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Water-Soluble Carbosilane Dendrimers: Synthesis Biocompatibility and Complexation with Oligonucleotides; Evaluation for Medical Applications

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Abstract: Novel amine- or ammonium-terminated carbosilane dendrimers of type *n*G-[Si{$OCH_2(C_6H_3)$-3,5-$(OCH_2CH_2NMe_2)_2$}]$_x$, *n*G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]$_x$ and *n*G-[Si{$(CH_2)_3NH_2$}]$_x$ or *n*G-[Si{$OCH_2(C_6H_3)$-3,5-$(OCH_2CH_2NMe_3{}^+I^-)_2$}]$_x$, *n*G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_3{}^+I^-$}]$_x$, and *n*G-[Si{$(CH_2)_3NH_3{}^+Cl^-$}]$_x$ have been synthesized and characterized up to the third generation by two strategies: 1) alcoholysis of Si–Cl bonds with amino alcohols and subsequent quaternization with MeI, and 2) hydrosilylation of allylamine with Si–H bonds of the dendritic systems and subsequent quaternization with HCl. Quaternized carbosilane dendrimers are soluble in water, although degradation is apparent due to hydrolysis of Si–O bonds. However, dendrimers containing Si–C bonds are water-stable. The biocompatibility of the second-generation dendrimers in primary cell cultures of peripheral blood mononuclear cells (PBMCs) and erythrocytes have been analyzed, and they show good toxicity profiles over extended periods. In addition, we describe a study on the interactions between the different carbosilane dendrimers and DNA oligodeoxynucleotides (ODNs) and plasmids along with a comparative analysis of their toxicity. They can form complexes with DNA ODNs and plasmids at biocompatible doses via electrostatic interaction. Also a preliminary transfection assay has been accomplished. These results demonstrate that the new ammonium-terminated carbosilane dendrimers are good base molecules to be considered for biomedical applications.

Keywords: carbosilanes · dendrimers · DNA · drug delivery · oligonucleotides

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Introduction

There is currently significant interest in dendrimers as a result of their potential applications, including light harvesting and energy transfer, nanoscale catalysis, chemical sensors, unimolecular micelles, enzyme mimics, encapsulation of guest molecules, molecular recognition, diagnostic agents, and gene and drug delivery.[1] In particular, the structural precision of dendrimers has motivated numerous studies aimed at biomedical applications. One of the most active research areas in dendrimer-based therapeutics is DNA transfection. This application is currently based on viral vectors as the most efficient gene-transfer agents. However, the use of viral vectors suffers from adverse effects, such as immune reaction against the viral vector or lymphoproliferative syndromes associated with oncogene dysregulation.[2] To overcome these drawbacks, nonviral vehicles such as cationic liposomes, polymers, and dendrimers have been developed. Once in contact with negatively charged DNA oligodeoxynucleotides (ODNs) or plasmids, cationic systems form electrostatic complexes with the nucleic acids, known as lipo-

plexes, polyplexes or dendriplexes. The use of liposomes for transfection purposes was first described in 1987.[3] Cationic lipids prepared for this purpose are commercially available (e.g., Cytofectin and Lipofectin). However, they have side-effects such as inflammatory lung reactions,[4] and transfection may fail in the presence of serum. The main drawback of the use of conventional degradable polymers as delivery agents, beside their polydispersity, is their thermodynamic instability, which results in a short in vivo lifetime of the active species.[5]

Dendrimers represent an alternative approach for the transfection of nucleic acids in a wide range of cells. The major advantage of dendrimers over other nonviral vehicles is their uniform structure and the versatility with which their skeletons and surfaces can be modified, which allows precise characterization of the nucleic acid/vector complex and a more accurate and systematic analysis of the transfection process. The first study on using dendritic macromolecules for transfection, reported in 1993, used polyamidoamine (PAMAM) dendrimers,[6] and since then extensive studies have been performed.[7] Good results are typically achieved with sixth- or seventh-generation dendrimers. However, the transfection efficiency can be increased two- to threefold when PAMAM is activated by heat treatment (e.g., Superfect (SF)).[8]

Another class of potential transfecting agents are phosphorus-containing dendrimers,[9] which can be synthesized up to the twelfth generation. The dendritic surface has been grafted with protonated or methylated terminal tertiary amines and examined as transfecting agent for the luciferase gene in 3T3 cells. The efficiency increased as a function of the dendrimer generation, although a constant value was reached between generations three and five. Furthermore, these dendrimers exhibit improved transfection efficiency when serum is present.

Other kinds of dendritic macromolecules, such as polypropylenimine (PPI),[10] and polylysine[11] dendrimers, have also been studied as potential DNA or ODN carriers. For instance, low-generation PPI dendrimers have also shown gene-transfection ability in vitro with low cytotoxicity, although at higher generations increased toxicity prohibits their use.

To our knowledge, no studies concerning the use of water-soluble carbosilane-based dendrimers as potential carriers for DNA or ODNs or for other biomedical applications have been published, although an in vitro biocompatibility study on poly(ethylene oxide)-grafted carbosilane dendrimers has recently been reported.[12] Only three synthetic studies of polycationic silane dendrimers have been reported previously,[13–15] and only one of them has medical implications.[15]

We recently developed a synthetic strategy for the preparation of new peripheral amine- and ammonium-terminated carbosilane dendrimers of type *n*G-[Si($OCH_2CH_2NMe_2$)$_y$]$_x$ and *n*G-[Si($OCH_2CH_2NMe_3^+I^-$)$_y$]$_x$, and a preliminary evaluation of their toxicity and complexation with ODNs was carried out, mainly on second-generation dendrimers 2G-[Si($OCH_2CH_2NMe_3^+I^-$)]$_8$ (**1**) and 2G-[Si($OCH_2CH_2NMe_3^+I^-$)$_2$]$_8$ (**2**).[16] Here we describe the synthesis of new water-soluble carbosilane dendrimers up to the third generation and analyze their biocompatibility in primary cell cultures of peripheral blood mononuclear cells (PBMCs) and erythrocytes. These data allow a comparative analysis of the toxicity of the different carbosilane macromolecules. In addition, we describe a study on complex formation between these dendrimers and ODNs. These studies provide essential insights into the potential of these carbosilane dendrimers as drug delivery systems, and in particular for their use in delivery of DNA (ODNs, plasmids).

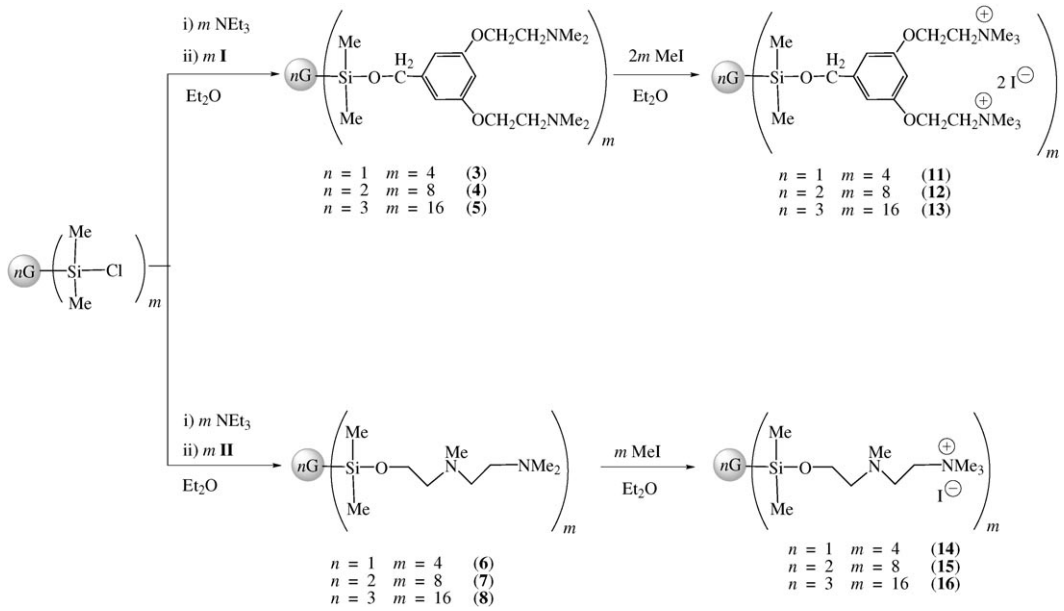
Results and Discussion

We previously prepared a family of new peripheral amine- and ammonium-terminated carbosilane dendrimers by alcoholysis of the well-known chlorosilane-terminated dendrimers[17] *n*G-($SiCl_y$)$_x$, with *N*,*N*-dimethylethanolamine and subsequent quaternization with MeI to afford dendrimers of type *n*G-[Si($OCH_2CH_2NMe_2$)$_y$]$_x$ and *n*G-[Si($OCH_2CH_2NMe_3^+I^-$)$_y$]$_x$, respectively.[16] However, all these systems are sensitive to hydrolysis by slow cleavage of the Si–O bonds. To study such behavior and to get better insight into the potential biomedical applications of the carbosilane systems, new families of dendrimers were prepared.

Amine-terminated carbosilane dendrimers: We studied the synthesis of new dendrimers with amino groups at their periphery. For this purpose two general strategies were developed: 1) an extension of the alcoholysis of dendritic Si–Cl bonds by using modified terminal amine fragments, and 2) hydrosilylation of allyl amines with dendritic Si–H bonds.

Dendrimers formed by alcoholysis of dendritic Si–Cl bonds: Chlorosilane-terminated dendrimers of type *n*G-($SiCl$)$_x$ (*n* = 1, 2 and 3; *x* = 4, 8 and 16) were synthesized as reported previously[17] and formed the starting materials for the preparation of new dendrimers by alcoholysis. Two different amino alcohols were used: 3,5-$(NMe_2CH_2CH_2O)_2C_6H_3CH_2OH$ (**I**) and $Me_2NCH_2CH_2N(Me)CH_2CH_2OH$ (**II**). The latter was obtained from commercial sources, and its selection was motivated by presence of a second amino group that, if it is not quaternized, may trap endosomal protons and reducing nucleases. The former was elected to restrict the hydrolysis process and was synthesized previously (see Experimental Section).

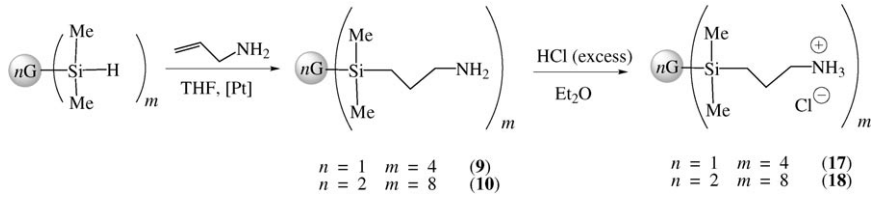
Chlorosilane-terminated dendrimers were treated with stoichiometric amounts of amino alcohol **I** or **II** in diethyl ether in the presence of an excess of NEt_3 to afford the corresponding amine-terminated dendrimers *n*G-[Si{OCH_2-(C_6H_3)-3,5-($OCH_2CH_2NMe_2$)$_2$}]$_x$ (*n* = 1, *x* = 4 (**3**); *n* = 2, *x* = 8 (**4**); *n* = 3, *x* = 16 (**5**)) and *n*G-[Si{$O(CH_2)_2N(Me)$-$(CH_2)_2NMe_2$}]$_x$ (*n* = 1, *x* = 4 (**6**); *n* = 2, *x* = 8 (**7**); *n* = 3, *x* = 16 (**8**)) in high yields as colorless or yellow oils (Scheme 1). All



Scheme 1.

these dendrimers are soluble in all common organic solvents but insoluble in water.

Dendrimers formed by hydrosilylation with dendritic Si–H bonds: SiH-terminated carbosilane dendrimers of type *n*G-$(SiH)_x$ ($n=1$ and 2; $x=4$ and 8) were synthesized as reported previously[17d,e] and used in the hydrosilylation of allylamine. The reactions were performed in ampoules with J Young valves using THF as solvent and the Speier catalyst[18] and heating the mixture at 120 °C for 4 h to afford the corresponding dendrimers *n*G-$[Si(CH_2)_3NH_2]_x$ ($n=1$, $x=4$ (**9**); $n=2$, $x=8$ (**10**)) in high yields as colorless oils (see Scheme 2). ^{1}H NMR spectroscopy was used to follow the



Scheme 2.

progress of the reactions by monitoring the loss of the Si–H resonance. The processes occurred exclusively by β-addition to give the $-SiCH_2CH_2CH_2NH_2$ group, and neither α-addition products nor *N*-silylated byproducts were detected in the ^{1}H NMR spectra of the crude products. All of these dendrimers are soluble in all common organic solvents but insoluble in water.

The NMR spectroscopic, MALDI-TOF MS and analytical data for compounds **3**–**10** are consistent with their proposed structures (Schemes 1 and 2).

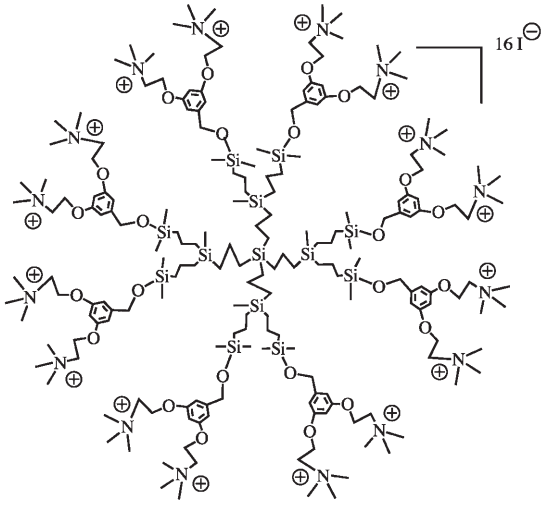
Ammonium-terminated carbosilane dendrimers: The ammonium-terminated dendrimers were prepared by adding MeI to parent dendrimers **3**–**8** in diethyl ether (see Scheme 1). Dendrimers **3**–**5** reacted with an excess of MeI to cleanly afford the corresponding quaternized derivatives *n*G-$[Si\{OCH_2(C_6H_3)$-3,5-$(OCH_2CH_2NMe_3{}^+I^-)_2\}]_x$ ($n=1$, $x=4$ (**11**); $n=2$, $x=8$ (**12**); $n=3$, $x=16$ (**13**)) as white solids. In the conversion of amine-terminated dendrimer **5** to **13**, some dimethylamino groups remained unquaternized. The ^{1}H NMR spectrum revealed that roughly 85 % of the amino groups were quaternized, even if we used a large excess of MeI and prolonged reaction times. The stoichiometric reaction of dendrimers **6**–**8** containing 4, 8 and 16 terminal $[O(CH_2)_2N(Me)(CH_2)_2NMe_2]$ units, respectively, with same number of equivalents of MeI gave the corresponding outermost quaternized terminal amine compounds *n*G-$[Si\{O(CH_2)_2N(Me)(CH_2)_2NMe_3{}^+\}I^-]_x$ ($n=1$, $x=4$ (**14**); $n=2$, $x=8$ (**15**); $n=3$, $x=16$ (**16**)), albeit with small amounts of doubly methylated branches (less than 1–3 % depending on the generation). Hence, minor chemical competition between the two types of amino groups per peripheral unit was observed, although the selectivity increased with increasing generation or peripheral hindrance of the dendrimers.

Dendrimers **9** and **10** were quaternized by adding stoichiometric amounts or an excess of HCl to produce *n*G-$[Si(CH_2)_3NH_3{}^+Cl^-]_x$ ($n=1$, $x=4$ (**17**); $n=2$, $x=8$ (**18**)) as white solids (see Scheme 2).

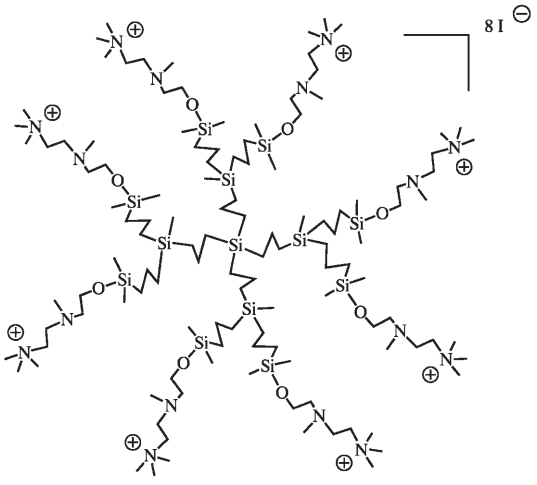
All compounds **11**–**18** are water-soluble, although solubility decreases with increasing generation. However, the den-

drimers with Si–O bonds decomposed slowly by hydrolysis of these bonds. This behavior was observed in dendrimers **1** and **2** with $-OCH_2CH_2NMe_2$ terminal units.[16] However, the hydrolysis rates of dendrimers **11**–**13** containing quaternized groups derived from amino alcohol **I** are considerably attenuated with respect to dendrimers with fragment **II** as peripheral groups, which are similar to those in **1** and **2**. In contrast, dendrimers **17** and **18** based on Si–C bonds are completely stable towards hydrolysis.

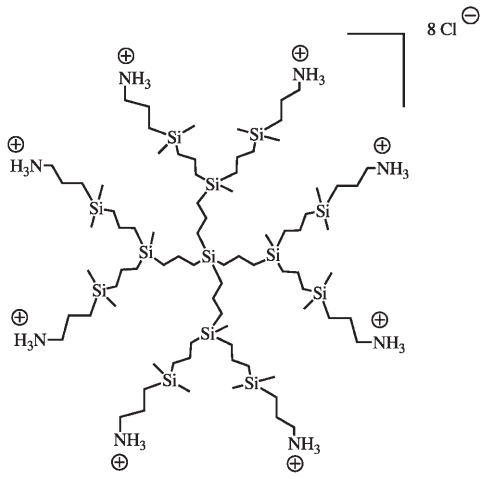
The NMR spectroscopic and analytical data of **11**–**18** are consistent with their proposed structures (Schemes 1–3). The ^{1}H NMR spectra were recorded in [D$_6$]DMSO at room temperature for dendrimers **11**–**16**, and in D_2O for dendrimers **17** and **18**. In these solvents the line widths of these spectra tended to be broader than those of derivatives soluble in common organic solvents. The ^{1}H and ^{13}C NMR spectra of the quaternized dendrimers exhibit identical resonance patterns to those observed in their neutral counterparts **3**–**10** for the carbosilane framework, although broader signals are seen with increasing generation (see Experimental Section and Supporting Information). In general for the ^{1}H NMR spectra, the quaternization of the amine groups results in deshielding of about $\Delta\delta = 1$ ppm for the geminal methylene and methyl groups directly bound to the charged



2G-[SiOCH$_2$-(C$_6$H$_3$)-3,5-(OCH$_2$CH$_2$NMe$_3^+$I$^-$)$_2$]$_8$ (**12**)



2G-[SiO(CH$_2$)$_2$N(Me)(CH$_2$)$_2$NMe$_3^+$I$^-$]$_8$ (**15**)



2G-[Si(CH$_2$)$_3$NH$_3^+$Cl$^-$]$_8$ (**18**)

Scheme 3. Molecular representations of ammonium-terminated carbosilane dendrimers **12**, **15**, and **18**.

nitrogen atoms, whereas small downfield shifts of around $\Delta\delta = 0.3$–0.4 ppm are found for the vicinal methylene groups. Beyond these positions, no displacement is observed for the chemical shift due to the positive charge on the nitrogen atoms. However, this effect is more evident in the compounds quaternized by MeI than those prepared with HCl. Analogous behavior is observed for the carbon atoms in the ^{13}C NMR spectra. Dendrimers **14**–**16** with outermost quaternized amines exhibit different ^{1}H and ^{13}C NMR patterns for singly and doubly methylated branches that facilitate their discrimination (see Experimental Section and Supporting Information). In any case, signals attributed to the monomethylated forms of the innermost quaternized amine were observed, even when a deficit of reagent was added. This strongly suggests that the quaternization process starts at the outermost amine and subsequently proceeds to the innermost when a slight excess of MeI is added. Attempts to carry out MALDI-TOF MS of these dendrimers failed probably due, inter alia, to solubility problems.

Toxicity evaluation of dendrimers: Quaternized second-generation carbosilane dendrimers **12**, **15**, and **18** were tested on a primary cell culture of peripheral blood mononuclear cells (PBMCs) from healthy donors as an initial screening for biocompatibility, and the values compared with preliminary data shown by dendrimers **1** and **2**.[16] First-generation dendrimer **14** was too water sensitive for toxicity evaluation, while third-generation dendrimers were not tested due to solubility problems.

Toxicity was evaluated by challenging PBMCs with increasing concentrations of the free quaternized carbosilane dendrimers, in order to obtain a range of biocompatibility. Toxicity was initially evaluated by 1) visual examination under a phase-contrast light microscope and 2) MTT toxicity assay.

According to microscopy studies (see Supporting Information), the carbosilane dendrimers best tolerated by PBMCs were **12** and **15**, whereas the dendrimer **18** showed the highest toxicity and dendrimers **1** and **2** had intermediate toxicity profiles. Superfect (SF) and a 4G-PAMAM showed higher toxicity than all second-generation carbosilane dendrimers. However, this is not surprising because of it is known that dendrimer toxicity increases with increasing generation.[1i,19]

In the MTT assay, cells treated with **12** and **15** showed higher mitochondrial activity (MA) (Figure 1 A). Dendrimer **18** was more toxic at 1 μM, but the toxicities of **1** and **2** strongly increase from 5 μM and beyond. However, taking into account the data from visual observation along with the MTT values as a whole, it is deduced that whilst **1** and **2** induced diminution of birefringence or formation of cell aggregates, **12** and **15** did not and are thus the most biocompatible systems for PBMCs. In addition, and in view of the results of this initial screening, one can conclude that the upper limit of the carbosilane dendrimer concentration to be further considered for biological assays should be between 1 and 5 μM.

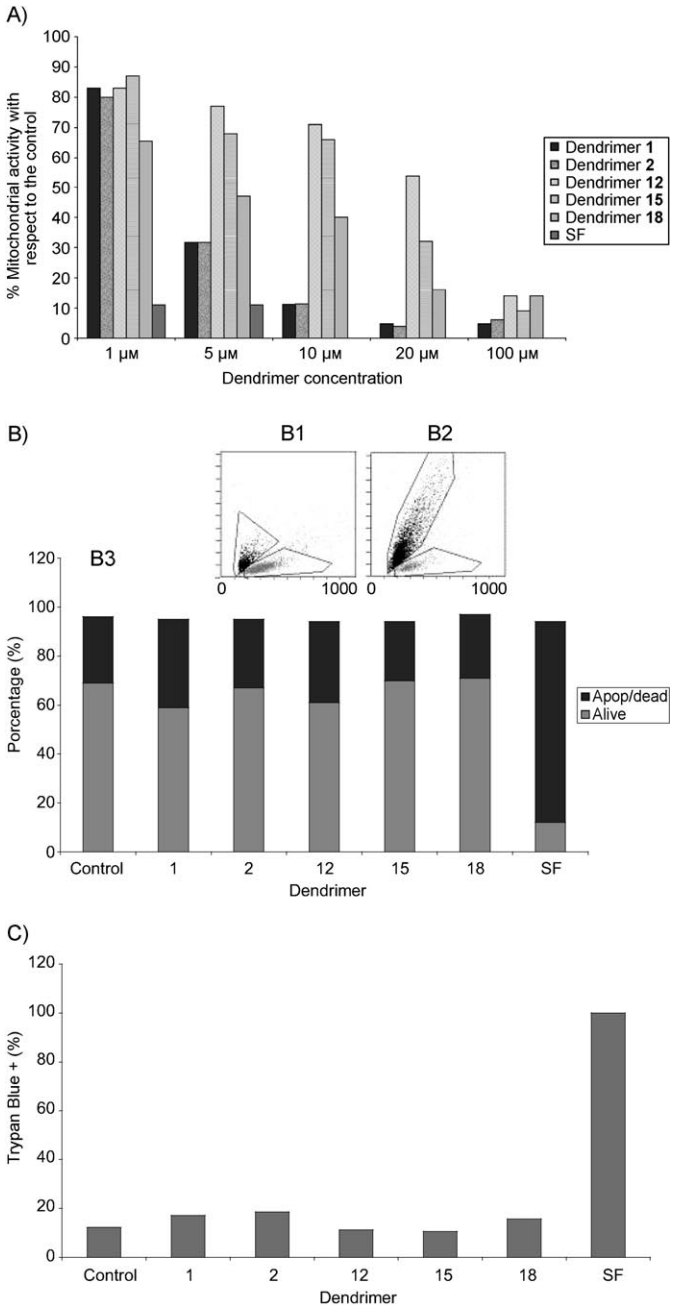


Figure 1. A) Quantification of mitochondrial activity (MA) of PBMCs by MTT test after 48 h of incubation with different dendrimer concentrations. MA is expressed as percentage with respect to the MA of the control (untreated cells). B) Flow cytometry of PBMCs after 72 h of incubation with dendrimers: B1) representation of cells treated with dendrimer **15**; B2) representation of cells treated with SF dendrimer; B3) evaluation of the percentage of cells with FW and SD corresponding to live cells against the percentage of cells corresponding to dead/apoptotic cells. Black areas denote dead cells, and grey areas live cells. C) Percentage of cells positive for Trypan Blue after 72 h of incubation with dendrimers. All experiments were assayed three times.

Because of the apparent discrepancy between MTT assays and the microscopic observations, which were also found for other polycationic dendrimers, such as those based on the phosphoramidothioate backbone,[20] toxicity of the carbosi-

lane dendrimers was studied by additional methods: flow cytometry, Trypan Blue (TB) uptake, DAPI staining and in vivo microscopy. In these studies, the incubation time was increased to 72 h, and the doses assayed for carbosilane dendrimers were in the range of 2–4 μM, while less than 1 μM was used for the reference SF PAMAM dendrimer.

Flow cytometry (FC): PBMCs treated with carbosilane dendrimers at these doses did not show significant changes in their forward (FW) and side (SD) light-scattering characteristics after 72 h of incubation. Moreover, the percentage of unviable cells was similar in all cases assayed to that of untreated cells (around 20%, see Figure 1B). On the other hand, PBMCs treated with SF dramatically increased their mortality.

Trypan Blue (TB) uptake: TB is excluded by viable cells but can penetrate cell membranes of dying or dead cells. When TB staining is negative, membrane integrity is present. The percentage of cells positive for TB staining for the wells treated with dendrimers **1**, **2**, **12**, **15**, and **18** was similar to that found in untreated cells (Figure 1C). However, cells treated with SF displayed significant mortality compared with control cells.

DAPI staining: An additional test of cell viability was provided by the staining of cell nuclei with the vital dye DAPI. Cell nuclei undergoing apoptosis or necrosis show reduced nuclear size, chromatin condensation and nuclear fragmentation, processes that can be easily detected by DAPI staining. Cells treated with all carbosilane dendrimers had similar appearances to untreated cells, showing rounded nuclei with homogeneously distributed chromatin. In contrast, cells treated with SF decreased dramatically in number. Furthermore, the cell pellet obtained for DAPI staining after centrifugation of PBMCs treated with SF was notably small. This reduction in cell number was confirmed by microscopy (see Supporting Information).

Time-lapse video microscopy: We monitored cell behavior by time-lapse and in vivo microscopy for cells treated with carbosilane dendrimers. In all cases, cells showed similar patterns of movement and migration to untreated control cells (for an example, see Supporting Information).

Therefore, from these four additional methods, all carbosilane dendrimers **1**, **2**, **12**, **15**, and **18** assayed under these conditions showed good biocompatibility on PBMC cells.

Toxicity was also evaluated on erythrocytes challenged with increased dendrimer concentrations by means of induction of hemagglutination along with hemoglobin release from the red blood cells. Dendrimers **1**, **2**, **12**, and **15** were used for this assay, and dendrimer **18** was not included because it was shown to be the most toxic for lymphocytes in some of the methods and concentrations employed before. A fourth-generation PAMAM dendrimer was also used for comparison. From visual examination, dendrimer **15** showed the lowest agglutination, while for dendrimer **12** morphologic changes were observed even at 1 μM concentration. Analogous results were detected for 4G PAMAM dendrimer (see Supporting Information).

The interaction of cationic dendrimers with negatively charged membranes was also studied by hemolysis experiments. The release of hemoglobin was used to quantify the membrane-damaging properties of the dendritic macromolecules. Lysis of red blood cells precludes direct intravenous delivery of the desired agents and often enhances their toxicity when administered by other routes. Hemolysis was recorded spectrophotometrically at 1 h and considered 100% in the positive control (cells treated with Triton X-100) and 7% in the negative control of untreated cells. The absorbances of the different wells were expressed as percentages with respect to the absorbance of the positive control well, and from this percentage, the 7% corresponding to the negative control was subtracted. A value of 10% was considered as the cut-off for toxicity.[19b] Carbosilane dendrimers **12** and **15** induced less hemoglobin release in the 1–5 μM concentration range, although at 5 μM, these two dendrimers slightly exceed 10% (see Figure 2). In spite of this good result, compound **12** induced hemagglutination, while **15** did not at this concentration (see Supporting Information). It can be said that polycation/DNA complexes are usually less cytotoxic than uncomplexed polycations.[21] Thus, the experimental conditions chosen here reflect the worst-case scenario for cytotoxicity. In conclusion, taking into account both hemagglutination and hemoglobin release from erithrocytes, as well as toxicity in lymphocytes, dendrimer **15** is the least harmful for both types of cell at higher concentrations and thus a good candidate as delivery agent.

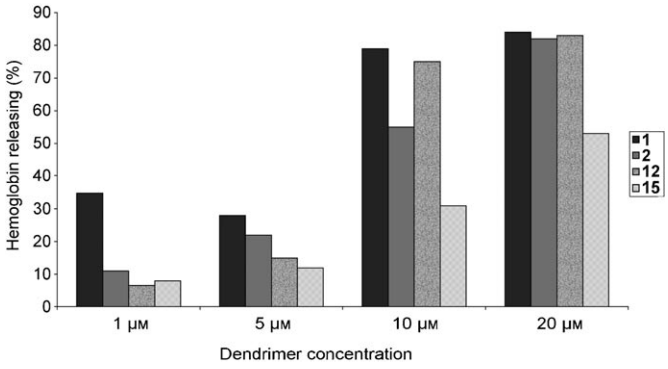


Figure 2. Hemolysis test: hemoglobin release after 1 h with different dendrimer concentrations. The experiment was assayed three times.

Antigenicity: lymphoproliferative assay: When a new macromolecule is under consideration for a potential biological application, it is important that it does not constitute an unspecific antigenic stimulus (unless desired for development of immunogens). Again we challenged PBMCs with different concentrations of carbosilane dendrimers **1**, **2**, **12**, and **15** looking for induction/noninduction of proliferation, compared with that achieved with an unspecific mithogenic stim-

uli such as phytohemagglutinin (PHA). From the data shown in Figure 3, none of the carbosilane dendrimers constituted an antigenic stimulus for PBMCs at the tested concentrations (2 and 5 μM).

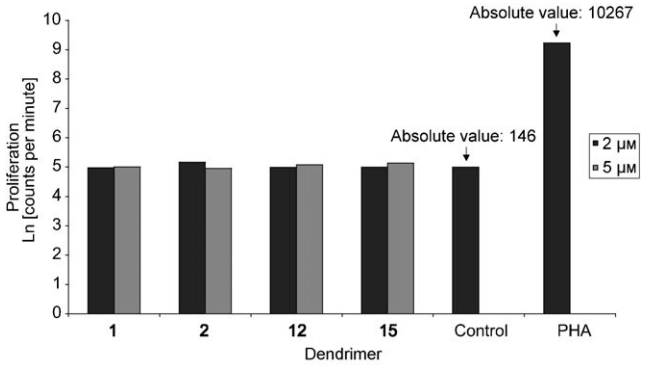


Figure 3. Lymphoproliferative assay: antigenicity of the carbosilane dendrimers in the proliferation of PBMCs exposed to two different concentrations of each dendrimer and compared with that achieved with phytohemagglutinin (PHA). Data expressed as the Napierian logarithm of the number of counts per minute. The experiment was assayed three times.

Dendriplex formation: Having evaluated the biocompatibility range for the new water-soluble ammonium carbosilane dendrimers, we studied their capacities for complexation with oligodeoxynucleotides (ODN) to give so-called dendriplexes. Quaternized second-generation dendrimers **12**, **15**, and **18** as well as elsewhere-prepared **1** and **2**,[16] where used for potential dendriplex formation. Dendriplex formation was studied by electrophoresis in agarose gels with fluoresceinated phosphorothioate ODNs. Mixtures were prepared with an excess of positive charge to provide a sufficient number of charges to interact with the ODN. Preliminary results[16] for dendrimer **1** at different electrostatic charge ratios (+)/(−) showed retardation of ODN migration in all cases, that is, the ODN is associated with the dendrimer, even at 2:1 charge ratio, for which the dendrimer concentration is in the aforementioned range of biocompatibility.[16] Consequently, the carbosilane dendrimers of this work were complexed with ODN at an electrostatic charge ratio (+)/(−) of 2/1. Figure 4 shows agarose gel electrophoresis of the mixtures of dendrimers **12**, **15**, or **18** and ODN in comparison with those formed with **1** and **2**. In the gel presented in Figure 4 A1, the dendrimers were diluted in water and immediately mixed with ODN and run in an agarose gel. In all cases the different carbosilane dendrimers retained the ODN during its migration, and this demonstrates successful complex formation. With regard to the hydrolysis problems presented by the Si−O-containing carbosilane dendrimers, we recently demonstrated that the terminal units released after hydrolytic scission were not able to retard ODN migration, and that the whole functionalized dendrimer was necessary to form complexes with DNA.[16] An analogous result was recently obtained in binding experiments on DNA with

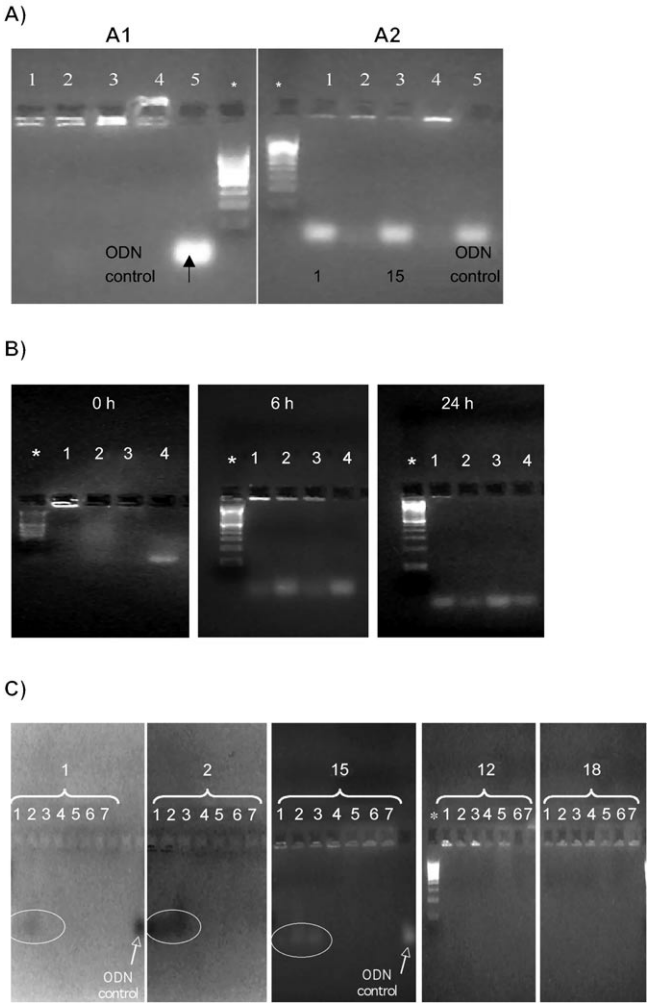


Figure 4. A) Electrophoresis of carbosilane dendrimer/ODN dendriplexes with (+)/(−)=2/1 on 3% agarose gel: 1) **1**/ODN; 2) **18**/ODN; 3) **15**/ODN; 4) **12**/ODN; 5) ODN only. A1) Dendrimers dissolved in water and immediately mixed with ODN; A2) dendrimers dissolved in water for 24 h and then mixed with ODN prior to electrophoresis. Asterisk denotes 100 bp DNA ladder as reference. B) Electrophoresis of carbosilane dendrimer/ODN dendriplexes with (+)/(−)=2/1, on 3% agarose gel at pH 7.4 at different incubation times: 1) **1**/ODN; 2) **2**/ODN; 3) **15**/ODN; 4) ODN only. Asterisk denotes 100 bp DNA ladder as reference. C) Electrophoresis of carbosilane dendrimer/ODN dendriplexes with (+)/(−)=2/1 on 3% agarose gel at different pHs: 1) pH 2.8; 2) pH 3.7; 3) pH 4.7; 4) pH 5.7; 5) pH 6.4; 6) pH 7.4; 7) pH 8.0. Asterisk denotes 100 bp DNA ladder as reference. The experiments were assayed three times.

spermine and low-molecular weight dendrons based on Newkome-type branching.[22] This finding confirms that the hydrolysis processes suffered by the carbosilane dendrimers are slow under highly dilute conditions. It is noteworthy that our results show that second-generation carbosilane dendrimers are capable of complexing ODNs to give [ODN/dendrimer] through electrostatic interactions, whereas higher generations of other dendrimer topologies have been used in the literature.[7,20,21] For the gel visualized in Figure 4 A2, the dendrimers were dissolved in water for 24 h and then mixed with ODN before electrophoresis. After this time, dendrimers **1** and **15** were not able to retain the ODN

during its migration, that is, the time dissolved in water affects the ability of the dendrimer to form complexes with the ODN. On the other hand, **12** and **18** were not affected to the same degree and preserved the capacity to retain the majority of the ODN during its migration. Thus, under these conditions dendrimers containing Si–O bonds lost their ability to bind ODNs after 24 h in water in dilute concentrations, except for dendrimer **12**. Likewise, dendrimer **18** is also water-stable because of the presence of robust Si–C bonds.

For water-sensitive dendrimers **1**, **2**, and **15**, a second type of electrophoretic migration assay was developed consisting of running dendriplexes in a gel after 0, 6, and 24 h of incubation in an atmosphere of 5% of CO_2 at 37 °C. As can see from Figure 4B, the three dendrimers released the ODNs progressively. It is possible to conclude that **1**, **2**, and **15** have the ability to release the ODN in a time-dependant way when they are dissolved in water. This feature suggests potential use in controlled release of ODNs and perhaps of other polyanionic drugs. The controlled release of active substances based on the chemical stability of the linker towards hydrolysis has been described, for example, in the case of phosphorus dendrimers on the basis of slow degradation of imine bonds.[23]

Dendriplex stability was also tested at different pH values. Blood physiological pH is 7.4, but anatomical or cellular locations with more acidic (stomach, endosome/lysosome) or basic (duodeni) pH exist. Dendriplexes were formed as usual and exposed to different solutions from acid to basic pH (2.8, 3.7, 4.7, 5.7, 6.4, 7.4 and 8.0) prior electrophoresis (see Figure 4C). Dendriplexes formed between **12** or **18** and ODN were stable at all tested pH values, whereas dendriplexes formed between **15** and ODN released the ODN at acid pH (<5.7). For dendrimers **1** and **2** the results were similar: dendriplexes **1**/ODN dissociated at pH <4.7, and dendriplexes **2**/ODN at pH <5.7. All dendriplexes were stable at basic pH (up to pH 8.0). Thus, in an acidic environment dendriplexes **1**/ODN, **2**/ODN and **15**/ODN would release the ODN, whereas under basic conditions they would remain stable. This opens new perspectives for applications that need pH-controlled ODN release.

Toxicity profiles of dendriplexes formed by carbosilane dendrimers **1**, **2**, **12**, **15**, and **18** were studied by some of the methods used for dendrimers alone: flow cytometry and Trypan Blue (TB) uptake (see Supporting Information). The data showed very similar values to those obtained for dendrimers without complexation to ODN, as has been reported elsewhere.[19]

In addition, the ammonium-terminated second-generation carbosilane dendrimers were able to complex with plasmids. As a demonstrative example, dendrimer **1** formed dendriplexes with the NfκB plasmid (which codifies Nf-kappaB protein involved in regulation of immune or inflammation responses). This was again true even at 2:1 charge ratio (see Figure 5). This plasmid has an approximate length of 5000 base pairs, determined on the basis of its comparative migration with the DNA ladder as reference. This result shows

Figure 5. Electrophoresis of dendrimer **1**/NfκB plasmid dendriplexes on 3% agarose gel: 1) (+)/(−)=2/1; 2) (+)/(−)=6/1; 3) (+)/(−)=10/1; 4) (+)/(−)=100/1; 5) and 6) plasmid only. Asterisk denotes 5000 bp DNA ladder as reference. The experiment was assayed three times.

that, regardless of the low generation, the carbosilane dendrimers have the capacity to bind large DNA molecules.

Finally, a preliminary study of the capacity of the dendriplex formed by **15** and the fluoresceinated ODN to penetrate into PBMCs was performed by confocal microscopy. Figure 6 shows the internalization and intracellular distribution of the nucleic material, with which the dendrimer does not seem to interfere. Research is in progress to elucidate the transfection process.

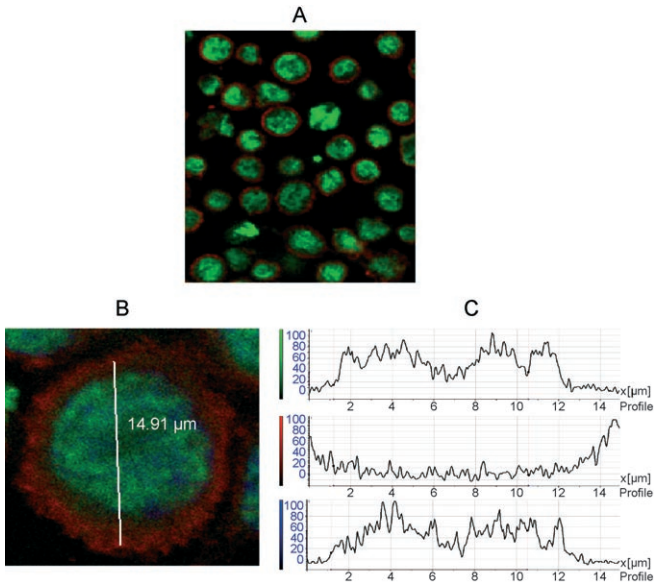


Figure 6. A) Confocal micrograph of internalization of **15**/ODN dendriplex after 48 h. B) Image of an isolated cell; white line denotes a section through the median plane *XY*. C) Plots of fluorescence emission through the section: green (fluoresceinated ODN), blue (cell nucleus) and red (cell membrane).

Conclusion

New families of amine- and ammonium-terminated carbosilane dendrimers have been synthesized by two strategies and fully characterized. The first entails alcoholysis of Si–Cl bonds by amino alcohols and subsequent quaternization with MeI. The second involves hydrosilylation of allylamine with Si–H bonds of dendrimers and subsequent quaternization with HCl. Quaternized carbosilane dendrimers are soluble in water, although degradation is apparent by hydrolysis of Si–O bonds. However, hydrolysis can be prevented or reduced, under very dilute conditions, by introduction of a rigid phenyl group as linker between the amino or ammonium groups and the Si–O bonds. Similarly, dendrimers containing Si–C bounds proved to be water-stable. For toxicity evaluation, distinct (but complementary) approaches were employed to evaluate membrane integrity, metabolic activity, apoptosis, morphology and cell movement. This approach allowed us to obtain a global picture of the potential toxic effects. Novel quaternized dendrimers of the second generation show good toxicity profiles in cell cultures over extended periods. Taking together the toxicity data for PBMCs and toxicity results for erythrocytes, the most biocompatible compound proved to be second-generation dendrimer **15**. Remarkably, in spite of their low generation, the carbosilane dendrimers are able to form complexes with DNA ODNs or even with plasmids by electrostatic interaction at biocompatible doses. In addition, the presence of Si–O bonds in the dendrimer architecture opens the way to use these dendritic macromolecules as drug delivery systems via an electrostatic approach with subsequent release by means of the hydrolytic process.

These results demonstrate that the new ammonium-terminated carbosilane dendrimers are a good base molecule to be considered for biomedical applications, such as drug carriers, controlled drug liberation (pH- or time-dependant, as in the case of **1**, **2**, and **15**), as antigen carriers or as vehicles for nucleic acids. Dendrimers **12** and **18** demonstrated very stable ODN binding, and hence they could be used to develop DNA-based devices such as microarrays.

The results of our biocompatibility and preliminary internalization assays highlight the possibility of developing transfection and immunomodulation experiments in PBMCs, and of studying the ability to interfere with the replication of pathogens such as viruses, bacteria or prions. These assays are currently being developed in our laboratories and will be the subject of future papers.

Experimental Section

General remarks: All manipulations of oxygen- or water-sensitive compounds were carried out under an atmosphere of argon using standard Schlenk techniques or an argon-filled glove box. Solvents were dried and freshly distilled under argon prior to use: hexane from sodium/potassium, toluene from sodium, tetrahydrofuran and diethyl ether from sodium benzophenone ketyl and dichloromethane over P_4O_{10}. Unless otherwise stated, reagents were obtained from commercial sources and used as received. *n*G-$(SiCl_x)_y$ and *n*G-$(SiH)_x$ dendrimers were prepared according to reported methods.[17] Superfect (SF) is an activated PAMAM dendrimer with 140 terminal primary amino groups and $M = 35000$ (Qiagen, Crawley, UK). G4 PAMAM dendrimer has $M = 14215$ and 64 surface primary amino groups (Aldrich Chemical Co., Milwaukee, WI).

^{1}H, ^{13}C, and ^{29}Si NMR spectra were recorded on Varian Unity VXR-300 and Varian 500 Plus Instruments. Chemical shifts (δ, ppm) were measured relative to residual ^{1}H and ^{13}C resonances for $CDCl_3$, [D_6]DMSO and D_2O used as solvents, and ^{29}Si chemical shifts were referenced to external $SiMe_4$ (0.00 ppm). C, H and N analyses were carried out with a Perkin-Elmer 240 C microanalyzer. ESI and APCI samples were prepared in acetonitrile or methanol, and spectra were recorded on a Termoquest Finnigan Automass Multi Mass Spectrometer. MALDI-TOF MS samples were prepared in a 1,8,9-trihydroxyanthracene (dithranol) matrix, and spectra were recorded on a Bruker Reflex II spectrometer equipped with a nitrogen laser emitting at 337 nm and operated in the reflection mode at an accelerating voltage in the range 23–25 kV.

Synthesis of [3,5-$(NMe_2CH_2CH_2O)_2$]$(C_6H_3)CH_2OH$ (I): 2-Chloro-*N*,*N*-dimethylethylamine hydrochloride (2.98 g, 20.78 mmol), K_2CO_3 (6.43 g, 46.75 mmol), KI (1.72 g, 10.39 mmol), and [18]crown-6 (0.54 g, 2 mmol) were added to an acetone solution (100 mL) of 3,5-dihydroxybenzyl alcohol (1.47 g, 10.39 mmol). The reaction mixture was refluxed for 48 h and, after removal of the solvent, the residue was extracted with CH_2Cl_2/H_2O (2×50 mL). The organic layer was dried with $MgSO_4$, the resulting solution evaporated under reduced pressure, and the residue washed with hexane (2×10 mL) to give **I** as a pale yellow oil (1.53 g, 50%). ^{1}H NMR ($CDCl_3$): $\delta = 6.48$ (m, 2H; C_6H_3), 6.36 (m, 1H; C_6H_3), 4.57 (s, 2H; CH_2OH), 3.99 (t, 4H; $CH_2OC_6H_3$), 2.65 (t, 4H; CH_2N), 2.60 (s, 1H; CH_2OH), 2.28 ppm (s, 12H; NMe_2); ^{13}C NMR ($CDCl_3$): $\delta = 159.9$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.8 (C_6H_3, C_{ipso} bonded to CH_2OSi), 105.1, 100.6 (C_6H_3), 65.8 ($CH_2OC_6H_3$), 64.9 (CH_2OH), 58.2 (CH_2N), 45.8 ppm (NMe_2); elemental analysis calcd (%) for $C_{15}H_{26}N_2O_3$: C 63.80, H 9.28, N 9.92; found: C 63.50, H 9.17, N 9.83.

Synthesis of 1G-[Si{$OCH_2(C_6H_3)$-3,5-$(OCH_2CH_2NMe_2)_2$}]$_4$ (3): A slight excess of NEt_3 (0.12 mL, 0.87 mmol) and 3,5-$(OCH_2CH_2NMe_2)_2$-$(C_6H_3)CH_2OH$ (0.22 g, 0.76 mmol) were added to a diethyl ether solution (50 mL) of first-generation chloro-terminated dendrimer 1G-Cl_4 (0.11 g, 0.19 mmol). The reaction mixture was stirred for 12 h at room temperature and then evaporated to dryness to remove residual NEt_3. The residue was extracted with Et_2O (50 mL) and filtered through Celite to remove the ammonium salt NEt_3·HCl. The resulting solution was evaporated under reduced pressure to give **3** as pale yellow oil (0.23 g, 80%). ^{1}H NMR ($CDCl_3$): $\delta = 6.45$ (m, 8H; C_6H_3), 6.36 (m, 4H; C_6H_3), 4.56 (s, 8H; CH_2OSi), 3.99 (t, 16H; $CH_2OC_6H_3$), 2.66 (t, 16H; CH_2N), 2.28 (s, 48H; NMe_2), 1.33 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.68 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.55 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.08 ppm (s, 24H; $SiMe_2$); ^{13}C NMR ($CDCl_3$): $\delta = 159.8$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.2 (C_6H_3, C_{ipso} bonded to CH_2OSi), 104.9, 100.2 (C_6H_3), 65.9 ($CH_2OC_6H_3$), 64.6 (CH_2OSi), 58.2 (CH_2N), 45.8 (NMe_2), 21.2 ($SiCH_2CH_2CH_2SiO$), 17.9, 17.2 ($SiCH_2CH_2CH_2SiO$), −1.89 ($SiMe_2$); ^{29}Si NMR ($CDCl_3$): $\delta = 0.49$ (G_0-Si), 18.7 ppm (G_1-Si); elemental analysis calcd (%) for $C_{80}H_{148}N_8O_{12}Si_5$: C 61.81, H 9.60, N 7.21; found C 62.10, H 9.82, N 7.30.

Synthesis of 2G-[Si{$OCH_2(C_6H_3)$-3,5-$(OCH_2CH_2NMe_2)_2$}]$_8$ (4): This dendrimer was prepared using a similar method to that described for **3**, starting from 2G-Cl_8 (0.27 g, 0.19 mmol), 3,5-$(OCH_2CH_2NMe_2)_2C_6H_3CH_2OH$ (0.43 g, 1.52 mmol) and NEt_3 (0.22 mL, 1.62 mmol) to obtain compound **4** as a pale yellow oil (0.54 g, 82%). ^{1}H NMR ($CDCl_3$): $\delta = 6.45$ (m, 16H; C_6H_3), 6.36 (m, 8H; C_6H_3), 4.56 (s, 16H; CH_2OSi), 3.99 (t, 32H; $CH_2OC_6H_3$), 2.67 (t, 32H; CH_2N), 2.29 (s, 96H; NMe_2), 1.33 (m, 24H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.69 (m, 16H; $SiCH_2CH_2CH_2SiO$), 0.55 (m, 32H; rest of CH_2 bonded to Si), 0.09 (s, 48H; $OSiMe_2$), −0.09 ppm (s, 12H; SiMe); ^{13}C NMR ($CDCl_3$): $\delta = 159.0$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.3 (C_6H_3, C_{ipso} bonded to CH_2OSi), 104.8, 100.1 (C_6H_3), 65.9 ($CH_2OC_6H_3$), 64.6 (CH_2OSi), 58.3 (CH_2N), 45.9 (NMe_2), 21.2 ($SiCH_2CH_2CH_2SiO$), 17.9, 17.2 and overlapping signals ($SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), −1.7 ($OSiMe_2$), −4.9 ppm (SiMe); ^{29}Si NMR ($CDCl_3$): $\delta = 0.93$ (G_1-Si),

18.7 ppm (G_2-Si), G_0-Si was not observed; elemental analysis calcd (%) for $C_{176}H_{332}N_{16}O_{24}Si_{13}$: C 61.78, H 9.78, N 6.55; found: C 62.51, H 9.90, N 6.75; MALDI-TOF-MS: m/z 3422.1 $[M+H]^+$ (calcd 3422.2).

Synthesis of 3G-[Si{OCH_2-(C_6H_3)-3,5-($OCH_2CH_2NMe_2$)$_2$}]$_{16}$ (5): This dendrimer was prepared using a similar method to that described for **3**, starting from 3G-Cl_{16} (0.07 g, 0.02 mmol), 3,5-($OCH_2CH_2NMe_2$)$_2$-(C_6H_3)CH_2OH (0.10 g, 0.35 mmol) and NEt_3 (0.06 mL, 0.43 mmol) to obtain compound **5** as a pale yellow oil (0.11 g, 69%). ^{1}H NMR ($CDCl_3$): $\delta=6.46$ (m, 32H; C_6H_3), 6.35 (m, 16H; C_6H_3), 4.56 (s, 32H; CH_2OSi), 3.97 (t, 64H; $CH_2OC_6H_3$), 2.67 (t, 64H; CH_2N), 2.28 (s, 192H; NMe_2), 1.33 (m, 56H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.69 (m, 32H; $SiCH_2CH_2CH_2SiO$), 0.55 (m, 80H; rest of CH_2 bonded to Si), 0.09 (s, 96H; $OSiMe_2$), −0.09 ppm (s, 36H; SiMe); ^{13}C NMR ($CDCl_3$): $\delta=160.0$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.2 (C_6H_3, C_{ipso} bonded to CH_2OSi), 104.3, 100.8 (C_6H_3), 65.9 ($CH_2OC_6H_3$), 64.6 (CH_2OSi), 58.3 (CH_2NMe_2), 45.8 (NMe_2), 21.1 ($SiCH_2CH_2CH_2SiO$), 17.8, 17.2 and overlapping signals ($SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), −1.8 ($OSiMe_2$), −4.9 ppm (SiMe); ^{29}Si NMR ($CDCl_3$): $\delta=0.94$ (G_1-Si and G_2-Si), 18.6 ppm (G_3-Si), G_0-Si was not observed; elemental analysis calcd (%) for $C_{368}H_{700}N_{32}O_{48}Si_{29}$: C 61.76, H 9.86, N 6.26; found: C 62.43, H 9.90, N 6.80.

Synthesis of 1G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]$_4$ (6): This dendrimer was prepared using a similar method to that described for **3**, starting from 1G-Cl_4 (0.31 g, 0.54 mmol), 2-{[2-(dimethylamino)ethyl]methylamino}ethanol (0.35 mL, 2.16 mmol), and NEt_3 (0.5 mL, 3.58 mmol) to obtain compound **6** as a colorless oil (0.3 g, 57%). ^{1}H NMR ($CDCl_3$): $\delta=$ 3.64 (t, 8H; CH_2O), 2.51 (m, 16H; $CH_2N(Me)CH_2$), 2.35 (t, 8H; CH_2NMe_2), 2.26 (s, 12H; NMe), 2.19 (s, 24H; NMe_2), 1.29 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.62 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.59 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.05 ppm (s, 24H; $SiMe_2$); ^{13}C NMR ($CDCl_3$): $\delta=$ 60.8 (CH_2O), 59.9, 56.2 ($CH_2N(Me)CH_2$), 57.5 (CH_2NMe_2), 45.9 (NMe_2), 43.3 (NMe), 21.2 ($SiCH_2CH_2CH_2SiO$), 17.8 ($SiCH_2CH_2CH_2SiO$), 17.2 ($SiCH_2CH_2CH_2SiO$), −2.0 ppm ($OSiMe_2$); ^{29}Si NMR ($CDCl_3$): $\delta=0.49$ (G_0-Si), 17.59 ppm (G_1-Si); elemental analysis calcd (%) for $C_{48}H_{116}N_8O_4Si_5$: C 57.09, H 11.58, N 11.10; found: C 57.60, H 11.72, N 11.20.

Synthesis of 2G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]$_8$ (7): This dendrimer was prepared using a similar method to that described for **3**, starting from 2G-Cl_8 (1.12 g, 0.77 mmol), 2-{[2-(dimethylamino)ethyl]methylamino}ethanol (1 mL, 6.16 mmol), and NEt_3 (1 mL, 7.17 mmol) to obtain compound **7** as a pale yellow oil (1.3 g, 72%). ^{1}H NMR ($CDCl_3$): $\delta=3.65$ (t, 16H; CH_2O), 2.51 (m, 32H; $CH_2N(Me)CH_2$), 2.30 (t, 16H; CH_2NMe_2), 2.26 (s, 24H; NMe), 2.20 (s, 48H; NMe_2), 1.31 (m, 24H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.63 (m, 16H; $SiCH_2CH_2CH_2SiO$), 0.59 (m, 32H; $SiCH_2$), 0.06 (s, 48H; $SiMe_2$), −0.10 ppm (s, 12H; SiMe); ^{13}C NMR ($CDCl_3$): $\delta=60.8$ (CH_2O), 59.9, 56.2 ($CH_2N(Me)CH_2$), 57.5 (CH_2NMe_2), 45.9 (NMe_2), 43.3 (NMe), 21.2 (CH_2SiO), 18.7–17.9 ($SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), −1.79 ($OSiMe_2$), −4.8 ppm (SiMe); ^{29}Si NMR ($CDCl_3$): $\delta=0.38$ (G_0-Si), 0.93 (G_1-Si), 17.58 ppm (G_2-Si); elemental analysis calcd (%) for $C_{112}H_{226}N_{16}O_8Si_{13}$: C 57.67, H 11.58, N 9.61; found: C 57.20, H 11.40, N 9.52; MALDI-TOF-MS: m/z 2332.8 $[M+H]^+$ (calcd 2332.8).

Synthesis of 3G-[Si{$O(CH_2)_2N(Me)(CH_2)_2NMe_2$}]$_{16}$ (8): This dendrimer was prepared using a similar method to that described for **3**, starting from 3G-Cl_{16} (0.49 g, 0.15 mmol), 2-{[2-(dimethylamino)ethyl]methylamino}ethanol (0.39 mL, 2.43 mmol) and NEt_3 (0.40 mL, 2.86 mmol) to obtain compound **8** as a pale yellow oil (0.51 g, 67%). ^{1}H NMR ($CDCl_3$): $\delta=3.65$ (t, 32H; CH_2O), 2.51 (m, 64H; $CH_2N(Me)CH_2$), 2.36 (t, 32H; CH_2NMe_2), 2.26 (s, 48H; NMe), 2.21 (s, 96H; NMe_2), 1.30 (m, 56H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.63 (m, 32H; $SiCH_2CH_2CH_2SiO$), 0.53 (m, 80H; rest of $SiCH_2$), 0.06 (s, 96H; $SiMe_2$), −0.10 ppm (s, 36H; SiMe); ^{13}C NMR ($CDCl_3$): $\delta=60.8$ (CH_2O), 60.0, 56.2 ($CH_2N(Me)CH_2$), 57.4 (CH_2NMe_2), 45.9 (NMe_2), 43.3 (NMe), 21.1 (CH_2SiO), 18.7–17.8 ($SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), −1.9 ($OSiMe_2$), −4.8 ppm (SiMe); ^{29}Si NMR ($CDCl_3$): $\delta=0.93$ (G_1-Si and G_2-Si), 17.58 ppm (G_3-Si), G_0-Si not observed; elemental analysis calcd (%) for $C_{240}H_{572}N_{32}O_{16}Si_{29}$: C 57.91, H 11.58, N 9.00; found: C 57.32, H 11.38, N 8.72.

Synthesis of 1G-[Si{$(CH_2)_3NH_2$}]$_4$ (9): Allylamine (1.5 mL, 19.99 mmol) and two drops of Speier's catalyst were added to a solution of first-generation hydrogen-terminated dendrimer 1G-H_4 (0.54 g, 1.23 mmol) in the minimum amount of THF (1 mL). The reaction mixture was heated at 120°C for 4 h and then evaporated to dryness to remove excess allylamine. The residue was dissolved in CH_2Cl_2 (10 mL) and filtered through Celite and active carbon to remove Pt. The resulting solution was evaporated under reduced pressure to give **9** as a colorless oil (0.23 g, 80%). ^{1}H NMR ($CDCl_3$): $\delta=2.63$ (t, 8H; CH_2N), 1.34 (m, 16H; $SiCH_2CH_2CH_2N$ and $SiCH_2CH_2CH_2Si$), 1.13 (s, 8H; NH_2), 0.54–0.44 (m, 24H; CH_2Si), −0.06 ppm (s, 24H; $SiMe_2$); ^{13}C NMR ($CDCl_3$): $\delta=45.7$ (CH_2N), 28.3 ($SiCH_2CH_2CH_2N$), 20.2, 18.6, 17.6, ($SiCH_2CH_2CH_2Si$) ,12.4 ($SiCH_2CH_2CH_2N$), −3.3 ppm ($SiMe_2$); ^{29}Si NMR ($CDCl_3$): $\delta=0.50$ (G_0-Si), 1.96 ppm (G_1-Si); elemental analysis calcd (%) for $C_{32}H_{80}N_4Si_5$: C 58.14, H 12.21, N 8.48; found: C 57.63, H 12.27, N 8.78; ESI MS: m/z 660.52 $[M+H]^+$ (calcd 661.52).

Synthesis of 2G-[Si{$(CH_2)_3NH_2$}]$_8$ (10): This dendrimer was prepared using a similar method to that described for **9**, starting from 2G-H_8 (0.42 g, 0.36 mmol), allylamine (2 mL, 26.7 mmol) and two drops of Speier's catalyst to obtain compound **10** as a colorless oil (0.32 g, 55%). ^{1}H NMR ($CDCl_3$): $\delta=2.63$ (t, 16H; CH_2N), 1.41–1.28 (m, 40H; $SiCH_2CH_2CH_2Si$ and $SiCH_2CH_2CH_2N$), 0.54–0.44 (m, 64H; CH_2Si), −0.06 (s, 48H; $SiMe_2$), −0.10 ppm (s, 12H; SiMe); ^{13}C NMR ($CDCl_3$): $\delta=45.7$ (CH_2N), 28.4 ($SiCH_2CH_2CH_2N$), 20.2, 18.6, 17.6 ($SiCH_2CH_2CH_2Si$), 12.5 ($SiCH_2CH_2CH_2N$), −3.1 ($SiMe_2$), −4.8 ppm (SiMe); ^{29}Si NMR ($CDCl_3$): $\delta=0.92$ (G_1-Si), 2.00 ppm (G_2-Si). The NMR data were assigned by analogy with the previously prepared model compound $Et_3SiCH_2CH_2CH_2NH_2$;[24] elemental analysis calcd (%) for $C_{80}H_{196}N_8Si_{13}$: C 58.75, H 12.08, N 6.85; found: C 58.16, H 11.95, N 6.58; ESI MS: $z=1$ not observed, $z=2$: m/z 818.44 $[M/2+H]^+$ (calcd 818.8). MALDI-TOF-MS: m/z 1636.3 $[M+H]^+$ (calcd 1636.3).

Synthesis of 1G-[Si{$OCH_2(C_6H_3)$-3,5-($OCH_2CH_2NMe_3^+I^-$)$_2$}]$_4$ (11): A solution of MeI in Et_2O (2M, 0.35 mL, 0.70 mmol) was added to a diethyl ether (10 mL) solution of **3** (0.10 g, 0.06 mmol). The resulting solution was stirred for 48 h at room temperature and then evaporated under reduced pressure to remove residual MeI. The residue was washed with Et_2O (2×5 mL) and dried under vacuum to give **11** as a white solid (0.14 g, 90%). ^{1}H NMR ([D$_6$]DMSO): $\delta=6.55$ (m, 12H; C_6H_3), 4.58 (s, 8H; CH_2OSi), 4.42 (t, 16H; $CH_2OC_6H_3$), 3.77 (t, 16H; CH_2N), 3.18 (s, 72H; NMe_3^+), 1.35 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.70 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.57 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.08 ppm (s, 24H; $SiMe_2$); ^{13}C NMR ([D$_6$]DMSO): $\delta=157.8$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.2 (C_6H_3, C_{ipso} bonded to CH_2OSi), 104.9, 99.6 (C_6H_3), 63.5 (CH_2NMe_2), 63.0 (CH_2OSi), 61.3 ($CH_2OC_6H_3$), 52.7 (NMe_3^+), 19.9 ($SiCH_2CH_2CH_2SiO$), 16.9, 16.1 ($SiCH_2CH_2CH_2SiO$), −2.4 ppm ($SiMe_2$); elemental analysis calcd (%) for $C_{88}H_{172}I_8N_8O_{12}Si_5$: C 39.29, H 6.44, N 4.17; found: C 38.90, H 6.24, N 4.09.

Synthesis of 2G-[Si{OCH_2-(C_6H_3)-3,5-($OCH_2CH_2NMe_3^+I^-$)$_2$}]$_8$ (12): This dendrimer was prepared by a similar method to that described for **11**, starting from **4** (0.08 g, 0.023 mmol) and a solution of MeI in Et_2O(2M, 0.30 mL, 0.6 mmol). Compound **12** was isolated as a white solid (0.11 g, 85%). ^{1}H NMR ([D$_6$]DMSO): $\delta=6.58$ (m, 24H; C_6H_3), 4.58 (s, 24H; CH_2OSi), 4.44 (t, 32H; $CH_2OC_6H_3$), 3.79 (t, 32H; CH_2N), 3.20 (s, 144H; NMe_3^+), 1.33 (m, 24H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), 0.67 (m, 24H; $SiCH_2CH_2CH_2SiO$), 0.54 (m, 32H; rest of CH_2 bonded to Si), 0.07 (s, 48H; $OSiMe_2$), −0.07 ppm (s, 12H; SiMe); ^{13}C NMR ([D$_6$]DMSO): $\delta=157.9$ (C_6H_3, C_{ipso} bonded to $OCH_2CH_2NMe_2$), 143.2 (C_6H_3, C_{ipso} bonded to CH_2OSi), 105.2, 99.6 (C_6H_3), 63.5 (CH_2NMe_2), 63.0 (CH_2OSi), 61.4 ($CH_2OC_6H_3$); 52.7 (NMe_3^+), 19.8 ($SiCH_2CH_2CH_2SiO$), 17.5, 16.8 and overlapping signals ($SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$ overlapping), −2.4 ($OSiMe_2$), −5.5 ppm (SiMe); elemental analysis calcd (%) for $C_{192}H_{380}I_{16}N_{16}O_{24}Si_{13}$: C 40.51, H 6.73, N 3.94; found: C 41.20, H 7.02, N 4.10.

Reaction of 3G-[Si{OCH_2-(C_6H_3)-3,5-($OCH_2CH_2NMe_2$)$_2$}]$_{16}$ (5) with MeI: The reaction of **5** with MeI by a similar method to that described for **11** afforded the dendrimer 3G-[Si{$OCH_2(C_6H_3)$-3,5-($OCH_2CH_2NMe_3^+I^-$)$_2$}]$_{16}$ (**13**). However, the NMR analysis revealed that not all the amino groups were quaternized: roughly 85% of the groups were methylated

based on integration of the corresponding signal of the outer $-OCH_2CH_2N-$ branch of the amino or ammonium groups. The NMR data of the quaternized branches are analogous to those given for dendrimer **12**.

Synthesis of 1G-[Si{O(CH$_2$)$_2$N(Me)(CH$_2$)$_2$NMe$_3^+$I$^-$}]$_4$ (14): This dendrimer was prepared using a similar method to that described for **11**, starting from **6** (0.043 g, 0.047 mmol) and 0.095 mL of a 2 M solution in Et_2O of MeI (0.19 mmol). Compound **14** was isolated as a white solid (0.49 g, 95%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.61$ (t, 8H; OCH_2), 3.47 (t, 8H; $CH_2NMe_3^+$), 3.11 (s, 36H; NMe_3^+), 2.76 (t, 8H; $N(Me)CH_2CH_2NMe_3^+I^-$), 2.49 (t, 8H; OCH_2CH_2N), 2.22 (s, 12H; NMe), 1.30 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.61 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.53 (m, 8H; $SiCH_2CH_2CH_2SiO$), 0.04 ppm (s, 24H; $OSiMe_2$); ^{13}C NMR ([D$_6$]DMSO): $\delta = 61.1$, 59.5, 58.3, 50.9 (methylene groups of $OCH_2CH_2N(Me)CH_2CH_2NMe_3^+$), 52.2 ($NMe_3^+$), 41.3 (NMe), 20.0 ($SiCH_2CH_2CH_2SiO$), 16.9, 16.1 ($SiCH_2CH_2CH_2SiO$), −2.5 ppm ($OSiMe_2$).

Synthesis of 2G-[Si{O(CH$_2$)$_2$N(Me)(CH$_2$)$_2$NMe$_3^+$I$^-$}]$_8$ (15): This dendrimer was prepared by a similar method to that described for **11**, starting from **7** (0.19 g, 0.08 mmol) and a solution of MeI in Et_2O (2 M, 0.34 mL, 0.64 mmol). Compound **15** was isolated as a white solid (0.49 g, 95%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.60$ (t, 16H; OCH_2), 3.42 (t, 16H; $CH_2NMe_3^+$), 3.11 (s, 72H; NMe_3^+), 2.76 (t, 16H; $N(Me)CH_2CH_2NMe_3^+$), 2.49 (t, 16H; OCH_2CH_2N), 2.22 (s, 24H; NMe) 1.30 (m, 24H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.59 (m, 16H; $SiCH_2CH_2CH_2SiO$), 0.51 (m, 32H; rest of CH_2Si groups), 0.03 (s, 48H; $OSiMe_2$), −0.11 ppm (s, 12H; SiMe); ^{13}C NMR ([D$_6$]DMSO): $\delta = 61.0$ ($CH_2NMe_3^+$), 59.6 (OCH_2), 58.3 (NCH_2CH_2O), 52.3 (NMe_3^+), 50.9 ($NCH_2CH_2NMe_3^+$), 41.3 (NMe), 20.0–16.8 (CH_2 groups of the carbosilane skeleton), −2.5 ($OSiMe_2$), −5.5 ppm (SiMe).

Synthesis of 3G-[Si{O(CH$_2$)$_2$N(Me)(CH$_2$)$_2$NMe$_3^+$I$^-$}]$_{16}$ (16): This dendrimer was prepared by a similar method to that described for **11**, starting from **8** (0.084 g, 0.017 mmol) and a solution of MeI in Et_2O (2 M, 0.13 mL, 0.27 mmol). Compound **16** was isolated as a white solid (0.49 g, 95%). ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.60$ (t, 32H; OCH_2), 3.44 (t, 32H; $CH_2NMe_3^+$), 3.12 (s, 144H; NMe_3^+), 2.76 (t, 32H; $N(Me)CH_2CH_2NMe_3^+$), 2.49 (t, 32H; OCH_2CH_2N), 2.22 (s, 48H; NMe), 1.28 (m, 56H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.51 (m, 112H; $SiCH_2CH_2CH_2SiO$ and $SiCH_2CH_2CH_2Si$), 0.03 (s, 96H; $OSiMe_2$), −0.11 ppm (s, 36H; SiMe); ^{13}C NMR ([D$_6$]DMSO): $\delta = 61.0$ ($CH_2NMe_3^+$), 59.6 (OCH_2), 58.3 (NCH_2CH_2O), 52.3 (NMe_3^+), 50.8 ($NCH_2CH_2NMe_3^+$), 41.3 (NMe), 20.0–16.8 (CH_2 groups of the carbosilane skeleton), −2.5 ($OSiMe_2$), −5.5 ppm (SiMe).

Synthesis of 1G-[Si{(CH$_2$)$_3$NH$_3^+$Cl$^-$}]$_4$ (17): A solution of HCl in Et_2O (1 M, 1.2 mL, 1.2 mmol) was added to a diethyl ether (40 mL) solution of **9** (0.17 g, 0.26 mmol). The resulting solution was stirred for 2 h at room temperature and then evaporated under reduced pressure to give **17** as a white solid in a quantitative yield. ^{1}H NMR (D_2O): $\delta = 2.74$ (t, 8H; CH_2N), 1.45 (m, 8H; $SiCH_2CH_2CH_2N$), 1.19 (m, 8H; $SiCH_2CH_2CH_2Si$), 0.38 (m, 24H; CH_2Si), −0.19 ppm (s, 24H; $SiMe_2$); ^{13}C NMR (D_2O): $\delta =$ 42.0 (CH_2N), 21.3 ($SiCH_2CH_2CH_2N$), 18.8, 18.0, 16.6 ($SiCH_2CH_2CH_2Si$), 11.2 ($SiCH_2CH_2CH_2N$), −4.4 ppm ($SiMe_2$); elemental analysis calcd (%) for $C_{32}H_{84}N_4Cl_4Si_5$: C 47.61, H 10.49, N 6.94; found: C 48.57, H 10.46, N 6.82.

Synthesis of 2G-[Si{(CH$_2$)$_3$NH$_3^+$Cl$^-$}]$_8$ (18): This dendrimer was prepared by a similar method to that described for **17**, starting from **10** (0.09 g, 0.05 mmol) and a solution of HCl in Et_2O (1 M, 0.6 mL, 0.6 mmol). Compound **18** was isolated as a white solid (0.06 g, 55%). ^{1}H NMR (D_2O): $\delta = 2.74$ (t, 16H; CH_2N), 1.47 (m, 16H; $SiCH_2CH_2CH_2N$), 1.16 (m, 24H; $SiCH_2CH_2CH_2Si$), 0.39 (m, 64H; CH_2Si), −0.16 (s, 48H; $SiMe_2$), −0.25 ppm (s, 12H; SiMe); ^{13}C NMR (D_2O): $\delta = 42.6$ (CH_2N), 21.8 ($SiCH_2CH_2CH_2N$), 20.5–17.0 ($SiCH_2CH_2CH_2Si$), 12.1 ($SiCH_2CH_2CH_2N$), −3.23 ($SiMe_2$), −4.17 ppm (SiMe); elemental analysis calcd (%) for $C_{80}H_{204}N_8Cl_8Si_{13}$: C 49.86, H 10.67, N 5.81; found: C 49.79, H 10.18, N 5.76.

PBMCs and oligonucleotide: Peripheral blood mononuclear cells (PBMCs) were derived from healthy voluntary donors, and obtained from leukophoresed blood by Ficoll gradient and elutriation centrifugation. The ODN sequence was 18 bases long and corresponded to an antisense (complementary) sequence of the HIV polypurine tract (PPT) element mRNA: 5′-fluoresceine-AAT TTT CTT TTC CCC CCT-3′. For treatment of PBMCs and oligonucleotide synthesis, see Supporting Information.

Formation of ODN/dendrimer complexes: Complex formation between dendrimers and ODNs was performed by an electrostatic approach. Ratios of ODN to dendrimer were based on the calculation of the electrostatic charge present on each component, for example, the number of phosphate groups in the ODN versus the number of terminal ammonium groups on the dendrimer. Dendrimers were diluted in sterile distilled water at 2 mg mL^{-1}. The ODN concentration for complexes with carbosilane dendrimers was 0.88 μM (2.57 μg), and for complexes with SF 0.34 μM (1 μg); the concentrations of the dendrimers were 3.93 μM (2.96 μg) for **1**, 1.99 μM (2.35 μg) for **2**, 1.96 μM (2.80 μg) for **12**, 3.94 μM (3.42 μg) for **15**, 3.98 μM (1.92 μg) for **18** and 0.68 μM for SF (final concentration in well). All complexes were formed in 60 μL of serum-free RPMI medium, with an incubation time of 20 min at room temperature. The concentration of DNA and SF in the complex was chosen according to the manufacturers instructions. In the same way, complexes between ODN and SF were formed according to these instructions.

Evaluation of ODN/dendrimer complex formation: Complex formation was assessed by evaluation of migration retardation of fluoresceinated ODNs or alternatively NFkappa-B plasmid during electrophoresis on 3% agarose gels. A 100 or 5000 bp DNA ladder was used respectively as reference (Gibco BRL).

pH gradient: To check stability of dendriplexes at different pH values, we employed different phosphate and acetate buffer solutions. For acid extreme of pH 2.8 an acidic solution (0.05 M glycine/HCl/0.1 M NaCl) was used.

Phase-contrast light microscopy: After incubation with dendrimers, changes in morphology and characteristics of PBMCs, such as cell membrane birefringence, were observed through a phase-contrast inverted microscope (Nikon TMS, Nikon, Japan) equipped with a 100X objective (Plan 10/0.30DL/Ph1, Nikon, Japan). Live PBMCs are bright, with a defined spherical shape, and float in the culture medium. Dead cells have a darker appearance and are mostly present in the bottom of the well. In addition, we assessed the presence or absence of cell aggregation.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay: This method was selected to analyze detrimental intracellular effects on mitochondria and metabolic activity. The colorimetric MTT test, based on the selective ability of viable cells to reduce MTT to purple formazan, relies on intact metabolic activity and is frequently used for cytotoxicity screening. After 48 h of incubation of PBMCs with different concentrations of dendrimers in a 96-well plate, culture medium containing the dendrimers was replaced with 200 μL of serum-free Optimem. 20 μL of sterile, filtered MTT (Sigma) stock solution in PBS (pH 7.4, 5 mg mL^{-1}) was added to each well to achieve a final concentration of 0.5 mg MTT per millilitre. After 4 h, unconverted dye was removed by aspiration and the formazan crystals were dissolved in dimethyl sulfoxide (200 μL per well; Merck, Darmstadt, Germany). The concentration of formazan was then determined spectrophotometrically in a plate reader at a wavelength of 570 nm (test) and 690 nm (reference). The spectrophotometer was calibrated to zero absorbance by using Optimem medium without cells. The percentage cell viability relative to control wells (cells with no dendrimer) was calculated by $([A]_{test}/[A]_{control}) \times 100$. Each dendrimer concentration was tested in triplicate, according to American Type Culture Collection (ATCC) directives.

Hemolysis test: The hemolytic and hemagglutinating activity of the carbosilane dendrimers was evaluated according to Parnham and Wetzig[25] and compared with that induced by a 4G PAMAM dendrimer. Erythrocytes were obtained from the bottom of the tube after PBMC extraction following blood centrifugation in Ficoll gradient. Erythrocytes were diluted with cold PBS (pH 7.4) to a convenient volume to make feasible their visualisation. This suspension of red blood cells was always freshly prepared and used within 24 h after collection. Carbosilane dendrimer solutions of different concentrations, also prepared in PBS buffer, were added to erythrocytes and were incubated for 60 min at 37 °C in a shaking water bath. The presence/absence of hemagglutination was observed

under a phase-contrast inverted microscope. In a second step, the release of hemoglobin was determined after centrifugation (1500 rpm) by photometric analysis of the supernatant at 540 nm. Complete hemolysis was achieved with 0.2% Triton X-100 to give the 100% control value. Less than 10% hemolysis was regarded as no-toxic-effect level in our experiments. The experiments were run in triplicate and were repeated twice.

Lymphoproliferative assay: PBMCs were incubated for one week with two different concentrations of each dendrimer in a 96-well plate (100000 cells per well seeded in 200 μL of complete RPMI medium with antibiotics, glutamine, and 10% of human AB serum). A well with untreated cells was included along with a positive control for proliferation (cells treated with 1 $\mu g\,mL^{-1}$ of phytohemagglutinin). PBMC proliferation was evaluated by incorporation of [^{3}H]thymidine into DNA during the last 16 h of culture. The cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested in glass-fiber filters by using an automatic cell harvester, and radioactivity incorporation was measured in a liquid scintillation spectrometer. The assay was carried out for triplicate cultures.

Flow cytometry (FC): Dead or dying cells were identified by their typical diminished forward (FW) and increased side (SD) light-scattering characteristics. In the FC analysis of PBMCs treated with the different dendrimers, we drew a gate around cells presenting FW and SD corresponding to live cells and another one around cells showing FW and SD of dead or dying cells. We then compared the percentage of cells included in the two gates. Cells with FW and SD corresponding to monocytes (less than 5%) were not included in the analysis. FC was performed in a Beckmann–Coulter flow cytometer.

Trypan Blue (TB) uptake: TB dye is excluded by viable cells but can penetrate cell membranes of dying or dead cells. When TB staining is negative, membrane integrity is present. Cells were treated with 0.6% TB (Sigma) for 5 min and then washed twice with PBS. At least 200 cells were counted under the microscope for each condition.

Microscopy

DAPI labeling: Cells were seeded on glass slides coated with Poly-L-lysine, fixed with 4% paraformaldehyde and treated with DAPI for 10 min. They were then washed three times with PBS and observed under a Leica TCS SP2 confocal microscope with excitation at 405 nm.

Time-lapse in vivo imaging: After 72 h of incubation with dendrimers or dendriplexes, cells were seeded in special chambers for in vivo microscopy at 37°C and 5% CO_2. One image was captured every 30 s over a period of 10 min. A time-lapse video was prepared from the images, and cell viability and motility were examined.

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