Amphiphilic Cationic Carbosilane–PEG Dendrimers: Synthesis and Applications in Gene Therapy

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Original article

Amphiphilic Cationic Carbosilane–PEG Dendrimers: Synthesis and Applications in Gene Therapy

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ABSTRACT

Here we synthesized carbosilane, generation 1 to 3, and PEG-based dendrons functionalized at the periphery with NHBoc groups and at the focal point with azide and alkyne moieties, respectively. The coupling of these two types of dendrons via click chemistry led to the formation of new hybrid dendrimers with two distinct moieties, the hydrophobic carbosilane and the hydrophilic PEG-based dendron. The protected dendrimers were transformed into cationic ammonium dendrimers. These unique amphiphilic dendrimers were studied as vectors for gene therapy against HIV in peripheral blood mononuclear cells (PBMC) and their performance was compared with that of a PEG-free carbosilane dendrimer. The presence of the PEG moiety afforded lower toxicities and evidenced a weaker interaction between dendrimers and siRNA when compared to the homodendrimer analogous. Both features, lower toxicity and lower dendriplex strength, are key properties for use of these vectors as carriers of nucleic material.

1. Introduction

In recent years, interest in treating diseases by gene therapy has grown as consequence of increased knowledge about the molecular pathways of diseases and the human genome. Gene therapy involves guiding functional genetic material, such as plasmids, nucleic acids (DNA and RNA) and oligonucleotides, to target cells [1]. Two types of gene delivery vectors can be distinguished, namely viral and non-viral. As a result of the intrinsic nature of viruses, the former have proved to be highly efficient, although they also show considerable disadvantages [2,3]. The second type is based on synthetic vectors, such as macromolecules, polymers and nanosystems, which can be rationally designed according to needs [4–11]. These vectors are required to interact with nucleic acids, usually forming electrostatic complexes, compacting them and preventing their degradation. Thus, various systems functionalized with cationic groups have been used for this purpose [12].

In the particular case of dendritic macromolecules, their characteristics, such as a well-defined size and structure, flexibility, monodispersity and multivalent molecular surface [13–22], have attracted attention for biomedical applications. In this regard, several types of cationic dendrimers containing different skeletons have been explored for gene therapy [6,10,23–31]. One such type comprises carbosilane dendrimers. Although these molecules are highly hydrophobic, water-soluble cationic carbosilane dendrimers have proved useful as non-viral vectors for gene therapy [32–36]. Their strong amphiphilic behavior differentiates them from other kinds of dendrimers with a more hydrophilic skeleton. These molecules are able to bind oligonucleotides and siRNA and transport them to the interior of a range of cell types [32–36]. In addition, these dendrimers protect nucleic material from degradation by serum proteins and nucleases. This protective feature is of fundamental importance as it ensures that nucleic material can

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exert an effect once inside the cell [34]. However, the major drawback of cationic dendrimers is their intrinsic toxicity. A strategy developed elsewhere to overcome this problem has been the binding of PEG moieties, an approach that also increases the solubility of the macromolecule [37–44].

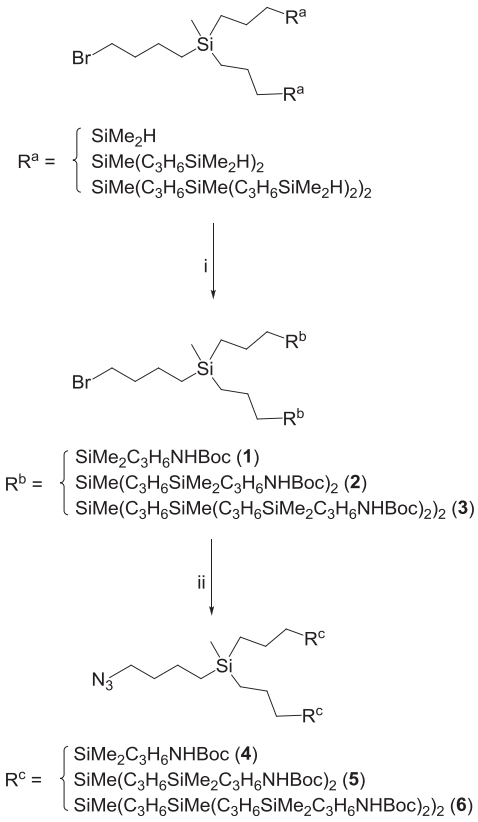
Taking these previous results into account, here we report the design and synthesis of Janus-type amphiphilic dendrimers composed of two types of dendrons, one based on the carbosilane moiety and the other on a PEG scaffold, both decorated with ammonium groups at their periphery. The synthesis of these dendrimers was performed by click chemistry (CuCAAC) using azide and alkyne groups at the focal point of carbosilane and PEG-based dendrons, respectively. Due to this composition, it is expected that these dendrimers increase their biocompatibility compared to carbosilane homodendrimers. This feature, along with complex formation with siRNA, cellular uptake, and HIV inhibition, was studied in primary peripheral blood mononuclear cells (PBMC).

2. Results and discussion

2.1. Synthesis of dendrons and Janus-type dendrimers

For the synthesis of carbosilane dendrons, as precursors we chose dendrons with a C–Br bond at the focal point and Si–H groups at the periphery [45]. We have previously reported the synthesis of carbosilane homodendrimers functionalized with amine $-NH_2$ groups at the periphery by hydrosilylation of allylamine with SiH-terminated dendrimers [35,45]. However, due to the chemical incompatibility of primary amine groups and C–Br bonds, this procedure is not adequate to obtain the dendrons required for preparation of the Janus-type dendrimers objective of this study. Therefore protection of the amine groups with Boc was necessary in order to use the same synthetic procedure. Thus, the reaction of Boc-protected allylamine C_3H_5NHBoc with 1–3 generation dendrons $BrG_n(SiH)_m$ ($n = 1, m = 2$; $n = 2, m = 4$; $n = 3, m = 8$) (Scheme 1) afforded the corresponding dendrons $BrG_n(NHBoc)_m$ ($n = 1, m = 2$ (**1**); $n = 2, m = 4$ (**2**); $n = 3, m = 8$ (**3**)) in moderate yields (*ca.* 65%). The reaction was followed by NMR spectroscopy, which showed the disappearance of the doublet corresponding to the methyl groups and the multiplet of the hydrogen atom of $SiMe_2H$ moieties. NMR spectroscopy also confirmed formation of these compounds (Figs. S3–S4 in Supporting Information (S.I.)). The new chain $-Si(CH_2)_3NH-$ introduced in dendrons **1**–**3** was clearly identified by $^1H-^1H$ TOCSY NMR spectra, which showed four resonances at about δ 4.55, 3.05, 1.25 and 0.45. ^{13}C NMR spectra presented one resonance at δ *ca.* 44.0 for the C–N atom of this chain and finally ^{29}Si NMR spectroscopy showed one resonance for the new outer $SiMe_2$ group δ *ca.* 2.0. The bromine atom at the focal point of compounds **1**–**3** was easily replaced by an azide function through the reaction with NaN_3 (Scheme 1), thus obtaining the dendrons $N_3G_n(NHBoc)_m$ ($n = 1, m = 2$ (**4**); $n = 2, m = 4$ (**5**); $n = 3, m = 8$ (**6**)) in high yield (*ca.* 85%). The introduction of this group was confirmed by NMR spectroscopy (Figs. S5–S8 in S.I.), which showed one resonance at δ *ca.* 3.25 in the ^1H NMR spectra and other resonance at δ *ca.* 51.0 in the ^{13}C NMR spectra for the new N_3CH_2 methylene group.

The alkyne PEG-based dendron consisted of a branching unit derived from diethylenetriaminepentaacetic acid (DTPA), to which PEG-chains of exact molecular weight (1-(*tert*-butoxycarbonylamino)-4,7,10-trioxa-13-tridecanamine) were incorporated. The DTPA derivative contains an alkyne moiety at its focal point instead of a carboxylic one, and was synthesized as follows (Scheme 2). Initially, *tert*-butyl bromoacetate was reacted with ethanolamine, after which HO/Br exchange using N-bromosuccinimide (NBS) and PPh_3 afforded compound **7** in good yield (71%), as confirmed by

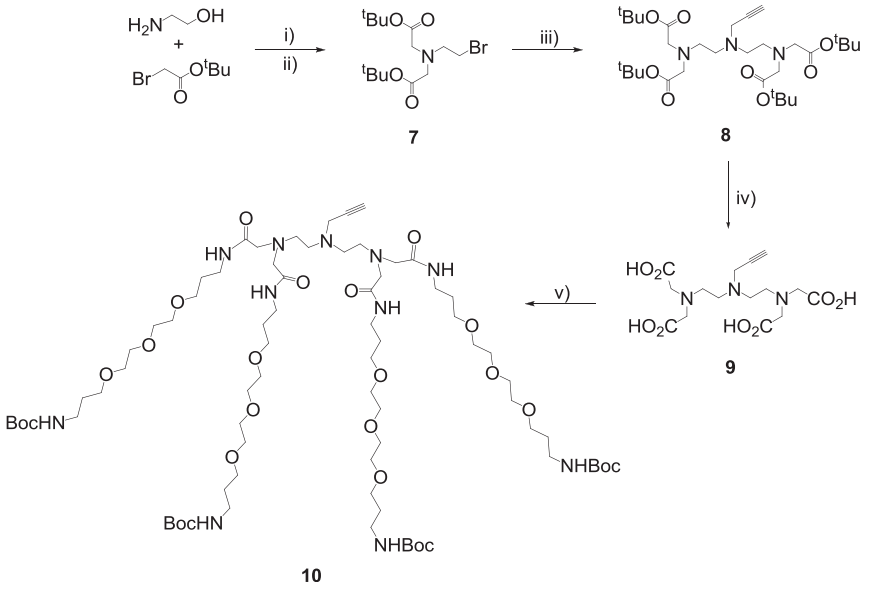


Scheme 1. Synthesis of generation 1–3 carbosilane dendrons $N_3G_n(NHBoc)_m$ ($n = 1$, $m = 2$ (**4**); $n = 2$, $m = 4$ (**5**); $n = 3$, $m = 8$ (**6**)). i) C_3H_5NHBoc, Pt Kardstedt's catalyst, THF, 60 °C, 12 h; ii) NaN_3, DMF, NaI, 80 °C, 12 h.

HPLC-MS and NMR (Figs. S9 and S10 in S.I.). The main NMR data for this compound in the ^1H NMR spectrum are the singlet at δ 3.48 for the CH_2CO group and the triplets at δ 3.13 and 3.48 for the $-N(CH_2)_2Br$ chain. The alkyne DTPA derivative **8** was then obtained by coupling two units of **7** to propargylamine (83% yield). This resulted to be the key step in the synthesis of the final dendron because we were able to minimize the overalkylation of the propargylamine. The best results were achieved using DIEA as base (2 eq.) and heating at 80 °C. The presence of the propargylamino fragment gave rise to a shifting to lower frequency of the $N(CH_2)_2N$ chain to δ 2.66 and 2.84 in the ^1H NMR spectrum (Figs. S11–S12 in S.I.).

Afterwards, the *tert*-butyl groups were removed under acidic conditions and the PEG-chains were introduced by amide bond formation, rendering the desired dendron **10** in satisfactory yield (84%), which was characterized by NMR (Figs. S13–S14 in S.I.), HPLC-MS and HR-MS. In this compound, the presence of the new amide groups were distinguished in ^1H NMR spectroscopy by means of one broad resonance at δ 7.51 for the NH protons, whereas the NH protons of the NHBoc groups were observed as a broad resonance at δ 5.10.

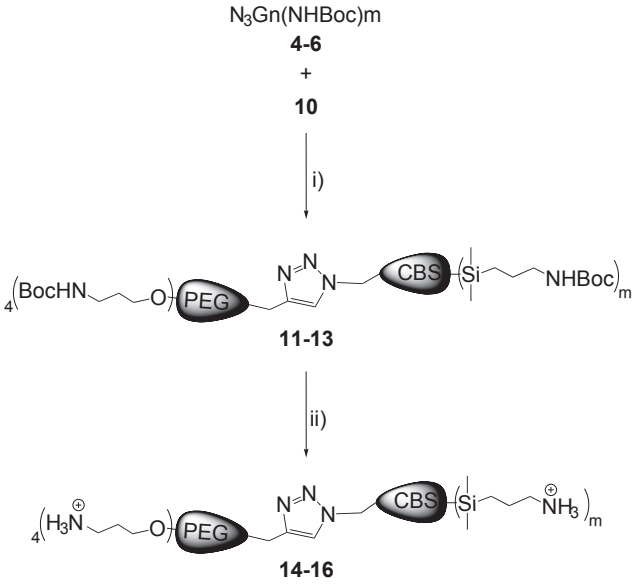
The coupling of azide carbosilane dendrons $N_3G_n(NHBoc)_m$ (**4**–**6**) with alkyne PEG-based wedge **10** was carried out in THF/H_2O in the presence of Cu^+ at 60 °C (Scheme 3), leading to the hybrid dendrimers **11**–**13**. The yield of this reaction was dependent on the generation of carbosilane dendron. While compounds **11** and **12** were obtained in acceptable yield (*ca.* 80%), compound **13** was isolated in very low yield (23%). The formation of amphiphilic dendrimers was confirmed by NMR spectroscopy (Figs. S15–S18 in S.I.). The ^1H NMR spectra showed one resonance at about δ 7.50 for the CH proton of the triazole ring and the ^{13}C NMR spectra showed



Scheme 2. Synthesis of **10**. i) $KHCO_3$, DMF, r. t., 22 h; ii) NBS, PPh_3, CH_2Cl_2, 0 °C, 2 h; iii) $NH_2C_3H_3$, DIPEA, CH_3CN, 24 h, 80 °C; iv) HCl/dioxane, 16 h, r. t.; v) Boc-1-amino-4,7,10-trioxa-13-tridecanamine, CH_2Cl_2:DMF (7:3, v:v), solid PyBOP, DIPEA (pH = 8), 1 h, r. t.

resonances at about δ 122 and δ 144 for the CH and *i*-C carbons of this ring, respectively. With respect to the two CH_2 groups bound to the triazole ring, the one stemming from the carbosilane dendron was observed about δ 4.30 in ^{1}H NMR spectroscopy and at about δ 50.0 in ^{13}C NMR spectroscopy, whereas the one derived from the PEG-based dendron was observed at around δ 3.70 in ^{1}H NMR spectroscopy and at about δ 46.0 in ^{13}C NMR spectroscopy.

Treatment of NHBoc dendrimers **11**–**13** with excess HCl in dioxane led to the corresponding amphiphilic cationic dendrimers **14**–**16** (Scheme 3, Fig. 1), which were isolated as white powders that were soluble in H_2O, in contrast to the starting compounds. The main NMR data (Figs. S19–S22 in S.I.) of these compounds are, on the one hand, the disappearance of the ^{t}Bu resonances of Boc groups and, on the other hand, the resonances for the NH_3^+ protons observed in the ^{1}H NMR spectra about δ 8.00 (DMSO-d_6). In addition, in Fig. 1, the analog carbosilane homodendrimer of generation 2, named as **17** [35], is also depicted for structural comparison.



Scheme 3. Synthesis of amphiphilic cationic dendrimers from carbosilane dendrons $N_3G_n(NHBoc)_m$ (**4**–**6**) and PEG-based dendron **10**. i) $CuSO_4$, NaAsc, NEt_3, THF/H_2O (6:2 v:v), 16 h, 60 °C; ii) HCl/dioxane, 5 h, r. t.

2.2. Biomedical assays

The potential application of amphiphilic dendrimers **14** and **15** for gene therapy, with 6 and 8 ammonium NH_3^+ groups respectively, was studied against HIV in PBMC and compared with that of the carbosilane homodendrimer **17** [35] with 8 NH_3^+ groups (Fig. 1). The very low yield obtained in the synthesis of the amphiphilic dendrimer **16** precluded its use in these studies.

First, we tested the toxicity of these dendrimers by MTT assays to measure mitochondrial metabolic activity (Fig. 2A). Compounds **14** and **15** were non-toxic up to 5 and 10 μM respectively, whereas carbosilane dendrimer **17** showed toxicity over 1 μM. Although it is clear that the presence of PEG fragments diminished the toxicity, the carbosilane moiety also might play a role, as the toxicity of **14** was slightly higher than that of **15**, with more cationic groups at the surface.

Also, the toxicity of dendriplexes was evaluated by MTT (Fig. 2B). These experiments showed again higher tolerance for dendriplexes formed by Janus-type dendrimers **14** and **15** than for carbosilane homodendrimer **17**. Dendriplexes formed by dendrimer **14** were biocompatible up to a siRNA:dendrimer ratio of 1:4 (−/+), dendriplexes containing **15** up to a ratio of 1:8 (−/+) and finally dendriplexes with **17** were non-toxic only below a ratio of 1:2 (−/+). These results are in agreement with the toxicity observed for dendrimers alone.

Electrophoresis analyses in agarose gel were performed to study the binding capacity of these dendrimers toward siRNA Nef (Fig. 3). Amphiphilic dendrimer **14** formed a complex with siRNA Nef from a ratio of 1:4 (−/+) at 2 h but released it after 24 h, being stable over time at a higher ratio. However, dendritic systems with 8 NH_3^+ groups, **15** and **17**, formed more stable complexes at those ratios in which the cargo was not released after 24 h. For amphiphilic dendrimer **15**, the dendriplex was formed from ratios 1:8 (−/+) but for carbosilane homodendrimer **17** stable dendriplexes were generated from ratios 1:1 (−/+). In any case, the presence of PEG units in

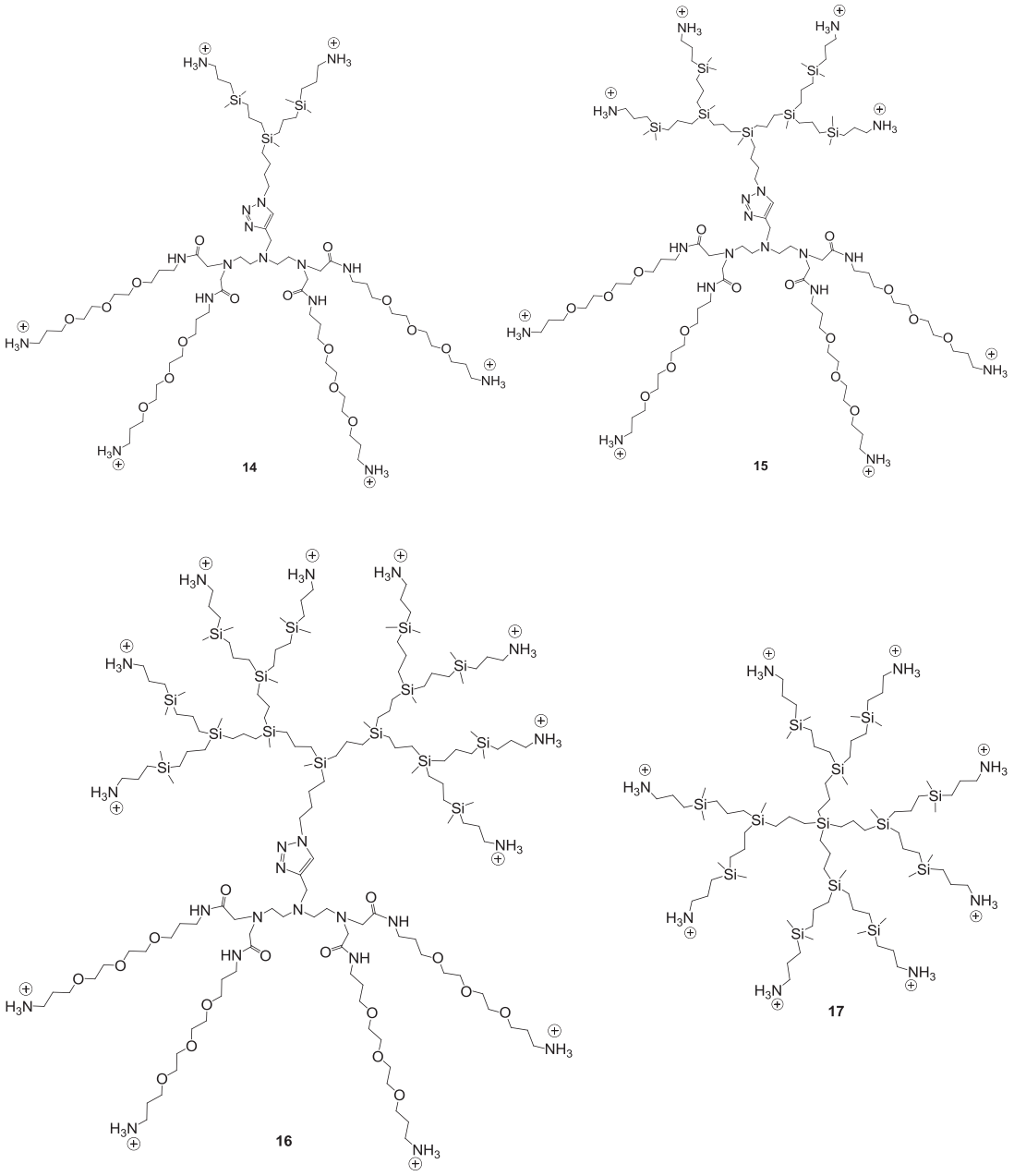


Fig. 1. Drawing of cationic amphiphilic dendrimers **14**–**16** described in this study and generation two carbosilane dendrimer **17** used as a reference model in biomedical assays.

these Janus-type dendrimers clearly weakened the interaction with siRNA.

In order to test the strength of the interaction between siRNA and dendrimers, heparin competition assays were performed (Fig. 4). The −/+ charge ratio of dendriplexes that has been used for this experiment corresponds with the best results found in the biocompatibility assays, 1:4 for compound **14**, 1:8 dendriplex for compound **15** and 1:2 dendriplex for compound **17**. Polyacrylamide gel electrophoresis (PAGE) was used to adequately visualize and quantify the siRNA released from the dendriplex as heparin concentration increased. It was found that such release process began at 0.1 U/μg for the three types of dendriplexes. However, the influence of PEG units was clear again, as higher retention was shown by free-PEG dendrimer **17**. Analogous dendritic systems to the homodendrimers **17** containing $-CH_2NMe_3^+$ at the periphery resulted to be inactive regarding HIV inhibition when dendriplexes were used [46]. This drawback was ascribed to the high stability shown by the dendriplexes, which enabled them to internalize their cargo but prevented their release inside the cell for *in vitro* experiments. The presence of PEG moieties weakened these interactions, which could be a key feature in transfection processes.

Because of the charge ratio is different for every dendrimer used, it seems difficult to compare among them in term of relative strength. However, it is also plausible that the difficulty on releasing the siRNA may increase on increasing the charge ratio at least until certain limits. Therefore, if a 1:4 charge ratio (−/+) were used for dendrimers **15** instead of 1:8, higher liberation of siRNA may be observed while the opposite would be true if dendrimer **17** were tested at such ratio, supporting again the assumption of the influence of PEG units on the biological properties of the Janus dendrimers.

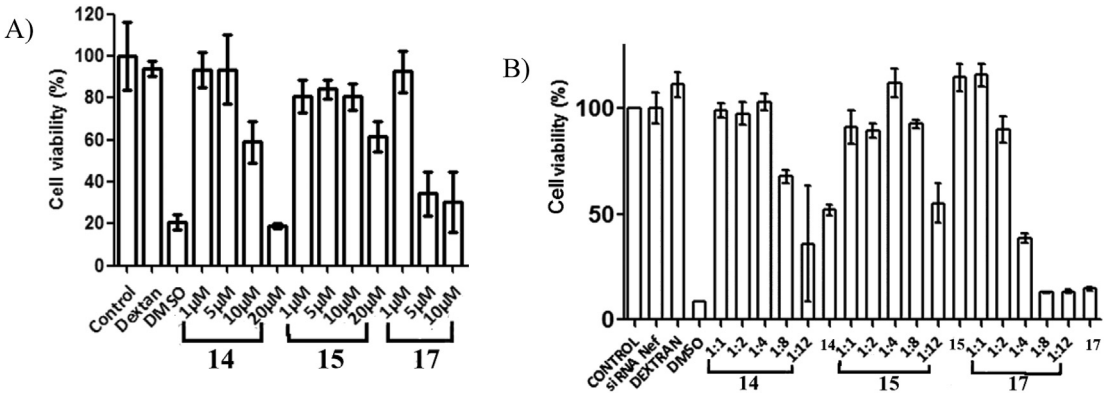


Fig. 2. Cytotoxicity (MTT) of dendrimers **14**, **15** and **17** (A) and the corresponding dendriplexes (B) at variable siRNA Nef:dendrimer charge ratios (−/+) in PBMC with siRNA Nef concentration of 25 nM. Dextran (5 μM) and DMSO (15%) were used as positive and negative controls respectively.

Zeta-potential and size measurement experiments have been carried out for dendriplexes formed by dendrimers **14**, **15** or **17** and siRNA-Nef (see Supporting Information). The differences between the charge ratio profiles of dendriplexes measured by Z-potential corroborate the intensity of interactions between de different dendrimers and siRNA. The lower zeta-potential values observed for **14** and **15** respecting **17** dendriplexes with siRNA show more efficiency in term of transfection and biocompatibility. Although, a positive charge on surface dendriplex facilitates cell absorption and mediates efficient endosomal uptake, high positive charges imply cytotoxicity and non-specific binding to others biological components. However, respecting particle size, no rational data were obtained for comparing among different dendriplexes, although a different profile can be discerned for **15** probably as result of a large excess of positive charges.

Finally, a post-treatment inhibitory study on PBMC by p24 ELISA assay was done to determine the capacity of dendrimer/siRNA Nef dendriplexes to interfere in HIV replication (Fig. 5). The efficiency of siRNA delivery is close related to the formation of p24 antigen. The siNef inhibit the formation of the viral Nef protein which is vital for the formation of new viral particles represented by the detection of the p24 antigen. The p24 protein is present in the HIV core; therefore quantification of this antigen is a method to evaluate the inhibition ability of any system once the cells are infected. Of the three systems used, the one formed with Janus-type dendrimer **15** gave the best results, showing inhibition of up to 50%. This result marks a clear difference with respect to those obtained for the dendriplexes formed with Janus-type dendrimer **14** and homo-dendrimer **17** at the chosen −/+ charge ratios. It is likely that the complex of siRNA with compound **15** may have a unique structure (in terms of zeta potential and size particles profiles), and/or larger excess of the dendritic compound might affect the efficiency of siRNA delivery. Thus, inhibitory capacity is more favorable in the case of dendriplex formed with **15** although the release of siRNA

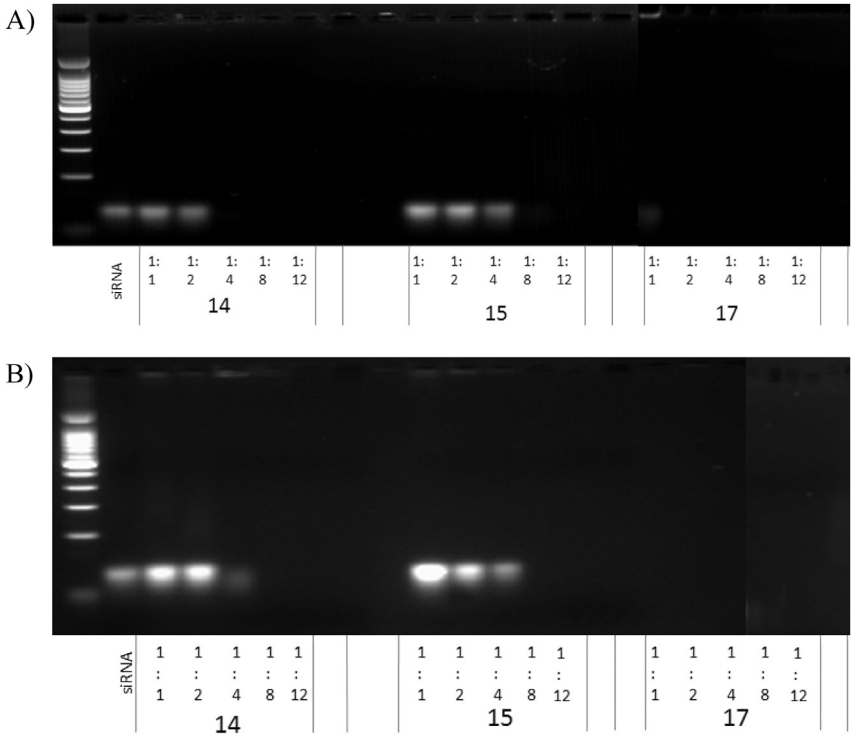


Fig. 3. Electrophoresis gel in agarose of dendriplexes at different siRNA Nef:dendrimer charge ratios after 2 h (3A) and 24 h (3B).

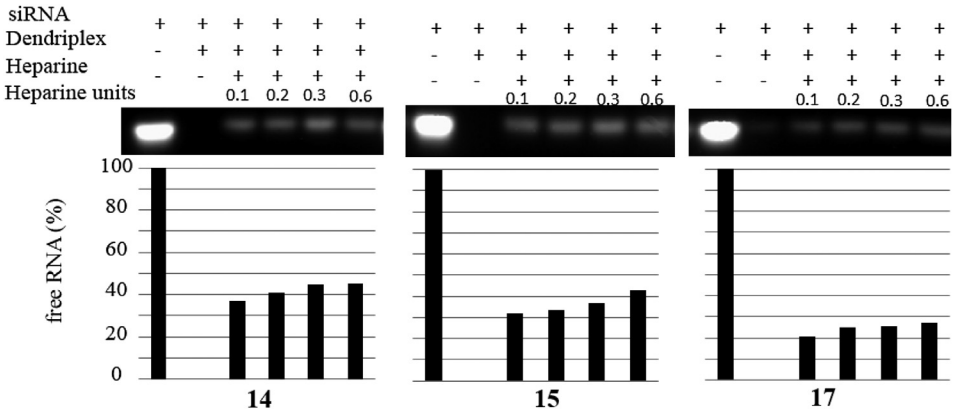


Fig. 4. Polyacrylamide gel electrophoresis of dendriplexes/heparin competition assays after 24 h. The $y-$ axis denotes free siRNA while concentration of heparin was measured in International Units of heparine per μg of siRNA (U/μg).

from **15** is lower than that observed from **14** (see Figs. 3 and 4). With respect to dendrimer **17**, the higher strength of the dendriplex probably is the major drawback of this system. Finally, dendriplex formed with **15** showed an activity similar to that of the polyplex formed with the well-known PEI system (at $-/+$ charge ratio 1:2) used as standard reference, which accounts for its effectiveness.

3. Conclusions

The click coupling of azide carbosilane dendrons (generations 1 and 2) and alkyne PEG-based dendron has been successfully used as building blocks to synthesize Janus-type cationic dendrimers functionalized with 6 and 8 ammonium NH_3^+ groups. However, for higher generations this reaction was not successful, as very low yield was obtained when the corresponding carbosilane dendron of generation 3 was used. The performance of these hetero-dendrimers **14** and **15** was compared with a carbosilane homo-dendrimer with 8 NH_3^+ groups (**17**) as gene delivery vectors against HIV in PBMC. The presence of the PEG moiety afforded lower toxicities than the homodendrimer analog, and the same trend was observed when the corresponding dendriplexes were used. With respect to dendriplex strength, electrophoresis analyses showed that dendrimers **14** and **15** formed dendriplexes at higher ratios than the homodendrimer **17**, thus evidencing a weaker interaction between dendrimers and siRNA. This finding was corroborated by the dendriplex/heparin competition assays. Both lower toxicity and lower dendriplex strength, ascribed to the presence of PEG moieties, could be crucial properties for their use as carriers of nucleic material, as Janus-type dendrimer **15** showed greater HIV inhibitory activity than homodendrimer **17** containing the same number of positive charges.

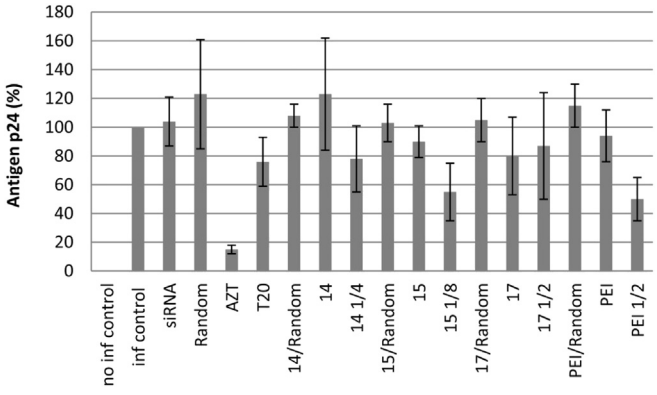


Fig. 5. HIV protein p24 ELISA test of infected PBMC after 48 h of treatment. AZT denotes zidovudine (replication inhibitor); T20 is enfuvirtide (fusion inhibitor); **14**, **15** and **17** mean inhibition in the presence of dendrimers alone below their cytotoxicity levels, 5, 10 and 1 μM respectively; **14** 1/4, **15** 1/8 and **17** 1/2 mean dendriplexes of the corresponding dendrimers at those $-/+$ charge ratio. Dendriplexes with random siRNA were formed at the same charge ratio than siRNA Nef. siRNA concentrations of 250 nM were used. Incubation time for dendriplex formation was 15 min and incubation time of PBMC with HIV virus was 1 h.

4. Experimental section

4.1. General considerations

All reactions were carried out under inert atmosphere and solvents were purified from appropriate drying agents when necessary (THF). NMR spectra were recorded on a Varian Unity VXR-300 (300.13 (^{1}H), 75.47 (^{13}C) MHz) or on a Bruker AV400 (400.13 (^{1}H), 100.60 (^{13}C), 79.49 (^{29}Si) MHz). Chemical shifts (δ) are given in ppm. ^{1}H and ^{13}C resonances were measured relative to solvent peaks, considering TMS = 0 ppm. ^{29}Si resonances were measured relative to external TMS. When necessary, assignment of resonances was done from HSQC, HMBC, COSY, TOCSY and NOESY NMR experiments. Elemental analyses were performed on a Perkin–Elmer 240C. Mass spectra were obtained from an Agilent 6210 (ESI), a Bruker Ultraflex III (MALDI-TOF) and a Thermo Scientific LTQ-FT Ultra (HRMS). Analytical HPLC was carried out on a Waters (Ireland) instrument comprising a Sunfire™ C18 reversed-phase analytical column, 3.5 μm, 4.6 × 100 mm, a separation module (Waters 2695), automatic injector, and photodiode array detector (Waters 2298). HPLC-MS analyses were carried out on a Waters (Ireland) instrument comprising a Sunfire™ C18 reversed-phase analytical column, 3.5 μm, 4.6 × 100 mm, a separation module (Waters 2695), automatic injector, photodiode array detector (Waters 2298), and a Waters micromass ZQ unit. The zeta potential measurements were carried out with a Malvern Nano ZS instrument. Compounds Karstedt's Pt catalyst, C_3H_5NHBoc, NaN_3, Na Ascorbate, tert-butyl bromoacetate, PPh_3, NBS, propargylamine (Aldrich), 1-(tert-butyloxycarbonyl-amino)-4,7,10-trioxa-13-tridecanamine (Iris Biotech) and $CuSO_4$ (Panreac) and Poly(-ethylene imine), PEI 25 KDa (Polyplus-transfection) were obtained from commercial sources. Compounds $BrG_n(SiH)_m$ ($n = 1$, $m = 2$; $n = 2$, $m = 4$; $n = 3$, $m = 8$) [45] and cationic carbosilane dendrimer **17** [35] were synthesized as published.

4.2. Synthesis of compounds

NMR data of dendrimers and dendrons of first generation and compounds **7**–**10** are labeled with letters. The corresponding labeled NMR spectra and structures are collected in Supporting Information.

4.2.1. Synthesis of BrG1(NHBoc)2 (**1**)

A solution of $BrG_1(SiH)_2$ (2.00 g, 5.24 mmol) and C_5H_3NHBoc (1.71 g, 10.90 mmol) in THF (5 mL) was stirred at 40 °C for 8 h in a teflon-valved ampoule in the presence of platinum Karstedt's catalyst. Afterwards the solvent was removed under vacuum, and **1** was purified by column chromatography (hexane:THF 5:2), which gave **1** as a colorless oil (2.88 g, 79%). NMR data ($CDCl_3$): ^{1}H NMR: −0.09 (s, 3H, Si*Me* (e)), −0.07 (s, 12H, Si*Me*$_2$ (i)), 0.43 (m, 6H, C*H*$_2$Si (j)), 0.52 (m, 8H, C*H*$_2$Si (f, h)), 1.23 (m, 6H, CH$_2$*CH*$_2$CH$_2$ (c, g)), 1.42 (s, 22H, SiCH$_2$*CH*$_2$CH$_2$N and ^{t}Bu (k, n)), 1.78 (m, 2H, BrCH$_2$*CH*$_2$CH$_2$CH$_2$ (b)), 3.06 (m, 4H, C*H*$_2$NH (l)), 3.40 (t, J = 6.80 Hz, 2H, BrC*H*$_2$ (a)), 4.54 (s, 2H, N*H* (m)). ^{13}C NMR: −5.1 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.0 (SiCH$_2$), 18.4, 18.6, 20.0 (SiCH$_2$ and SiCH$_2$*C*H$_2$), 22.4 (BrCH$_2$CH$_2$*C*H$_2$CH$_2$), 22.6 (BrCH$_2$CH$_2$CH$_2$*C*H$_2$), 24.6 (SiCH$_2$CH$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 33.7 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 36.3 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 43.8 (*C*H$_2$N), 79.9 (*C*Me$_3$), 155.9 (NH*C*OO). ^{29}Si NMR: 1.8 (SiMe), 2.0 (SiMe$_2$). MS (MALDITOF): 695.3656 [M + H$^+$]. Anal. Calc. for $C_{31}H_{67}BrN_2O_4Si_3$ (696.04): C, 53.46; H, 7.90; N, 4.02; Obt.: C, 53.32; H, 7.79; N, 3.94.

4.2.2. Synthesis of BrG2(NHBoc)4 (**2**)

Following the procedure described above for **1**, from $BrG_2(SiH)_4$ (1.50 g, 1.99 mmol) and C_5H_3NHBoc (1.31 g, 8.35 mmol) in THF (3 mL) afforded **2** as a colorless oil (1.68 g, 61%). NMR data ($CDCl_3$): ^{1}H NMR: −0.12 (s, 6H, Si*Me*), −0.09 (s, 3H, SiMe), −0.07 (s, 24H, Si*Me*$_2$), 0.42 (m, 10H, C*H*$_2$Si), 0.52 (m, 24H, C*H*$_2$Si), 1.23 (m, 14H, CH$_2$*CH*$_2$CH$_2$), 1.42 (s, 44H, SiCH$_2$*CH*$_2$CH$_2$N and ^{t}Bu), 1.78 (m, 2H, BrCH$_2$*CH*$_2$CH$_2$CH$_2$), 3.05 (m, 8H, C*H*$_2$NH), 3.39 (t, J = 6.80 Hz, 2H, BrC*H*$_2$), 4.57 (s, 4H, N*H*). ^{13}C NMR: −5.1 (Si*Me*), −5.0 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.0 (SiCH$_2$), 18.4, 18.8, 18.9, 20.0 (SiCH$_2$ and SiCH$_2$*C*H$_2$), 22.5 (BrCH$_2$CH$_2$*C*H$_2$CH$_2$ and BrCH$_2$CH$_2$CH$_2$*C*H$_2$), 24.6 (SiCH$_2$CH$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 33.6 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 36.4 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 43.8 (*C*H$_2$N), 79.0 (*C*Me$_3$), 155.9 (NH*C*OO). ^{29}Si NMR: 1.7 (SiMe), 2.1 (SiMe$_2$). MS (MALDITOF): 1381.8389 [M + H$^+$]. Anal. Calc. for $C_{65}H_{141}BrN_4O_8Si_7$ (1383.34): C, 56.44; H, 10.27; N, 4.05; Obt.: C, 56.26; H, 10.49; N, 4.27.

4.2.3. Synthesis of BrG3(NHBoc)8 (**3**)

Following the procedure described above for **1**, from $BrG_3(SiH)_8$ (1.10 g, 0.73 mmol) and C_5H_3NHBoc (0.97 g, 6.16 mmol) afforded **3** as a colorless oil (1.27 g, 63%) after size exclusion chromatography with THF. NMR data ($CDCl_3$): ^{1}H NMR: −0.11 (s, 21H, Si*Me*), −0.07 (s, 48H, Si*Me*$_2$), 0.43 (m, 18H, C*H*$_2$Si), 0.52 (m, 56H, C*H*$_2$Si), 1.23 (m, 30H, CH$_2$*CH*$_2$CH$_2$), 1.42 (s, 88H, SiCH$_2$*CH*$_2$CH$_2$N and ^{t}Bu), 1.78 (m, 2H, BrCH$_2$*CH*$_2$CH$_2$CH$_2$), 3.04 (m, 16H, C*H*$_2$NH), 3.39 (t, J = 6.80 Hz, 2H, BrC*H*$_2$), 4.58 (s, 4H, N*H*). ^{13}C NMR: −4.9 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.6 (SiCH$_2$), 18.4, 18.5, 18.8, 19.0, 20.0 (SiCH$_2$ and SiCH$_2$*C*H$_2$), 22.5 (BrCH$_2$CH$_2$*C*H$_2$CH$_2$ and BrCH$_2$CH$_2$CH$_2$*C*H$_2$), 24.6 (SiCH$_2$CH$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 33.6 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 36.4 (BrCH$_2$CH$_2$CH$_2$CH$_2$), 43.8 (*C*H$_2$N), 78.9 (*C*Me$_3$), 156.0 (NH*C*OO). ^{29}Si NMR: 1.7 (SiMe), 2.1 (SiMe$_2$). Anal. Calc. for $C_{133}H_{289}BrN_8O_{16}Si_{15}$ (2757.95): C, 57.92; H, 10.96; N, 4.06; Obt.: C, 57.81; H, 10.49; N, 3.88.

4.2.4. Synthesis of N3G1(NHBoc)2 (**4**)

A solution of **1** (1.50 g, 2.16 mmol) and NaN_3 (0.56 g, 8.62 mmol) in DMF (20 mL) was stirred at 80 °C for 16 h in a teflon-valved ampoule. Afterwards the solvent was removed under vacuum and extraction with Et_2O and brine was carried out. The organic phase was dried with $MgSO_4$ and filtered, and the solvent was removed under vacuum to give **4** as a colorless oil (1.28 g, 87%). NMR data ($CDCl_3$): ^{1}H NMR: −0.09 (s, 3H, Si*Me* (e)), −0.07 (s, 12H, Si*Me*$_2$ (i)), 0.43 (m, 6H, C*H*$_2$Si (d, j)), 0.51 (m, 8H, C*H*$_2$Si (f, h)), 1.27 (m, 6H, CH$_2$*CH*$_2$CH$_2$ (c, g)), 1.42 (s, 22H, SiCH$_2$*CH*$_2$CH$_2$N (k) and ^{t}Bu (p)), 1.60 (m, 2H, N$_3$CH$_2$*CH*$_2$ (b)), 3.05 (m, 4H, C*H*$_2$NH (l)), 3.25 (t, J = 6.8 Hz, 2H, N$_3$C*H*$_2$ (a)), 4.54 (s, 2H, N*H* (m)). ^{13}C NMR: −5.1 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.6 (SiCH$_2$), 18.4, 18.6, 20.0 (SiCH$_2$) and (SiCH$_2$*C*H$_2$), 21.2 (N$_3$CH$_2$CH$_2$*C*H$_2$CH$_2$), 24.6 (SiCH$_2$*C*H$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 32.7 (N$_3$CH$_2$*C*H$_2$), 43.8 (*C*H$_2$N), 51.1 (N$_3$CH$_2$), 79.0 (*C*Me$_3$), 155.9 (NH*C*OO). ^{29}Si NMR: 1.7 (SiMe), 2.0 (SiMe$_2$). MS (MALDITOF): 658.4531 [M + H]$^+$. Anal. Calc. for $C_{31}H_{67}N_5O_4Si_3$ (658.15): C, 56.57; H, 10.26; N, 10.64; Obt.: C, 56.42; H, 10.13; N, 10.21.

4.2.5. Synthesis of N3G2(NHBoc)4 (**5**)

Following the procedure described above for **4**, from **2** (1.22 g, 0.88 mmol) and NaN_3 (0.23 g, 3.53 mmol) afforded **5** as a colorless oil (1.03 g, 87%). NMR data ($CDCl_3$): ^{1}H NMR: −0.09 (s, 3H, Si*Me*), −0.09 (s, 6H, Si*Me*), −0.07 (s, 24H, Si*Me*$_2$), 0.43 (m, 10H, C*H*$_2$Si), 0.51 (m, 24H, C*H*$_2$Si), 1.27 (m, 14H, CH$_2$*CH*$_2$CH$_2$), 1.42 (s, 44H, SiCH$_2$*CH*$_2$CH$_2$N and ^{t}Bu), 1.60 (m, 2H, N$_3$CH$_2$*CH*$_2$), 3.05 (m, 8H, C*H*$_2$NH), 3.25 (t, J = 6.8 Hz, 2H, N$_3$C*H*$_2$), 4.54 (s, 4H, N*H*). ^{13}C NMR: −5.1 (Si*Me*), −5.0 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.5 (SiCH$_2$), 18.4, 18.5, 18.8, 18.9, 20.0 (SiCH$_2$ and SiCH$_2$*C*H$_2$), 21.2 (N$_3$CH$_2$CH$_2$*C*H$_2$CH$_2$), 24.6 (SiCH$_2$*C*H$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 32.6 (N$_3$CH$_2$*C*H$_2$), 43.8 (*C*H$_2$N), 51.1 (N$_3$CH$_2$), 78.9 (*C*Me$_3$), 155.9 (NH*C*OO). ^{29}Si NMR: 1.7 (SiMe), 2.0 (SiMe$_2$). MS (MALDITOF): 1344.9293 [M + H]$^+$. Anal. Calc. for $C_{65}H_{141}N_7O_8Si_7$ (1345.46): C, 58.02; H, 10.56; N, 7.29; Obt.: C, 58.21; H, 10.33; N, 7.11.

4.2.6. Synthesis of N3G3(NHBoc)8 (**6**)

Following the procedure described above for **4**, from **3** (1.05 g, 0.38 mmol) and NaN_3 (0.062 g, 0.95 mmol) afforded **6** as a white wax (0.82 g, 80%). NMR data ($CDCl_3$): ^{1}H NMR: −0.11 (s, 21H, Si*Me*), −0.07 (s, 48H, Si*Me*), 0.43 (m, 18H, C*H*$_2$Si), 0.51 (m, 56H, C*H*$_2$Si), 1.27 (m, 30H, CH$_2$*CH*$_2$CH$_2$), 1.42 (s, 88H, SiCH$_2$*CH*$_2$CH$_2$N) and (^{t}Bu), 1.60 (m, 2H, N$_3$CH$_2$*CH*$_2$), 3.05 (m, 16H, C*H*$_2$NH), 3.25 (t, J = 6.8 Hz, 2H, N$_3$C*H*$_2$), 4.59 (bs, 8H, N*H*). ^{13}C NMR: −5.1 and −5.0 (Si*Me*), −3.4 (Si*Me*$_2$), 12.4 (SiCH$_2$CH$_2$CH$_2$N), 13.5 (SiCH$_2$), 18.4, 18.5, 18.8, 18.9, 20.0 (SiCH$_2$) and (SiCH$_2$*C*H$_2$), 21.2 (N$_3$CH$_2$CH$_2$*C*H$_2$CH$_2$), 24.6 (SiCH$_2$CH$_2$CH$_2$NH), 28.4 (*C*Me$_3$), 32.6 (N$_3$CH$_2$*C*H$_2$), 43.8 (*C*H$_2$N), 51.1 (N$_3$CH$_2$), 78.9 (*C*Me$_3$), 156.0 (NH*C*OO). ^{29}Si NMR: 1.7 (SiMe), 2.1 (SiMe$_2$). Anal. Calc. for $C_{133}H_{289}N_{11}O_{16}Si_{15}$ (2720.06): C, 58.73; H, 10.71; N, 5.66; Obt.: C, 58.99; H, 10.87; N, 5.31.

4.2.7. Synthesis of 2-[bis-(tert-butyloxycarbonylmethyl)amino] ethyl bromide (**7**)

$KHCO_3$ was added (6.18 g, 61.8 mmol, 2.5 eq.) to a solution of tert-butyl bromoacetate (8.05 mL, 54.5 mmol, 2.2 eq.) in 50 mL of anhydrous DMF. The suspension was cooled at 0 °C, ethanolamine (1.48 mL, 24.5 mmol, 1 eq.) was added via a syringe over a 5-min period, and the solution was stirred at 0 °C for 30 min. The reaction mixture was then allowed to warm to room temperature and stirred for 22 h. After this time, most of the DMF was evaporated under vacuum and 50 mL of saturated $NaHCO_3$ was added. The resulting mixture was extracted with diethyl ether (3 × 50 mL), and the combined organic phases were washed with 50 mL of brine, dried with Na_2SO_4, and evaporated to afford the crude product *N,N*-bis[(*tert*-butoxycarbonyl)methyl]-2-hydroxyethylamine (7.41 g) as an oil. This crude product was dissolved in CH_2Cl_2 and solid PPh_3 (8.66 g, 33.0 mmol, 1.3 eq.) was added to the solution that was cooled at 0 °C. After that, solid NBS (5.87 g, 33.0 mmol, 1.3 eq.) was

added over a 5-min period and the mixture was stirred for 2 h at 0 °C. After this time the solvent was evaporated and a semisolid residue was obtained, which was then triturated with diethyl ether. The solid was filtered, washed with ether and discarded, and the ether phase was evaporated to afford an oily residue, which was purified by flash chromatography on silica (5 → 30% diethyl ether in hexane) yielding **7** (6.18 g, 71%).

NMR data of *N,N*-bis[(*tert*-butoxycarbonyl)methyl]-2-hydroxyethylamine ($CDCl_3$): ^{1}H NMR: 1.45 (s, 18H, ^{t}Bu), 2.82 (t, J = 4.89 Hz, 2H, $HOCH_2CH_2$), 3.38 (s, 2H, NCH_2CO), 3.46 (t, J = 4.98 Hz, 4H, $HOCH_2CH_2$), and 3.64 (bs, OH); ^{13}C NMR: 27.9 (CMe_3), 56.5 (NCH_2CO), 56.9 ($HOCH_2CH_2$), 58.2 ($HOCH_2CH_2$), 81.3 (CMe_3), 171.2 (NCH_2CO). MS: Theoretical mass for $C_{14}H_{27}O_5N$: 289.2; Mass found by HPLC-MS: 290.1 (M + 1).

NMR data of **7** ($CDCl_3$): ^{1}H NMR: 1.46 (s, 18H, ^{t}Bu (d)), 3.13 (t, J = 7.82 Hz, 2H, $BrCH_2CH_2$ (b)), 3.44 (t, J = 7.82 Hz, 2H, $BrCH_2CH_2$ (a)), and 3.48 (s, 4H, NCH_2CO (c)). ^{13}C NMR: 28.1 (CMe_3), 30.2 ($BrCH_2CH_2$), 56.5 (NCH_2CO), 56.6 ($BrCH_2CH_2$), 81.2 (CMe_3), 170.5 (NCH_2CO). MS: Theoretical mass for $C_{14}H_{26}BrO_4N$: 351.1; Mass found by HPLC-MS: 352.0 (M + 1).

4.2.8. Synthesis of 4-(prop-2-ynyl)-1,1,7,7-tetra(tert-butoxycarbonylmethyl)-1,4,7-triazaheptane (**8**)

Propargylamine (0.103 mL, 1.61 mmol) and DIEA (1.285 mL, 7.0 mmol) were added to a solution of bromoderivative **7** (1.250 g, 3.55 mmol) in 100 mL of acetonitrile. The resulting mixture was stirred at 80 °C for 24 h. Afterwards the acetonitrile was evaporated, and the resulting oil was dissolved in ethyl acetate. The organic phase was washed three times with an aqueous solution of 5% $NaHCO_3$, dried over $MgSO_4$ and evaporated. The yellow oil obtained was flash chromatographed on basic aluminum oxide (5 → 20% ethyl acetate in hexane), affording compound **8** (0.80 g, 83%). NMR data ($CDCl_3$): ^{1}H NMR: 1.45 (s, 36H, ^{t}Bu (f)), 2.15 (t, J = 2.2 Hz, 1H, NCH_2CCH (a)), 2.66 (t, J = 6.9 Hz, 4H, $NCH_2CH_2NCH_2CH_2N$ (c)), 2.84 (t, 6.9 Hz, 4H, $NCH_2CH_2NCH_2CH_2N$ (d)), 3.46 (s, 8H, NCH_2CO (e)), 3.54 (d, J = 2.2 Hz, 2H, NCH_2CCH (b)); ^{13}C NMR: δ: 28.2 (CMe_3), 42.0 (NCH_2CCH), 51.7 ($NCH_2CH_2NCH_2CH_2N$), 52.1 ($NCH_2CH_2NCH_2CH_2N$), 56.1 (NCH_2CO), 73.0 (NCH_2CCH), 78.7 (NCH_2CCH), 80.8 (CMe_3), 170.7 (NCH_2CO). MS: Theoretical mass for $C_{31}H_{55}N_3O_8$: 597.4. Experimental mass detected by LC-MS: 598.0 (M + 1). Experimental mass detected by HRMS: 598.4060 (M + 1).

4.2.9. Synthesis of 4-(prop-2-ynyl)-1,1,7,7-tetra(carboxymethyl)-1,4,7-triazaheptane (**9**)

Compound **8** (0.400 g, 0.66 mmol) was dissolved in 4.0 M HCl in dioxane (10 mL, 40 mmol) and the mixture was stirred overnight at room temperature. HCl and dioxane were then removed by evaporation and the resulting crude product was coevaporated with dioxane (×3), affording compound **9** as the corresponding hydrochloride salt (0.360 g), which was used for the next step without further purification.

4.2.10. Synthesis of alkyne-functionalized PEG dendron **10**

Solid PyBOP (1.510 g, 2.90 mmol) and 1-(*tert*-butyloxycarbonylamino)-4,7,10-trioxa-13-tridecanamine (0.930 g, 2.90 mmol) dissolved in 10 mL of CH_2Cl_2 were added to a solution of compound **9** in 500 mL of anhydrous CH_2Cl_2:DMF (7:3, v:v). The pH was adjusted to 8 with DIEA. After 1 h of stirring at room temperature, the solvents were removed in vacuo. The resulting crude product was dissolved in 100 mL of CH_2Cl_2 and washed with 5% $NaHCO_3$ (3 × 100 mL). The organic phase was evaporated, and the resulting crude product dissolved in CH_2Cl_2 (10 mL), was transferred to a 50-mL tube, and then hexane (40 mL) was added. The mixture was stirred vigorously and centrifuged. The supernatant was discarded, and the remaining oily precipitate corresponded to pure compound **10** (0.877 g, 84% yield). NMR data ($CDCl_3$): ^{1}H NMR: δ 1.43 (s, 36H, ^{t}Bu (r)), 1.78 (m, 16H, $NHCH_2CH_2CH_2O$ (h)), 2.26 (t, J = 2.2 Hz, 1H, NCH_2CCH (a)), 2.64 (m, 8H, $NCH_2CH_2NCH_2CH_2N$ (c, d)) 3.21 (m, 16H, $NCH_2CH_2CH_2O$ (g,p)), 3.34 (m, 8H, NCH_2CO (e)), 3.39 (d, J = 1.6 Hz, 2H, NCH_2CCH (b)), 3.53 (m, 16H, $NHCH_2CH_2CH_2O$ (n, i)), 3.57–3.66 (m, 32H, OCH_2CH_2O (j–m)), 5.10 (bs, NH (q)), 7.51 (bs, NH (f)). ^{13}C NMR ($CDCl_3$): δ 28.4 (CMe_3), 29.5 ($OCONHCH_2CH_2CH_2O$), 29.7 ($NCH_2CONCH_2CH_2CH_2O$), 37.1 ($NCH_2CONCH_2CH_2CH_2O$), 38.4 ($OCONHCH_2CH_2CH_2O$), 46.2 (NCH_2CCH), 51.7 ($NCH_2CH_2NCH_2CH_2N$), 53.2 ($NCH_2CH_2NCH_2CH_2N$), 59.0 (NCH_2CO), 69.4 ($OCONHCH_2CH_2CH_2O$), 69.5 ($NCH_2CONHCH_2CH_2CH_2O$), 70.2 (OCH_2CH_2O), 73.9 (CMe_3), 77.8 (NCH_2CCH), 78.9 (NCH_2CCH), 156.0 ($OCONHCH_2CH_2CH_2O$), 170.7 ($NCH_2CONCH_2CH_2CH_2O$). MS: Theoretical mass for $C_{75}H_{143}N_{11}O_{24}$: 1582.03. Experimental mass detected by LC-MS: 792.64 ((M + 2)/2). Experimental mass detected by HRMS: 1583.0358 (M + 1).

4.2.11. Synthesis of dendrimer **11**

Compounds **4** (0.069 g, 0.104 mmol) and **10** (0.150 g, 0.095 mmol) were stirred under argon atmosphere in a mixture of THF/H_2O (6/2 ml) at 40 °C in the presence of $CuSO_4$ (7% mol), Na Ascorbate (15% mol) and NEt_3 (20% mol) for 16 h. Afterwards, the reaction was treated with few drops of NH_4OH (30%) and the product was extracted with AcOEt against a saturated water solution of NH_4Cl. The organic phase was dried with $MgSO_4$, the solvent was removed under vacuum, and the remaining oil was washed with hexane (10 mL) to give compound **11** as yellowish oil (0.176 g, 83%). NMR data ($CDCl_3$): ^{1}H NMR: −0.07 (s, 15H, Si*Me* (e, i)), 0.47 (m, 6H, CH_2Si (d,j)), 0.52 (m, 8H, CH_2Si (f, h)), 1.23 (m, 6H, $CH_2CH_2CH_2$ (c, g)), 1.41 (m, 58H, $SiCH_2CH_2CH_2N$ and ^{t}Bu (k, n, r′)), 1.73 (m, 16H, $OCH_2CH_2CH_2N$ (o′)), 1.89 (m, 2H, $N{-}NCH_2CH_2$ (b)), 2.54 (t, J = 7.3 Hz, 2H, NCH_2CH_2N (c′)), 2.68 (t, J = 7.3 Hz, 2H, NCH_2CH_2N (d′)), 3.04 (m, 4H, CH_2NH (l)), 3.16 (m, 16H, CH_2NH and CH_2CO), 3.28 (m, 8H, CH_2NH), 3.45–3.60 (m, 48H, OCH_2 (j′–m′)), 3.70 (s, 2H, $C_{sp}CH_2N$ (b′)), 4.31 (t, J = 6.8 Hz, 2H, CH_2NN (a)), 4.64 (br. s, 2H, NH (m)), 5.05 (br. s, 4H, NH (q′)), 7.50 (s, 1H, $C_{sp}H$ (a′)), 7.75 (br. s, 4H, NH (f′)). ^{13}C NMR: −5.1 (SiMe), −3.3 ($SiMe_2$), 12.4 ($SiCH_2CH_2CH_2N$), 13.6 ($SiCH_2$), 18.4, 18.7, 20.0, 21.2 ($SiCH_2$ and $SiCH_2CH_2$), 24.7 ($SiCH_2CH_2CH_2NH$), 28.5 (CMe_3), 29.6 and 29.7 ($NHCH_2CH_2CH_2O$), 34.3 ($N{-}NCH_2CH_2$), 37.0 (CH_2NH), 38.5 (CH_2NH), 43.8 (CH_2NH), 46.3 ($C_{sp}CH_2N$), 50.1 (CH_2NN), 51.4 (NCH_2CH_2N), 52.7 (NCH_2CH_2N), 58.9 (CH_2CO), 69.4, 69.5, 70.2, 70.5 (OCH_2), 78.9 (CMe_3), 122.7 (CH═*C*), 143.5 (*C*H═C), 156.1 (NH*C*OO), 170.9 ($NHCOCH_2$). Anal. Calc. for $C_{106}H_{210}N_{16}O_{28}Si_3$ (2241.15): C, 56.81; H, 9.44; N, 10.00; Obt.: C, 56.59; H, 9.26; N, 9.74.

4.2.12. Synthesis of dendrimer **12**

Following the procedure described for **11**, reaction of **5** (0.147 g, 0.109 mmol) and **10** (0.150 g, 0.095 mmol) at 40 °C afforded **12** as yellowish oil (0.222 g, 80%). NMR data ($CDCl_3$): ^{1}H NMR: −0.11 (s, 9H, Si*Me*), −0.07 (s, 24H, Si*Me*), 0.47 (m, 10H, CH_2Si), 0.52 (m, 24H, CH_2Si), 1.23 (m, 14H, $CH_2CH_2CH_2$), 1.41 (m, 80H, $SiCH_2CH_2CH_2N$ and ^{t}Bu), 1.73 (m, 16H, $OCH_2CH_2CH_2N$), 1.89 (m, 2H, $N{-}NCH_2CH_2$), 2.54 (t, J = 7.3 Hz, 2H, NCH_2CH_2N), 2.68 (t, J = 7.3 Hz, 2H, NCH_2CH_2N), 3.04 (m, 8H, CH_2NH), 3.16 (m, 16H, CH_2NH, CH_2CO), 3.28 (m, 8H, CH_2NH), 3.45–3.60 (m, 48H, OCH_2), 3.70 (s, 2H, $C_{sp}CH_2N$), 4.30 (t, J = 6.8 Hz, 2H, CH_2NN), 4.64 (br. s, 4H, NH), 5.05 (br. s, 4H, NH), 7.48 (s, 1H, $C_{sp}H$), 7.73 (br. s, 4H, NH). ^{13}C NMR: −5.2 (SiMe), −4.9 (SiMe), −3.4 ($SiMe_2$), 12.4 ($SiCH_2CH_2CH_2N$), 15.3 ($SiCH_2$), 18.4, 18.7, 18.8, 19.0, 20.0, 21.2 ($SiCH_2$ and $SiCH_2CH_2$), 24.6 ($SiCH_2CH_2CH_2NH$), 28.5 (CMe_3), 29.7 ($NHCH_2CH_2CH_2O$), 34.3 ($N{-}NCH_2CH_2$), 37.0 (CH_2NH), 38.4 (CH_2NH), 43.8 (CH_2NH), 46.3 ($C_{sp}CH_2N$), 50.1 (CH_2NN), 51.4 (NCH_2CH_2N), 52.7 (NCH_2CH_2N), 58.7 (CH_2CO), 69.3, 69.5, 70.2, 70.4, 70.5 (OCH_2), 78.9 (CMe_3), 122.7 (CH═*C*), 143.5 (*C*H═C), 156.1

(NHCOO), 170.9 (NHCOCH$_2$). Anal. Calc. for $C_{140}H_{284}N_{18}O_{32}Si_7$ (2928.45): C, 57.42; H, 9.77; N, 8.61; Obt.: C, 57.31; H, 9.55; N, 8.44.

4.2.13. Synthesis of dendrimer **13**

Following the procedure described for **11**, reaction of **6** (0.151 g, 0.056 mmol) and **10** (0.080 g, 0.051 mmol) at 60 °C afforded **13** as yellowish oil (0.050 g, 23%), after washing with hexane (5 ml) and purification through size-exclusion chromatography. NMR data ($CDCl_3$): ^{1}H NMR: −0.07 (s, 15H, Si*Me*), 0.47 (m, 6H, C*H*$_2$Si), 0.52 (m, 8H, C*H*$_2$Si), 1.23 (m, 6H, CH$_2$C*H*$_2$CH$_2$), 1.41 (m, 122H, CH$_2$C*H*$_2$CH$_2$N and ^{t}Bu), 1.73 (m, 16H, OCH$_2$C*H*$_2$CH$_2$N), 1.89 (m, 2H, N−NC*H*$_2$), 2.54 (t, J = 7.3 Hz, 2H, NCH$_2$C*H*$_2$N), 2.68 (t, J = 7.3 Hz, 2H, NC*H*$_2$CH$_2$N), 3.04 (m, 16H, C*H*$_2$NH), 3.16 (m, 16H, C*H*$_2$NH, C*H*$_2$CO), 3.28 (m, 8H, C*H*$_2$NH), 3.45−3.60 (m, 48H, OC*H*$_2$), 3.70 (s, 2H, C$_{sp}$C*H*$_2$N), 4.30 (t, J = 6.8 Hz, 2H, C*H*$_2$NN), 4.64 (br. s, 8H, NH), 5.05 (br. s, 4H, NH), 7.48 (s, 1H, C$_{sp}$H), 7.73 (br. s, 4H, NH). ^{13}C NMR: −5.1 (SiMe), −3.4 (SiMe$_2$), 12.3 (SiCH$_2$CH$_2$CH$_2$N), 18.3, 18.7, 18.8, 19.0, 19.7 (SiCH$_2$ and SiCH$_2$CH$_2$), 23.3 (SiCH$_2$CH$_2$CH$_2$NH), 28.3 (C*Me*$_3$), 29.5 (NHCH$_2$CH$_2$CH$_2$O), 34.3 (N−NCH$_2$CH$_2$), 36.8 (*C*H$_2$NH), 38.3 (*C*H$_2$NH), 43.6 (*C*H$_2$NH), 46.3 (C$_{sp}$CH$_2$N), 50.1 (*C*H$_2$NN), 51.4 (NCH$_2$*C*H$_2$N), 52.7 (N*C*H$_2$CH$_2$N), 58.7 (*C*H$_2$CO), 69.1−70.4 (O*C*H$_2$), 78.7 (*C*Me$_3$), 129.4 (CH=*C*), 142.4 (*C*H=C), 155.8 (NH*C*OO), 155.9 (NH*C*OO), 170.7 (NH*C*OCH$_2$). Anal. Calc. for $C_{208}H_{432}N_{22}O_{40}Si_{15}$ (4303.06): C, 58.06; H, 10.12; N, 7.16; Obt.: C, 57.81; H, 9.98; N, 7.02.

4.2.14. Synthesis of dendrimer **14**

Compound **11** (0.150 g, 0.067 mmol) was mixed in CH_2Cl_2/H_2O (5 mL each) and then excess HCl was added (8.04 mmol), and the suspension was stirred at ambient temperature for 3 h. Afterwards, volatiles were removed under vacuum, and the solid was washed with Et_2O (2 × 15 ml) and finally ultrafiltrated with a membrane of MWCO = 500 to give compound **14** as white powder (0.113 g, 91%). NMR data (DMSO-d$_6$): ^{1}H NMR: −0.05 (s, 3H, Si*Me* (e)), −0.02 (s, 12H, Si*Me*$_2$ (i)), 0.55 (m, 14H, C*H*$_2$Si (f, h, j)), 1.30 (m, 10H, CH$_2$C*H*$_2$CH$_2$ and SiCH$_2$C*H*$_2$CH$_2$N (c, g, k)), 1.65−1.85 (m, 22H, CH$_2$C*H*$_2$CH$_2$N (b, h′, o′)), 2.73 (m, 2H, NCH$_2$C*H*$_2$N (c′)), 2.80 (m, 2H, NCH$_2$C*H*$_2$N (d′)), 3.05 (m, 2H, NC*H*$_2$CH$_2$N (d′)), 3.15−3.40 (m, 20H, C*H*$_2$NH and C*H*$_2$NH (l, n′, p′)), 3.50−3.90 (m, 58H, C*H*$_2$CO and OC*H*$_2$ (b′, j′−m′)), 4.35 (m, 2H, C*H*$_2$NN (a)), 7.67 (m, 1H, C$_{sp}$H (a′)), 8.14 (br. S, 30H, NH$_3^+$ (m, q′)), 8.74 (br. s, 4H, NH (f′)). ^{13}C NMR: −5.4 (SiMe), −3.9 (SiMe$_2$), 11.1 (SiCH$_2$CH$_2$CH$_2$N), 13.6 (SiCH$_2$), 17.5, 18.7, 21.1, 21.8, 22.5 (SiCH$_2$ and SiCH$_2$C*H*$_2$), 28.7, 29.2, (NCH$_2$CH$_2$CH$_2$O), 35.0 (N−NCH$_2$C*H*$_2$), 36.2 (*C*H$_2$NH), 41.1 (*C*H$_2$NH$_3$), 46.3 (C$_{sp}$CH$_2$N), 55.2 (NCH$_2$C*H*$_2$N), 59.3 (*C*H$_2$CO), 68.8−69.5 (OCH$_2$), 122.7 (CH=*C*), 143.5 (*C*H=C), 165.8 (NH*C*OCH$_2$). Anal. Calc. for $C_{76}H_{168}Cl_6N_{16}O_{16}Si_3$ (1859.22): C, 49.10; H, 9.11; N, 12.05; Obt.: C, 48.77; H, 8.90; N, 11.91.

4.2.15. Synthesis of dendrimer **15**

Following procedure described for **14**, reaction of **12** (0.150 g, 0.051 mmol) and excess HCl in Et_2O (12.24 mmol) afforded **15** as white powder (0.111 g, 90%). NMR data (DMSO-d$_6$): ^{1}H NMR: −0.07 (s, 33H, Si*Me* and Si*Me*$_2$), 0.55 (m, 30H, C*H*$_2$Si), 1.30 (m, 14H, CH$_2$C*H*$_2$CH$_2$ and SiCH$_2$C*H*$_2$CH$_2$N), 1.50−1.87 (m, 26H, CH$_2$C*H*$_2$CH$_2$N), 2.70 and 2.80 (m, 8H, C*H*$_2$NH$_3^+$), 3.05 (m, 4H, NC*H*$_2$CH$_2$N), 3.30−3.67 (br. s, 66H, C*H*$_2$NH, C$_{sp}$C*H*$_2$N, C*H*$_2$CO and OC*H*$_2$), 4.24 (m, 2H, C*H*$_2$NN), C$_{sp}$H not observed, 8.14 (br. s, 36H, NH$_3^+$), CONH not observed. ^{13}C NMR: −5.4 (SiMe), −3.9 (SiMe$_2$), 11.1 (SiCH$_2$CH$_2$CH$_2$N), 13.6 (SiCH$_2$), 17.5−22.1 (SiCH$_2$ and SiCH$_2$C*H*$_2$), 28.7, 29.2, (NCH$_2$CH$_2$CH$_2$O), 35.4 (*C*H$_2$), 41.1 (*C*H$_2$NH$_3$), 59.3 (*C*H$_2$CO), 68.8−69.5 (OCH$_2$), not observed (CH=*C*, *C*H=C and 165.8 NHCOCH$_2$). Anal. Calc. for $C_{100}H_{228}Cl_8N_{18}O_{16}Si_7$ (2419.21): C, 49.65; H, 9.50; N, 10.42; Obt.: C, 49.33; H, 9.31; N, 10.24.

4.2.16. Synthesis of dendrimer **16**

Following procedure described for **14**, reaction of **13** (0.075 g, 0.017 mmol) and excess HCl in Et_2O (8.16 mmol) afforded **16** as white powder (0.055 g, 89%). NMR data (DMSO-d$_6$): ^{1}H NMR: −0.07 (s, 69H, Si*Me* and Si*Me*$_2$), 0.55 (m, 74H, C*H*$_2$Si), 1.30 (m, 30H, CH$_2$C*H*$_2$CH$_2$ and SiCH$_2$C*H*$_2$CH$_2$N), 1.50−1.87 (m, 34H, CH$_2$C*H*$_2$CH$_2$N), 2.65−2.85 (br. s, 24H, C*H*$_2$NH$_3^+$), 3.05 (m, 4H, NC*H*$_2$CH$_2$N), 3.30−3.67 (br. s, 66H, C*H*$_2$NH, C$_{sp}$C*H*$_2$N, C*H*$_2$CO and OC*H*$_2$), 4.24 (m, 2H, C*H*$_2$NN), C$_{sp}$H not observed, 8.14 (br. s, 48H, NH$_3^+$), CONH not observed. The ^{13}C NMR spectrum of this compound was not done due to the difficult to obtain a sufficient amount. Anal. Calc. for $C_{148}H_{348}Cl_{12}N_{22}O_{16}Si_{15}$ (3539.20): C, 50.23; H, 9.91; N, 8.71; Obt.: C, 50.01; H, 9.71; N, 8.62.

4.3. Z potential and size measurements

The particle size and zeta-potential of the complexes were measured using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25 °C in a disposable Malvern plastic cuvette. The initial RNA concentration used was 250 nM. These samples were prepared and measured in 10 mmol/L Na-phosphate buffer, pH 7.4, which was filtered through 22 nm filter paper prior to use.

4.4. siRNA

All siRNA sequences were chosen from previously published results [34,47,48] and had inhibited HIV replication in experiments using transiently transfected cells. They were purchased from Dharmacon. Inc. (Lafayette, CO). The sequence of the siRNA was siNEF sense: GUGCCUGGCUAGAAGCACAdTdT, antisense: UGUGCUUCUAGCCAGGCACdTdT. The siRNA siNEF labeled with the fluorochrome cyanine 3 (Cy3) on the 5′ end of the sense strand was used to detect entry of siRNA into cells. In siRNA functionality experiments, a siRNA of random sequence was used as a negative control to test for sequence-specific effects (siRandom). This siRNA was siCONTROL Non-Targeting siRNA #2 and was designed and screened by Dharmacon to have no silencing effect on any human, mouse or rat genes.

4.5. Primary cell cultures

Blood samples were obtained from healthy anonymous donors from the transfusion centers of Albacete and Madrid, following national guidelines. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll-Hypaque density gradient (Rafer, Spain) following the current procedures of the Spanish HIV HGM BioBank [49]. PBMC were activated with the mitogen phytohemagglutinin (PHA) (2 μg/ml) and maintained in RPMI 1640 complete growth medium supplemented with 10% FBS and antibiotics along with interleukin-2 (20 U/ml).

4.6. Dendriplex and polyplex formation

Dendriplexes or polyplex were formed by mixing equal volumes of dendrimer (or PEI) and siRNA dissolved in OPTIMEM® I free of serum or antibiotics at concentrations depending on the −/+ charge ratio and molar concentration desired [34,48], with an incubation time of 15 min.

4.7. Heparin exclusion experiments

These experiments were used to test the strength of the binding between dendrimer and siRNA. They were carried out by mixing dendriplexes (−/+ ratio 4) of **14**, (−/+ ratio 8) **15** or (−/+ ratio 2) **17** with varying concentrations of Heparin (0.1, 0.2, 0.3, and 0.6 U/μg siRNA). The mixture was run on a 2% agarose gel, containing 1 μl/ml

of GelRed (Biotium), for 2 h at 90 V. The gels were photographed and the bands were quantified using the program ImageJ (1.38×, NIH, USA).

4.8. Cytotoxicity (MTT assay)

PBMC were seeded in 96-well plates in OPTIMEM® I medium containing 10% FBS (1.5 × 105 cells in 190 μl/well) and submitted to 10 μl treatment of siRNA alone or complexed with **14**, **15** or **17** at varying −/+ charge ratios. 20 h later, 20 μl MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) substrate solution (5 mg/ml) was added to the cells to measure mitochondrial activity. After 4 h, the supernatant was removed and the crystals formed were dissolved in 200 μl DMSO, and absorbance was measured at 550 nm with a reference of 690 nm. All points were performed in triplicate. Dextran (5 μM) and DMSO (15%) were used as positive and negative controls respectively.

4.9. HIV inhibition

PBMC stimulated with PHA for up to 72 h were infected with HIV NL4-3 at 0.05 or 0.01 MOI, respectively. The cells were washed twice with warm medium before being plated and treated with siRNA (250 nM) or dendriplexes at varying concentrations and −/+ charge ratios. The dendriplex treatment was applied to the infected cells within an hour of termination of the incubation with HIV. The dendriplex was formed in serum- and antibiotic-free OPTIMEM® I and incubated for 15 min at RT for the complex to form prior to its addition to the plated cells. Samples were collected every 24 h starting 24 h after treatment. They were then spun, and the supernatant was collected and assayed for viral concentration using the HIV protein p24 ELISA kit, following the manufacturer's instructions. The dilution factors depended on the MOI and the time point at which the sample was collected but ranged from 10^{-1} to 10^{-4}. Analogous procedure was conducted with the polyplex generated with PEI at −/+ charge ratio of 1:2 and used as control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.061.

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