n=2$, 13 μmol; $n=3$, 6 μmol; $n=4$, 3 μmol; $n=5$, 1.5 μmol) in distilled water (30 mL). The precipitate was recovered by centrifugation, dissolved in chloroform, then the organic layer was dried over Na_2SO_4, filtered and evaporated to dryness.

Dendrimer 3-[G$_1$]: Yield 80%; $^{31}P\{^1H\}$ NMR ($CDCl_3$): $\delta=8.2$ (P_0), 68.3 (P_1); ^1H NMR (CD_2Cl_2): $\delta=0.9$ (t, $^3J_{HH}=7.0$ Hz, 72H; CH_2CH_3), 2.3–2.5 (m, 72H; CH_2-N(CH_2-CH_3)$_2$), 2.9 (m, 24H; CH_2-N-P_1), 3.1 (d, $^3J_{HP1}=9.4$ Hz, 18H; CH_3-N-P_1), 4.0 (m, 12H; N-H), 6.9 (d, $^3J_{HH}=8.5$ Hz, 12H; C_0^2-H), 7.5 (s, 6H; CH=N), 7.5 (d, $^3J_{HH}=8.5$ Hz, 12H; C_0^3-H); $^{13}C\{^1H\}$ NMR ($CDCl_3$): $\delta=11.4$ (s, CH_2CH_3), 30.5 (d, $^2J_{CP1}=10.7$ Hz, CH_3-N-P_1), 38.4 (s, CH_2-N-P_1), 46.3 (s, CH_2CH_3), 52.9 (d, $^3J_{CP1}=7.8$ Hz, CH_2-CH_2-N-P_1), 120.8 (s, C_0^2), 127.3 (s, C_0^3), 132.7 (s, C_0^4), 135.4 (d, $^3J_{CP1}=12.5$ Hz, CH=N), 150.4 (d, $^2J_{CP0}=5.1$ Hz, C_0^1).

Dendrimer 3-[G$_2$]: Yield 95%; $^{31}P\{^1H\}$ NMR ($CDCl_3$): $\delta=8.4$ (P_0), 62.9 (P_1), 68.1 (P_2); ^1H NMR (CD_2Cl_2): $\delta=0.9$ (t, $^3J_{HH}=7$ Hz, 144H; CH_2CH_3), 2.2–2.5 (m, 144H; CH_2-N(CH_2CH_3)$_2$), 2.9 (m, 48H; CH_2-N-P_2), 3.0 (d, $^3J_{HP2}=9.2$ Hz, 36H; CH_3-N-P_2), 3.2 (d, $^3J_{HP1}=10$ Hz, 18H; CH_3-N-P_1), 4.0 (brm, 24H; N-H), 6.9–7.1 (m, 36H; C_0^2-H, C_1^2-H), 7.4–7.7 (m, 54H; CH=N, C_0^3-H, C_1^3-H); $^{13}C\{^1H\}$ NMR ($CDCl_3$): $\delta=11.4$ (s, CH_2CH_3), 30.6 (d, $^2J_{CP2}=10.8$ Hz, CH_3-N-P_2), 32.9 (d, $^2J_{CP1}=11.8$ Hz, CH_3-N-P_1), 38.3 (s, CH_2-N-P_2), 46.3 (s, CH_2CH_3), 52.9 (d, $^3J_{CP2}=7.8$ Hz, CH_2-CH_2-N-P_2), 121.1 (s, C_0^2, C_1^2), 127.4 (s, C_1^3), 128.1 (s, C_0^3), 132.0 (s, C_0^4), 133.1 (s, C_1^4), 135.1 (d, $^3J_{CP2}=12.4$ Hz, CH=N), 138.6 (d, $^3J_{CP1}=15.4$ Hz, CH=N), 150.3 (d, $^2J_{CP1}=7.3$ Hz, C_1^1), 151.1 (s, C_0^1).

Dendrimer 3-[G$_3$]: Yield 95%; $^{31}P\{^1H\}$ NMR ($CDCl_3$): $\delta=8.5$ (P_0), 62.9 ($P_{1,2}$), 68.1 (P_3); ^1H NMR (CD_2Cl_2): $\delta=0.9$ (t, $^3J_{HH}=6.4$ Hz, 288H; CH_2CH_3), 2.2–2.5 (brm, 288H; CH_2-N(CH_2CH_3)$_2$), 2.9 (brm, 96H; CH_2-N-P_3), 3.0 (d, $^3J_{HP3}=9.2$ Hz, 72H; CH_3-N-P_3), 3.2–3.4 (brm, 54H; CH_3-N-$P_{1,2}$), 4.0 (brs, 48H; N-H), 6.9–7.3 (brm, 84H; C_0^2-H, C_1^2-H, C_2^2-H), 7.4–7.7 (brm, 126H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H); $^{13}C\{^1H\}$ NMR ($CDCl_3$): $\delta=11.4$ (s, CH_2CH_3), 30.6 (d, $^2J_{CP3}=10.3$ Hz, CH_3-N-P_3), 32.5 (d, $^2J_{CP}=12.7$ Hz, CH_3-N-$P_{1,2}$), 38.4 (s, CH_2-N-P_3), 46.4 (s, CH_2CH_3), 53.0 (d, $^3J_{CP3}=7.9$ Hz, CH_2-CH_2-N-P_3), 121.4 (s, C_0^2, C_1^2, C_2^2), 127.5 (s, C_2^3), 128.2 (s, C_0^3, C_1^3), 132.0 (s, C_0^4), 132.3 (s, C_1^4), 133.1 (s, C_2^4), 135.2 (d, $^3J_{CP3}=12.1$ Hz, CH=N), 138.8

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(d, $^3J_{CP}$ = 12.1 Hz, CH=N), 150.4 (d, $^2J_{CP2}$ = 6.8 Hz, C_2^1), 151.2 (d, $^2J_{CP}$ = 6.3 Hz, C_0^1, C_1^1).

Dendrimer 3-[G$_4$]: Yield 95%; $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ = 8.4 (P_0), 62.4 ($P_{1,2,3}$), 68.0 (P_4); ^1H NMR (CD_2Cl_2): δ = 0.8 (brs, 576H; CH_2CH_3), 2.4 (brs, 576H; CH_2-N(CH_2CH_3)$_2$), 2.6–3.4 (brm, 462H; CH_3-N-$P_{1,2,3,4}$, CH_2-N-P_4), 4.0 (brm, 96H; N-H), 7.0–7.7 (m, 450H; C_6H_4, CH=N); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 11.6 (s, CH_2CH_3), 30.6 (d, $^2J_{CP4}$ = 10.4 Hz, CH_3-N-P_4), 33.0 (d, $^2J_{CP}$ = 12.6 Hz, CH_3-N-$P_{1,2,3}$), 38.5 (s, CH_2-N-P_4), 46.4 (s, CH_2CH_3), 53.1 (d, $^3J_{CP4}$ = 7.7 Hz, CH_2-CH_2-N-P_4), 121.5 (s, C_3^2), 121.8 (s, C_0^2, C_1^2, C_2^2), 127.5 (s, C_3^3), 128.3 (s, C_0^3, C_1^3, C_2^3), 132.2 (s, C_0^4, C_1^4, C_2^4), 133.2 (s, C_3^4), 135.1 (d, $^3J_{CP4}$ = 12.0 Hz, CH=N), 138.7 (brs, CH=N), 150.5 (d, $^2J_{CP2}$ = 7.4 Hz, C_3^1), 151.3 (brm, C_0^1, C_1^1, C_2^1).

Dendrimer 3-[G$_5$]: Yield 95%; $^{31}P\{^1H\}$ NMR ($CDCl_3$): δ = 62.4 ($P_{1,2,3,4}$), 68.0 (P_5); ^1H NMR (CD_2Cl_2): δ = 1.0 (brs, 1152H; CH_2CH_3), 2.4 (brs, 1152H; CH_2-N(CH_2CH_3)$_2$), 2.7–3.5 (brm, 942H; CH_3-N-$P_{1,2,3,4,5}$, CH_2-N-P_5), 4.1 (m, 192H; N-H), 7.0–7.8 (m, 930H; C_6H_4, CH=N); $^{13}C\{^1H\}$ NMR ($CDCl_3$): δ = 11.5 (s, CH_2CH_3), 30.5 (d, $^2J_{CP5}$ = 11.1 Hz, CH_3-N-P_5), 33.0 (d, $^2J_{CP}$ = 12.3 Hz, CH_3-N-$P_{1,2,3,4}$), 38.4 (s, CH_2-N-P_5), 49.4 (s, CH_2CH_3), 53.0 (d, $^3J_{CP5}$ = 8.1 Hz, CH_2-CH_2-N-P_5), 121.4 (s, C_4^2), 121.7 (s, C_0^2, C_1^2, C_2^2, C_3^2), 127.4 (s, C_4^3), 128.1 (s, C_0^3, C_1^3, C_2^3, C_3^3), 132.1 (s, C_0^4, C_1^4, C_2^4, C_3^4), 133.1 (s, C_4^4), 135.1 (d, $^3J_{CP5}$ = 12.1 Hz, CH=N), 138.7 (brs, CH=N), 150.5 (d, $^2J_{CP3}$ = 6.6 Hz, C_4^1), 151.2 (d, $^2J_{CP}$ = 6.2 Hz, C_0^1, C_1^1, C_2^1, C_3^1).

General procedure for the synthesis of methylated dendrimers 4-[G$_1$]–4-[G$_5$] with iodides as counterions: A solution of the neutral dendrimers **3-[G$_n$]** (100 mg; n = 1, 36 μmol; n = 2, 15 μmol; n = 3, 7 μmol; n = 4, 3.3 μmol; n = 5, 1.6 μmol) and methyl iodide (n = 1, 27 μL, 0.43 mmol; n = 2, 22 μL, 0.36 mmol; n = 3, 21 μL, 0.34 mmol; n = 4, 20 μL, 0.32 mmol; n = 5, 19 μL, 0.31 mmol) in DMF (15 mL) was stirred at room temperature overnight. The solution was then evaporated to dryness. The resulting paste was washed with 20 mL of a pentane/ether mixture (1/1, v/v) to afford the methylated dendrimers **4-[G$_n$]** as yellow powders.

Dendrimer 4-[G$_1$]: Yield 90%; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.3 (P_0), 68.1 (P_1); ^1H NMR ([D$_6$]DMSO): δ = 1.3 (t, $^3J_{HH}$ = 6.4 Hz, 72H; CH_2CH_3), 3.1 (s, 36H; $^+$N-CH_3), 3.2 (d, $^3J_{HP1}$ = 10.4 Hz, 18H; CH_3-N-P_1), 3.3–3.7 (brs, 96H; CH_2), 5.5 (brm, 12H; N-H), 7.1 (d, $^3J_{HH}$ = 8.1 Hz, 12H; C_0^2-H), 8.0 (s, 6H; CH=N), 8.0 (d, $^3J_{HH}$ = 8.1 Hz, 12H; C_0^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.8 (s, CH_2CH_3), 32.6 (d, $^2J_{CP1}$ = 9.5 Hz, CH_3-N-P_1), 34.9 (s, CH_2-N-P_1), 47.3 (s, $^+$N-CH_3), 56.4 (s, CH_2CH_3), 58.8 (s, CH_2-CH_2-N-P_1), 120.7 (s, C_0^2), 128.3 (s, C_0^3), 133.2 (s, C_0^4), 137.4 (d, $^3J_{CP1}$ = 14.2 Hz, CH=N), 149.9 (s, C_0^1).

Dendrimer 4-[G$_2$]: Quantitative yield; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.3 (P_0), 61.7 (P_1), 68.1 (P_2); ^1H NMR ([D$_6$]DMSO): δ = 1.3 (t, $^3J_{HH}$ = 6.2 Hz, 144H; CH_2CH_3), 3.1 (s, 72H; $^+$N-CH_3), 3.2–3.6 (m, 246H; CH_3-N-$P_{1,2}$, CH_2), 5.7 (brs, 24H; N-H), 7.0–7.4 (m, 36H; C_0^2-H, C_1^2-H), 7.7–8.2 (m, 54H; CH=N, C_0^3-H, C_1^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.8 (s, CH_2CH_3), 32.2 (d, $^2J_{CP2}$ = 8.9 Hz, CH_3-N-P_2), 33.5 (d, $^2J_{CP1}$ = 12.9 Hz, CH_3-N-P_1), 34.9 (s, CH_2-N-P_2), 47.3 (s, $^+$N-CH_3), 56.4 (s, CH_2CH_3), 58.8 (d, $^3J_{CP2}$ = 3.3 Hz, CH_2-CH_2-N-P_2), 121.3 (s, C_1^2, C_0^2), 128.2 (s, C_1^3), 128.6 (s, C_0^3), 132.3 (s, C_0^4), 133.2 (s, C_1^4), 137.1 (d, $^3J_{CP2}$ = 12.3 Hz, CH=N), 140.7 (brs, CH=N), 150.1 (d, $^2J_{CP1}$ = 6 Hz, C_1^1), 150.5 (s, C_0^1).

Dendrimer 4-[G$_3$]: Quantitative yield; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): δ = 6.9 (P_0), 61.9 ($P_{1,2}$), 68.1 (P_3); ^1H NMR ([D$_6$]DMSO): δ = 1.3 (brs, 288H; CH_2CH_3), 3.1 (s, 144H; $^+$N-CH_3), 3.1–3.6 (m, 510H; CH_3-N-$P_{1,2,3}$, CH_2), 5.5 (brs, 48H; N-H), 7.0–7.4 (m, 84H; C_0^2-H, C_1^2-H, C_2^2-H), 7.7–8.2 (m, 126H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.7 (s, CH_2CH_3), 32.2 (d, $^2J_{CP3}$ = 9.4 Hz, CH_3-N-P_3), 33.5 (m, CH_3-N-$P_{1,2}$), 34.9 (s, CH_2-N-P_3), 47.3 (s, $^+$N-CH_3), 56.4 (s, CH_2CH_3), 58.8 (d, $^3J_{CP3}$ = 4.8 Hz, CH_2-CH_2-N-P_3), 121.0 (brs, C_0^2, C_1^2, C_2^2), 128.5 (brs, C_0^3, C_1^3, C_2^3), 132.3 (s, C_0^4, C_1^4), 133.2 (s, C_2^4), 137.1 (d, $^3J_{CP3}$ = 12.1 Hz, CH=N), 141.0 (brs, CH=N), 150.1 (d, $^2J_{CP2}$ = 6.2 Hz, C_2^1), 150.7 (brs, C_0^1, C_1^1).

Dendrimer 4-[G$_4$]: Quantitative yield; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): δ = 6.3 (P_0), 61.6 ($P_{1,2,3}$), 68.0 (P_4); ^1H NMR ([D$_6$]DMSO): δ = 1.3 (brs, 576H; CH_2CH_3), 3.1 (s, 288H; $^+$N-CH_3), 3.1–3.6 (m, 1038H; CH_3-N-$P_{1,2,3,4}$, CH_2), 5.5 (brs, 96H; N-H), 7.1–8.5 (m, 450H; C_6H_4, CH=N); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.8 (s, CH_2CH_3), 32.2 (d, $^2J_{CP4}$ = 9.5 Hz, CH_3-N-P_4), 33.6 (d, $^2J_{CP}$ = 7.6 Hz, CH_3-N-$P_{1,2,3}$), 34.9 (s, CH_2-N-P_4), 47.3 (s, $^+$N-CH_3), 56.4 (s, CH_2CH_3), 58.7 (d, $^3J_{CP4}$ = 4.8 Hz, CH_2-CH_2-N-P_4), 121.2 (brs, C_0^2, C_1^2, C_2^2, C_3^2), 128.2 (brs, C_0^3, C_1^3, C_2^3 , C_3^3), 132.2 (s, C_0^4, C_1^4, C_2^4), 133.2 (s, C_3^4), 137.1 (d, $^3J_{CP4}$ = 9.4 Hz, CH=N), 140.8 (brs, CH=N), 150.0 (d, $^2J_{CP2}$ = 6.2 Hz, C_3^1), 150.7 (brs, C_0^1, C_1^1, C_2^1).

Dendrimer 4-[G$_5$]: Quantitative yield; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): δ = 61.6 ($P_{1,2,3,4}$), 68.0 (P_5); ^1H NMR ([D$_6$]DMSO): δ = 1.3 (brs, 1152H; CH_2CH_3), 3.1 (s, 576H; $^+$N-CH_3), 3.1–3.6 (m, 2094H; CH_3-N-$P_{1,2,3,4,5}$, CH_2), 5.4 (m, 192H; N-H), 7.2–8.5 (m, 930H; C_6H_4 , CH=N); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.8 (s, CH_2CH_3), 32.3 (brs, CH_3-N-P_5), 33.5 (brs, CH_3-N-$P_{1,2,3,4}$), 34.9 (s, CH_2-N-P_5), 47.3 (s, $^+$N-CH_3), 56.4 (s, CH_2CH_3), 58.8 (d, $^3J_{CP}$ = 5.0 Hz, CH_2-CH_2-N-P_5), 121.3 (brs, C_0^2, C_1^2, C_2^2, C_3^2, C_4^2), 128.2 (brs, C_0^3, C_1^3, C_2^3, C_3^3, C_4^3), 132.2 (s, C_0^4, C_1^4, C_2^4, C_3^4), 133.2 (s, C_4^4), 137.2 (d, $^3J_{CP4}$ = 9.7 Hz, CH=N), 141.2 (brs, CH=N), 150.1 (d, $^2J_{CP2}$ = 6.0 Hz, C_4^1), 150.7 (brs, C_0^1, C_1^1, C_2^1, C_3^1).

General procedure for the synthesis of methylated dendrimers 5-[G$_1$]–5-[G$_5$] with acetate as counterions: Strong anion exchange resin AG1-X8 (n = 1, 1.24 g; n = 2, 1.13 g; n = 3, 1.06 g; n = 4, 1.03 g; n = 5, 1 g) was added to a suspension of the methylated dendrimers (iodide form) **4-[G$_n$]** (100 mg; n = 1, 22 μmol; n = 2, 10 μmol; n = 3, 4.7 μmol; n = 4, 2.3 μmol; n = 5, 1.1 μmol) in distilled water (n = 1, 25 mL; n = 2, 23 mL; n = 3, 22 mL; n = 4, 21 mL; n = 5, 20 mL), and the mixture was stirred gently for one hour. The resulting paste was washed with pentane/ether (20 mL, 1/1, v/v) mixture to afford the methylated dendrimers (acetate form) **5-[G$_n$]** as white powders. It should be noted that we observed that 8 to 10% of the terminal branches are modified during the counterion exchange process with the resin; these were tentatively attributed to a "demethylated form", since the NMR data were similar to that of neutral tertiary amine dendrimers **3-[G$_n$]** (these minor terminal branches have been indicated by an asterisk in the NMR data reported below and the indicated yields correspond to the overall dendrimer).

Dendrimer 5-[G$_1$]: Yield 90%; $^{31}P\{^1H\}$ NMR (CD_3OD): δ = 7.8 (P_0), 69.8 (P_1*), 70.3 (P_1); ^1H NMR ([D$_6$]DMSO): δ = 1.0 (t, $^3J_{HH}$ = 6.9 Hz, 6H; CH_2CH_3*), 1.3 (t, $^3J_{HH}$ = 6.5 Hz, 66H; CH_2CH_3), 1.7 (s, 33H; CH_3COO^-), 2.5 (m, 6H; CH_2*-N(CH_2*CH_3)$_2$), 3.1 (s, 33H; $^+$N-CH_3), 3.2 (d, $^3J_{HP1}$ = 8.3 Hz, 18H; CH_3-N-P_1), 3.3–3.7 (m, 90H; CH_2), 7.1 (d, $^3J_{HH}$ = 8.3 Hz, 12H; C_0^2-H), 7.6 (brm, 12H; N-H), 7.8 (s, 6H; CH=N), 7.8 (d, $^3J_{HH}$ = 8.3 Hz, 12H; C_0^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.6 (s, CH_2CH_3), 11.9 (s, CH_2C*H_3), 25.6 (s, CH_3COO^-), 32.2 (d, $^2J_{CP1}$ = 9.5 Hz, CH_3-N-P_1), 38.8 (s, CH_2-N-P_1), 46.6 (s, C*H_2CH_3), 47.0 (s, $^+$N-CH_3), 54.5 (s, C*H_2-CH_2-N-P_1), 56.2 (s, CH_2CH_3), 59.1 (d, $^3J_{CP1}$ = 5.3 Hz, CH_2-CH_2-N-P_1), 120.6 (s, C_0^2), 127.9 (s, C_0^3), 133.7 (s, C_0^4), 135.8 (d, $^2J_{CP0}$ = 10.8 Hz, CH=N), 149.8 (s, C_0^1), 173.7 (s, CH_3COO^-); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 284 nm (1.2×10^5).

Dendrimer 5-[G$_2$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): δ = 8.4 (P_0), 62.3 (P_1), 69.5 (P_2*), 70.1 (P_2); ^1H NMR ([D$_6$]DMSO): δ = 1.0 (brt, 12H; CH_2CH_3*), 1.3 (brs, 132H; CH_2CH_3), 1.7 (s, 66H; CH_3COO^-), 2.5 (brm, 12H; CH_2*-N(CH_2*CH_3)$_2$), 3.1 (s, 66H; $^+$N-CH_3), 3.2–3.6 (m, 234H; CH_3-N-$P_{1,2}$, CH_2), 7.1–7.3 (brm, 36H; C_0^2-H, C_1^2-H), 7.6 (brs, 24H; N-H), 7.7 (s, 12H; CH=N), 7.8–8.1 (brm, 42H; CH=N, C_0^3-H, C_1^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.6 (s, CH_2CH_3), 11.9 (s, CH_2C*H_3), 25.6 (s, CH_3COO^-), 32.1 (d, $^2J_{CP2}$ = 9.3 Hz, CH_3-N-P_2), 33.2 (d, $^2J_{CP1}$ = 13.4 Hz, CH_3-N-P_1), 35.0 (s, CH_2-N-P_2), 46.6 (s, C*H_2CH_3), 47.0 (s, $^+$N-CH_3), 54.5 (s, C*H_2-CH_2-N-P_2), 56.2 (s, CH_2CH_3), 59.1 (d, $^3J_{CP2}$ = 5.3 Hz, CH_2-CH_2-N-P_2), 121.3 (s, C_0^2, C_1^2), 127.9 (s, C_1^3), 128.5 (s, C_0^3), 132.3 (s, C_0^4), 133.8 (s, C_1^4), 135.7 (brs, CH=N), 149.8 (d, $^2J_{CP1}$ = 6.5 Hz, C_1^1), 150.7 (s, C_0^1), 173.7 (s, CH_3COO^-); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 284 nm (4.1×10^5).

Dendrimer 5-[G$_3$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): δ = 8.4 (P_0), 62.3 ($P_{1,2}$), 69.5 (P_3*), 70.1 (P_3); ^1H NMR ([D$_6$]DMSO): δ = 1.0 (brt, 30H; CH_2CH_3*), 1.3 (brs, 258H; CH_2CH_3), 1.7 (s, 129H; CH_3COO^-), 2.5 (brm, 30H; CH_2*-N(CH_2*CH_3)$_2$), 3.1 (s, 129H; $^+$N-CH_3), 3.1–3.6 (m, 480H; CH_3-N-$P_{1,2,3}$, CH_2), 7.1–7.5 (brm, 108H; C_0^2-H, C_1^2-H, C_2^2-H, N-H), 7.5–8.1 (brm, 126H; CH=N, C_0^3-H, C_1^3-H, C_2^3-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): δ = 7.6 (s, CH_2CH_3), 11.9 (s, CH_2C*H_3), 25.2 (s, CH_3COO^-), 32.1 (d, $^2J_{CP3}$ = 9.0 Hz, CH_3-N-P_3), 33.2 (brm, CH_3-N-$P_{1,2}$), 35.0 (s, CH_2-N-P_3), 46.6 (s, C*H_2CH_3), 47.1 (s, $^+$N-CH_3), 54.5 (s, C*H_2-CH_2-N-P_3), 56.3 (s, CH_2CH_3), 59.1 (d, $^3J_{CP3}$ = 5.7 Hz, CH_2-CH_2-N-P_3), 121.3 (brs, C_0^2, C_1^2, C_2^2), 128.0 (s, C_2^3), 128.5 (s, C_0^3, C_1^3), 132.4 (s, C_0^4, C_1^4), 133.8 (s, C_2^4), 136.3 (brd, C_2^4-CH=N), 141.0 (brm, CH=N), 150.1 (d, $^2J_{CP2}$ = 6.3 Hz, C_2^1), 151.0 (d, $^2J_{CP}$ = 7.1 Hz, C_0^1, C_1^1), 173.7 (s, CH_3COO^-); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 286 nm (7.4×10^5).

Dendrimer 5-[G$_4$]: Yield 95%; $^{31}P\{^1H\}$ NMR (CD_3OD): δ = 8.2 (P_0), 62.2 ($P_{1,2,3}$), 69.9 (P_4); ^1H NMR ([D$_6$]DMSO): δ = 1.0 (brm, 60H; CH_2CH_3*), 1.3 (brs, 516H; CH_2CH_3), 1.7 (s, 258H; CH_3COO^-), 2.5 (m, 60H; CH_2*-N(CH_2*CH_3)$_2$), 3.1 (s, 258H; $^+$N-CH_3), 3.1–3.6 (m, 978H; CH_3-N-$P_{1,2,3,4}$,

CH_2), 7.0 – 8.2 (m, 546 H; C_6H_4, CH=N, N-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): $\delta = 7.6$ (s, CH_2CH_3), 11.9 (s, $CH_2C^*H_3$), 24.7 (s, CH_3COO^-), 32.1 (d, $^2J_{CP4} = 9.6$ Hz, CH_3-N-P_4), 33.2 (d, $^2J_{CP} = 11.2$ Hz, CH_3-N-$P_{1,2,3}$), 35.0 (s, CH_2-N-P_4), 46.6 (s, $C^*H_2CH_3$), 47.0 (s, $^+$N-CH_3), 54.5 (d, $^3J_{CP4} = 3.9$ Hz, C^*H_2-CH_2-N-P_4), 56.3 (s, CH_2CH_3), 59.2 (d, $^3J_{CP4} = 5.7$ Hz, CH_2-CH_2-N-P_4), 121.2 (s, C_3^2), 121.6 (s, C_0^2, C_1^2, C_2^2), 127.9 (s, C_3^3), 128.5 (s, C_0^3, C_1^3, C_2^3), 132.3 (s, C_0^4, C_1^4, C_2^4), 133.7 (s, C_3^4), 135.8 (br s, C_3^4-CH=N), 141.0 (br s, CH=N), 149.9 (d, $^2J_{CP3} = 6.3$ Hz, C_3^1), 151.0 (br m, C_0^1,C_1^1, C_2^1), 173.7 (s, CH_3COO^-); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 288 nm (1.6×10^6).

Dendrimer 5-[G$_5$]: Yield 95 %; $^{31}P\{^1H\}$ NMR ([D$_6$]DMSO): $\delta = 62.2$ ($P_{1,2,3,4}$), 69.9 (P_5); ^{1}H NMR ([D$_6$]DMSO): $\delta = 1.0$ (m, 114 H; CH_2CH_3*), 1.3 (m, 1038 H; CH_2CH_3), 1.7 (s, 519 H; CH_3COO^-), 2.5 (m, 114 H; CH_2*-N(CH_2*CH_3)$_2$), 3.1 (s, 519 H; $^+$N-CH_3), 3.1 – 3.6 (m, 1980 H; CH_3-N-$P_{1,2,3,4,5}$, CH_2), 7.0 – 8.2 (m, 1122 H; C_6H_4, CH=N, N-H); $^{13}C\{^1H\}$ NMR ([D$_6$]DMSO): $\delta = 7.6$ (s, CH_2CH_3), 11.9 (s, $CH_2C^*H_3$), 24.7 (s, CH_3COO^-), 32.1 (d, $^2J_{CP5} = 9.6$ Hz, CH_3-N-P_5), 33.2 (d, $^2J_{CP} = 11.2$ Hz, CH_3-N-$P_{1,2,3,4}$), 35.0 (s, CH_2-N-P_5), 46.6 (s, $C^*H_2CH_3$), 47.0 (s, $^+$N-CH_3), 54.5 (d, $^3J_{CP5} = 3.9$ Hz, C^*H_2-CH_2-N-P_5), 56.3 (s, CH_2CH_3), 59.2 (d, $^3J_{CP5} = 5.7$ Hz, CH_2-CH_2-N-P_5), 121.2 (s, C_0^2, C_1^2, C_2^2, C_3^2, C_4^2), 127.9 (s, C_4^3), 128.5 (s, C_0^3, C_1^3, C_2^3, C_3^3), 132.3 (s, C_0^4, C_1^4, C_2^4, C_3^4), 133.7 (s, C_4^4), 135.9 (br s, C_4^4-CH=N), 141.0 (br s, CH=N), 149.9 (d, $^2J_{CP3} = 6.5$ Hz, C_4^1), 151.0 (br m, C_0^1, C_1^1, C_2^1, C_3^1), 173.7 (s, CH_3COO^-); UV/Vis (H_2O): λ_{max} (ε, $M^{-1}cm^{-1}$) = 284 nm (3.7×10^6).

Transfection experiments

Chemicals: Linear 22 KDa polyethyleneimine (ExGen500) was purchased from Euromedex (Souffelweyersheim, France).

Cell lines and cell culture: NIH 3T3 murine fibroblasts were purchased from ATCC (Rockville, MA, USA) and grown in Dulbecco's modified Eagle medium (DMEM). Cell culture media was supplemented with 10 % FCS (fetal calf serum, D. Dutscher, Brumath, France), L-glutamine (2 mM; Gibco-BRL), penicillin (100 units mL^{-1}; Gibco-BRL) and streptomycin (100 μg mL^{-1}; Gibco-BRL). Cells were maintained at 37 °C in a 5 % CO_2 humidified atmosphere. When 80 % confluent, they were detached with saline trypsine-EDTA (Gibco, BRL) and grown in new flasks at a 1/10 dilution.

Plasmids: pCMV-luc, encoding the *Photinus pyralis* luciferase under the control of the cytomegalovirus enhancer/promoter, was kindly given by Dr M. Scarpa (CRIBI, Padova, Italy). Plasmid was purified from *E.coli* strain XL blue using Qiagen columns (Rockford, USA).

Cell transfection: Adherent cells were seeded in 24-well plates (Costar, D. Dutscher, France) the day before transfection in order to reach 60 – 70 % confluence during transfection. All experiments were done in triplicate. Prior to transfection, cells were rinsed and culture medium (1 mL) with and without 10 % FCS was added in each well. Two μg of the desired plasmid (from a ca. 1.5 mg mL^{-1} solution in 10 mM Tris/1 mM EDTA buffer pH 7.4) were diluted into NaCl (0.15 M, 50 μL).

The ratio N/P (nitrogen/phosphate) corresponds to the amount of polymer necessary to have one amino group (43 Da = mean M_w for PEI; for dendrimers the amine, nitrogen molarity was calculated by dividing the M_w by the number of charges of each generation) per phosphate of nucleic acid (330 Da mean M_w).[12] The desired amount of linear PEI (ExGen500) and dendrimers (from 10 mM of amine nitrogen stock solutions of PEIs and dendrimers in sterile MilliQ water) were diluted into NaCl (0.15 M, 50 μL), vortexed gently, and spun down. Fifteen minutes later, the cationic vector was added all at once to the plasmid solution (and not the reverse order, see ref. [12]), mixed, vortexed, and spun down. The amounts and volumes given above refer to a single well and were actually threefold larger and distributed in three wells. After ca. 10 min the resulting mixture was added to the cells and the cell supernatant was uniformly distributed by a gentle horizontal hand rotation. Immediately after, the cell culture dish was centrifuged (Sigma 3 K10, Bioblock, France) for 5 min at 1500 rpm (ca. 280 g). After 2 – 3 h, fetal calf serum (110 μL) was added in the serum-free wells. The cells were cultured for a 24 h period and then tested for reporter gene expression.

Luciferase assay: Luciferase gene expression was measured by luminescence. The culture medium was discarded and cell lysate harvested upon cell incubation for 30 min at room temperature in Lysis Reagent 1 × (Promega, USA). The lysate was vortexed gently and centrifuged for 5 min at 14000 rpm (ca. 17530 g) at 4 °C. Lysate (20 μL) was diluted into luciferase reaction buffer (100 μL; Promega) and the luminescence was measured for 10 seconds (Mediators PhL, Wien, Austria). The results were expressed as light units per mg of cell protein (BCA assay, Pierce).

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