Fuentes Paniagua, M.E., Serramia , M.J., Sánchez-Nieves Fernández, J., Alvarez , S., Muñoz Fernández, M.A., Gómez Ramírez, R. & Mata De La Mata, Francisco J. De La 2015, "Fluorescein labelled cationic carbosilane dendritic systems for biological studies", European Polymer Journal, vol. 71, pp. 61-72.

Available at http://dx.doi.org/10.1016/j.eurpolymj.2015.07.043

Fluorescein Labelled Cationic Carbosilane Dendritic Systems for Biological Studies

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Abstract

Cationic carbosilane dendrimers and dendrons labelled with one fluorescein unit have been synthesized. For dendrimers (generations 1-3), a random procedure was followed by successive addition of two types of thiol compounds to vinyl terminated derivatives, first one with $–NH_3Cl$ and second one with $–NMe_2HCl$ functions, subsequent reaction with FITC and finally by quaternization with MeI. For dendrons, the use of compounds with a $-NH_2$ group at the focal point and NMe_2 functions at the periphery allowed us to obtain the corresponding fluoresceinated cationic derivatives. The toxicity of these dendritic molecules was studied by MTT and their interaction with siRNA Nef by electrophoresis. Finally, second generation dendrimer and its dendriplexes with siRNA Nef were chosen as a model to analyze their *in vivo* biodistribution in a model of BALB/c mice. The highest levels for dendriplexes were found in spleen, and liver, followed by those in lymph nodes, while lower levels were found in kidneys. This distribution is in accordance with long circulation times.

Keywords: labelled dendrimers and dendrons, carbosilane, gene therapy, HIV, biodistribution.

1. Introduction

Nanomedicine can be defined as the use of macromolecules on the nanoscale range for diagnostics and/or therapeutics. As an example, nanotechnology-based systems can be used to deliver therapeutic entities such as small-molecule drugs, peptides, proteins, and nucleic acids, either alone or in combinations.[1-3] The biological transport processes are affected by: i) the physical attributes of the nanocarriers, as for example size, shape, and flexibility; ii) their chemical characteristics, including for instance the incorporation of active ligands for recognition by and triggering of biological receptors;[4] as well as iii) their biologic response, drug toxicity, bioavailability, cell death, and modification of drug pharmacokinetics.[5-7]

Polymers and macromolecular aggregates have been vastly employed to effectively protect, transport and deliver drugs to target sites.[8-14] Among them, the characteristics of dendritic macromolecules, as well-defined size and structure, flexibility, monodispersity and multivalent molecular surface,[15-22] make them very attractive for biomedical applications.[23-31] Furthermore, their multivalency can be employed to obtain multitasking therapeutics. The presence of a director group altogether with adequate active functions have served for treating infectious diseases,[32, 33] or cancer[34] by targeting particular tissues.

Gene therapy is the transportation of functional genetic material, such as plasmids, nucleic acids (DNA and RNA) and oligonucleotides to the intracellular milieu in order to treat or prevent disesases.[35] The instability of nucleic acids in the bloodstream requires the use of carriers, which compact them and prevent their degradation. For this purpose, macromolecular systems are used and particularly cationic derivatives that are able to form nanoconjugates with nucleic acids through electrostatic interactions. Hence, cationic dendrimers can be employed for this application.[36-44] The understanding of their behaviour in cellular uptake and biodistribution should be very helpful to determine the border of their application and the need to introduce modifications on the molecule to improve their behaviour. It has been reported that circulation time, route of elimination, and organ accumulation strongly depends on dendrimers generation (size), charge and terminal groups.[45-47] This knowledge is of prime importance for

the rationzalization of dendriplexes design and utility, and is evaluated with compounds decorated with chromophore units.

Our group is interested in biomedical applications of carbosilane dendrimers and water-soluble cationic derivatives have been used as delivery vectors,[48-52] binding oligonucleotides and siRNA and transporting them to the interior of a range of cell types.[48-52] Cationic carbosilane dendrimers with two ammonium groups and a Si-O bond per branch were able to successfully transfect human astrocytes crossing an in vitro blood brain barrier (BBB) model.[53] However, these dendrimers were water unstable due to the presence of this Si-O bond very close to the dendrimer surface, making difficult their manipulation, modification and hence, their widespread application.[51] Other related systems with a Si-C bond instead of the Si-O bond were obtained by hydrosilylation reactions, although they were not as effective as RNA transfecting agents.[54] For those reasons, we have looked for dendrimers decorated with one cationic charge per branch and presenting non hydrolysable bonds. As synthetic strategy we have chosen the thiol-ene addition that also facilitates the access to heterofunctionalized systems.[55] Moreover, we have demonstrated that a second generation cationic dendrimer so synthesized and labelled with a fluorescein moiety form a stable dendriplex with siRNA Nef and crosses the BBB in a BALB/c mouse model, without histological and morphological changes in different brain regions.[56]

In view of these results, herein we have spread our research on heterofunctionalized dendritic systems to the preparation of new dendrimers and dendrons with a fluorescein fragment on the periphery or at the focal point, respectively. These systems have been evaluated as carriers in gene therapy by studying their interaction with siRNA Nef and their biodistribution *in vivo* in a model of BALB/c mice (both dendrimer and dendriplex). For this last goal we have chosen the second generation dendrimer labelled with fluorescein.

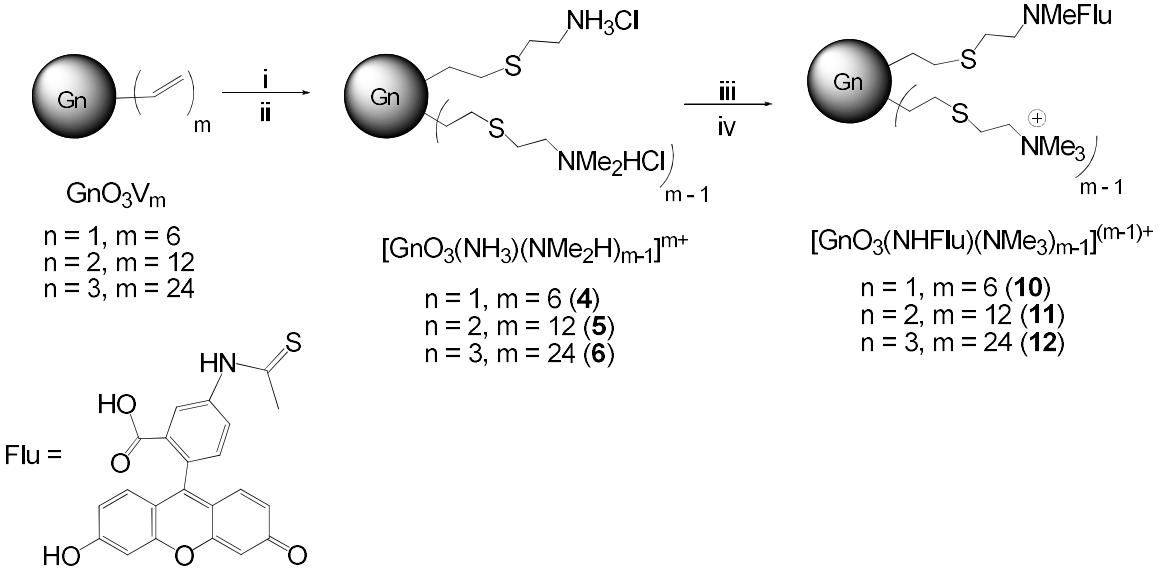
2. Results and Discussion

2.1. Synthesis of FITC labelled dendrimers and dendrons.

To obtain dendrimers and dendrons with cationic moieties $–NMe_3^+$ and with a fluorescein group we followed a random approach for dendrimers and a controlled procedure for dendrons. In both cases, the synthesis was performed from compounds containing one $–NH_2$ group and several $–NMe_2$ functions, employing the typical addition of a $–NH_2$ moiety to the isothiocyanate function of modified FITC (5'-isothiocyanatefluorescein) and then quaternization with MeI of dimethylamino ($–NMe_2$) peripheral groups. The amine groups on the periphery of carbosilane dendrimers and dendrons were introduced by thiol-ene click chemistry, which is a simple procedure to achieve this goal in carbosilane dendritic molecules with high yields.[57-60]

The reaction of vinyl dendrimers GnO_3V_m (n = 1, m = 6; n = 2, m = 12; n = 3, m =24)[59] with one equivalent of cysteamine hydrochloride $HS(CH_2)_2NH_3Cl$, under UV irradiation, allowed the introduction of one of this functions on the periphery of dendrimers (Scheme 1). The compounds $[GnO_3(NH_3)V_{m-1}]^+$ (n = 1, m = 6 (**1**); n = 2, m = 12 (**2**); n = 3, m = 24 (**3**)) thus formed were analyzed by NMR spectroscopy. The ^1H NMR spectra clearly showed the formation of the new chain $Si(CH_2)_2S$ by mean of the resonances at δ ca. 0.9 and δ ca. 2.5 for the methylene bonded to the Si and S atom, respectively.[55] Also, in this spectra were observed the resonances of the peripheral chain $S(CH_2)_2N$ at δ ca. 2.9 and δ ca. 3.2. The integral relationship between these resonances and those belonging to the remaining vinyl functions confirms the introduction of approximately one group. Next, addition of excess of the second thiol, $HS(CH_2)_2NMe_2{\cdot}HCl$, transformed the remaining vinyl functions and the corresponding dendrimers $[GnO_3(NH_3)(NMe_2H)_{m-1}]^{m+}$ (n = 1, m = 6 (**4**); n = 2, m = 12 (**5**); n = 3, m = 24 (**6**)) were obtained (Scheme 1). The monitorization of the reaction by NMR spectroscopy showed the disappearance of the vinyl groups. However, in the ^1H NMR spectra the methylene groups belonging to both type of peripheral chains (CH_2-NH_3^+ and CH_2-NMe_2H^+) were not distinguished due to overlapping of the signals. On the other hand, ^{13}C NMR spectra showed the resonances corresponding for the outer chains becoming from the different thiols employed. In the ^{15}N NMR spectroscopy, the only resonance observed was that corresponding to the cationic $–NMe_2H^+$ group.

Following the same protocol for second generation dendrimer $G_2O_3V_{12}$, but modifying the ratio of thiols $HS(CH_2)_2NH_3Cl/HS(CH_2)_2NMe_2HCl$, the heterofunctionalized cationic dendrimers $[G_2O_3(NH_3)_p(NMe_2H)_{12-p}]^{12+}$ (p = 2 (**5b**); p = 4 (**5c**) were prepared (Scheme S1, Supporting Information). These compounds showed analogous NMR data to those of derivatives **4-6**. These compounds could be useful to introduce simultaneously additional functionalities on dendritic periphery apart from a dye, such as a drug or a targeting moiety.

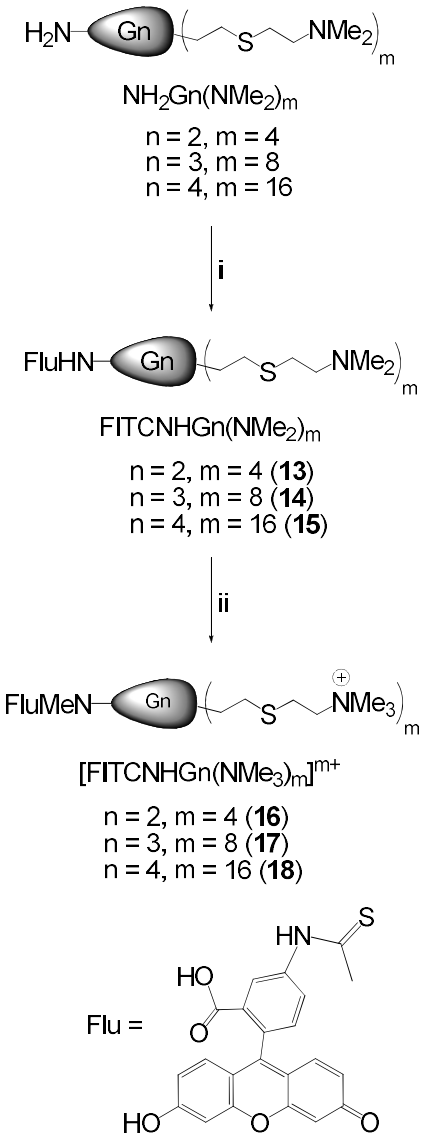


Scheme 1. Synthesis of generation 1-3 cationic carbosilane dendrimers labelled with fluorescein $[GnO_3(NMeFlu)(NMe_3)_{m-1}]^{m+}$ (n = 1, m = 6 (**10**); n = 2, m = 12 (**11**); n = 3, m =24 (**12**)). i) 1 eq. $HS(CH_2)_2NH_3Cl$, DMPA, UV; ii) excess $HS(CH_2)_2NMe_2HCl$, DMPA, UV; iii) FITC, excess NEt_3; iv) MeI.

The neutral labelled derivatives $[GnO_3(NMeFlu)(NMe_2)_{m-1}]$ (n = 1, m = 6 (**7**); n = 2, m = 12 (**8**); n = 3, m = 24 (**9**)) were formed by addition of 5'-fluorescein isothiocyanate (FITC) to compounds **4-6** (Scheme 1), in the presence of excess NEt_3. The finalization of the reaction was checked by the negative result of Kaiser's test. 2D-DOSY-NMR experiments were especially useful to confirm the formation of these compounds as just one diffusion coefficient for dendrimer and fluorescein fragments was observed. However, for the initial mixture of dendrimer and FITC two different diffusion values were obtained, being clearly higher the one belonging to the FITC molecule due to its smaller size. Afterward, to

compounds **7-9**, without purification, slight excess of MeI was added to furnish $[GnO_3(NMeFlu)(NMe_3)_{m-1}]^{(m-1)+}$ (n = 1, m = 6 (**10**); n = 2, m = 12 (**11**); n = 3, m = 24 (**12**)). Dendrimers **10-12** were obtained as orange solids soluble in DMSO and water with high yields (> 80 %). NMR spectroscopy confirmed formation of compounds **10-12**, mainly by the shifting to higher frequency of the resonances belonging to the groups bound to the cationic nitrogen atoms (CH_2 and Me). The addition of MeI also produced methylation of the nitrogen atom that acts as linker to the dendritic structure ($C(S)N(Me)CH_2$, *ca*. 2.80 ppm in ^1H NMR spectra and *ca*. 42.0 ppm in ^{13}C NMR spectra, respectively; Figure S5 in Supporting Information). This transformation does not modify the fluorescence properties of the fluorescein moiety. As for neutral compounds, 2D-DOSY-NMR spectra showed a unique diffusion coefficient for the whole molecule, confirming the bonding of the cromophore in these dendrimers (Figure S6, Supporting Information)

For the synthesis of related dendrons we started from compounds $NH_2Gn(NMe_2)_m$ (n = 2, m = 4; n = 3, m = 8; n = 4, m = 16) with $–NH_2$ and $–NMe_2$ groups at the focal point and on the periphery respectively.[59] From them, and employing the same methodology as described for dendrimers **10-12**, the corresponding dendrons $[(FluN(Me))Gn(NMe_3^+)_m]^{m+}$ (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m = 16 (**18**)) were obtained as orange solids soluble in DMSO and water. Again, Kaiser's tests and 2D-DOSY-NMR spectra confirmed the disappearance of the $–NH_2$ moiety and formation of cationic derivatives containing the fluorescein fragment (Figure S7, Supporting Information). Furthermore, 2D-TOCSY NMR spectroscopy showed the presence of the new chain formed at the focal point (Figure S8, Supporting Information). Also in this case methylation of the fluorescein nitrogen atom bonded to the dendron was observed.



Scheme 2. Synthesis of generation 2-4 cationic carbosilane dendrons labelled with fluorescein (Flu) $[(FluN(Me))Gn(NMe_3)_m]^{m+}$ (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m =16 (**18**)). i) FITC, NEt_3;ii) MeI.

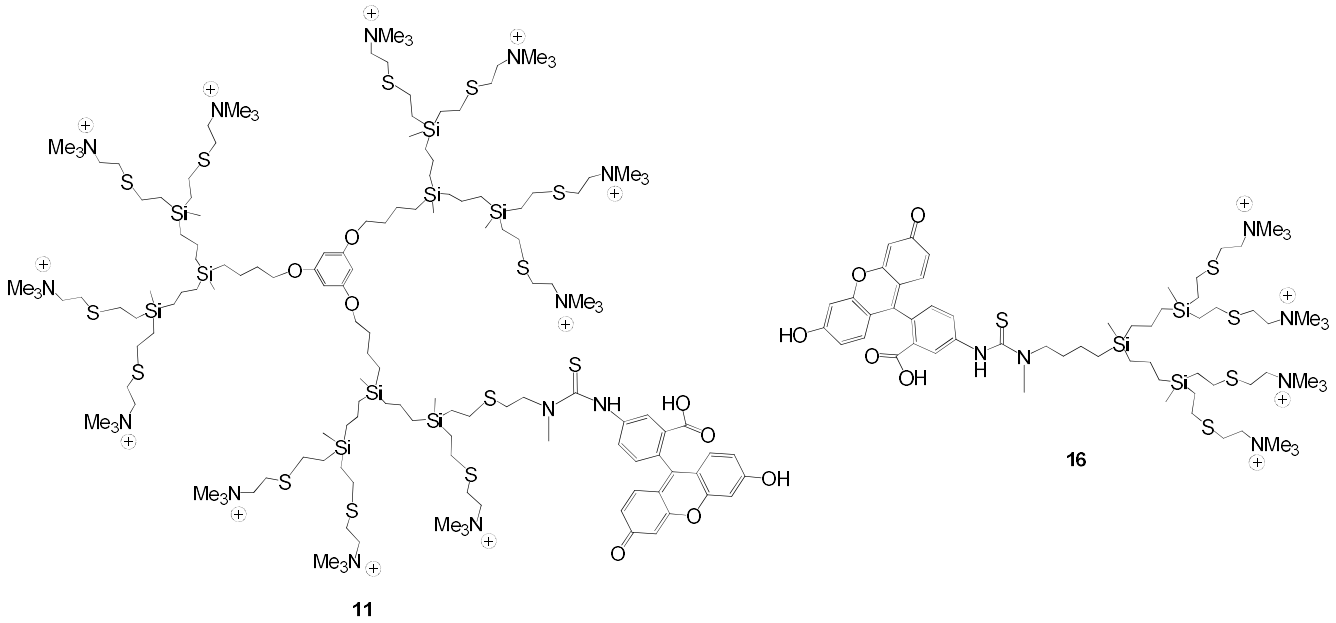


Figure 1. Drawing of cationic fluorescein carbosilane dendrimer and dendron of second generation (iodide anions are omitted for clarity).

2.2. Biological assays

Toxicitiy and dendriplex formation

Previously to biodistribution studies we have evaluated the toxicity of non-labelled dendrimers $[GnO_3(NMe_3)_m]^{m+}$ (n = 1, m = 6 (**19**); n = 2, m = 12 (**20**); n = 3, m =24 (**21**)),[55] labelled dendrimer $[G2O_3(NMeFlu)(NMe_3)_{11}]^{11+}$ (**11**), and labelled dendrons $[(FluN(Me))Gn(NMe_3)_m]^{m+}$ (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m =16 (**18**)), as well as dendriplexes formation with siRNA Nef and toxicity of them. These assays were carried out in peripheral blood mononuclear cells (PBMC), as primary cells.

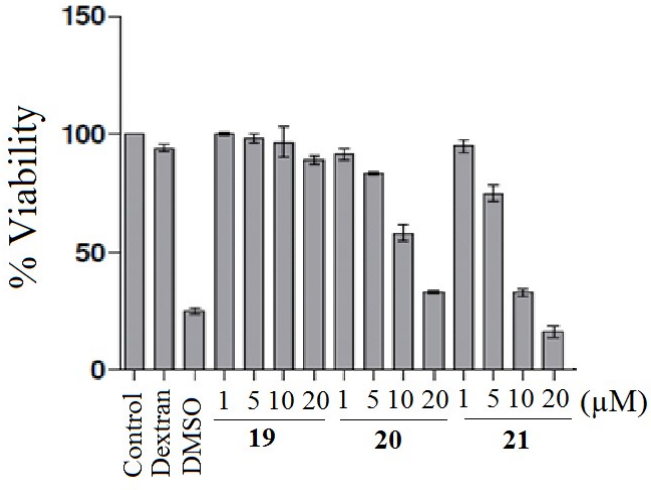


Figure 2. Cytotoxicity (MTT) of $[GnO_3(NMe_3)_m]^{m+}$ (n = 1, m = 6 (**19**); n = 2, m = 12 (**20**); n = 3, m =24 (**21**)) in PBMC after 48 h of treatment with the different nanosystems at various concentrations. Data are presented as percent of living cells compared with control cells as 100% of viable cells. Data were obtained from three independent experiments.

Metabolic activity assays of dendrimers **19-21** alone were tested by MTT (Figure 2). Generation-dependent data were obtained, with cellular viability over 80%, below 20 μM for **19**, 5 μM for **20**, and 1 μM for **21**. The interaction between dendrimers **19-21** and siRNA Nef was determined by gel electrophoresis at time of 24 h (Figure 3). From this study, complete dendriplex formation was observed for +/− ratios of 12/1 for **19** and **20** and of 8/1 for **21**. Furthermore, cell viability of these dendriplexes measured by MTT analyses showed that they were not toxic at the concentration chosen for further analyses (100 nM of siRNA Nef corresponding to 8.4 μM for **19**, 4.2 μM for **20** and 1.4 μM for **21**) (Figure S13, Supporting Information).

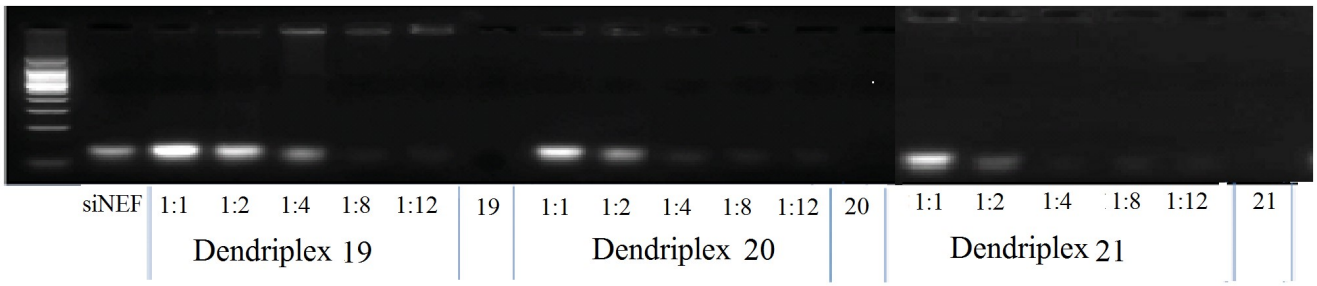


Figure 3. Dendriplex stability. Electrophoresis gel in agarose of dendriplexes $[GnO_3(NMe_3)_m]^{m+}$/siRNA

Nef at different siRNA Nef:dendrimer charge ratios after 24 h (n = 1, m = 6 (**19**); n = 2, m = 12 (**20**); n = 3, m =24 (**21**)).

Cationic dendrimer $[G_2O_3(NMeFlu)(NMe_3)_{11}]^{11+}$ (**11**) was chosen to compare the behaviour of non-labelled and labelled dendrimers with siRNA Nef described in this report, as usually second generation carbosilane dendrimers present the best balance between toxicity and activity. Mitochondrial activity measured by the MTT assay in PBMCs after treatment with $[G_2O_3(NMeFlu)(NMe_3)_{11}]^{11+}$ (**11**) alone for 48 hours remained higher than the toxicity limit of 80% up to 5 μM concentration (Figure 4). This value is the same than that found for the corresponding non-labelled dendrimer $[G_2O_3(NMe_3)_{12}]^{12+}$. Gel electrophoresis also showed a similar behavior to that of non-labelled dendrimer **20** in the case of interaction between dendrimer $[G_2O_3(NMeFlu)(NMe_3)_{11}]^{11+}$ (**11**) and siRNA (Figure 5). Previously, an analogous behaviour of these dendrimers was observed in primary NHA (normal human astrocytes).[56]

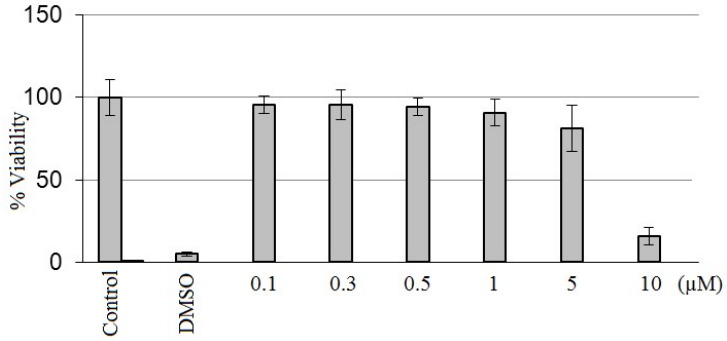


Figure 4. Cytotoxicity (MTT) of $[G_2O_3(NMeFlu)(NMe_3)_{11}]^{11+}$ (**11**) in PBMC after 48 h. Data are presented as percent of living cells compared with control cells as 100% of viable cells. Data are of three independent experiments.

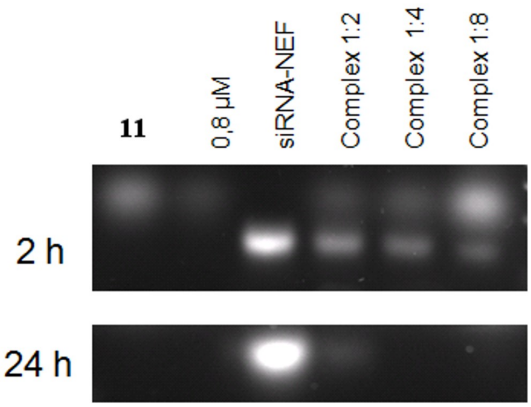


Figure 5. Dendriplex stability. Electrophoresis gel in agarose of dendriplexes **11**/siRNA Nef at different siRNA Nef:dendrimer charge ratios after 2 h and 24 h.

In a similar way than for dendrimers, toxicity and dendriplex formation was studied for fluorescein derivatized dendrons $[(FluN(Me)Gn(NMe_3)_m]^{m+}$ (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m =16 (**18**)). The metabolic activity assays of dendrons **16-18** on PBMCs by MTT showed also generation-dependent values, with cellular viability over 80% below 20 µM for **16**, 10 µM for **17**, and 5 µM for **18** (Figure 6). With respect to the ability to complex siRNA Nef, the electrophoretic analysis revealed weaker interactions between dendrons and siRNA than those observed for dendrimers. In the case of fourth generation dendron $[(FluN(Me))G_4(NMe_3)_{16}]^{16+}$ (**18**), higher stability was observed as consequence of its higher charge, although dendriplex formation was only observed when using +/− ratios as higher as 24/1(Figure 7) at long incubation times (48 h).

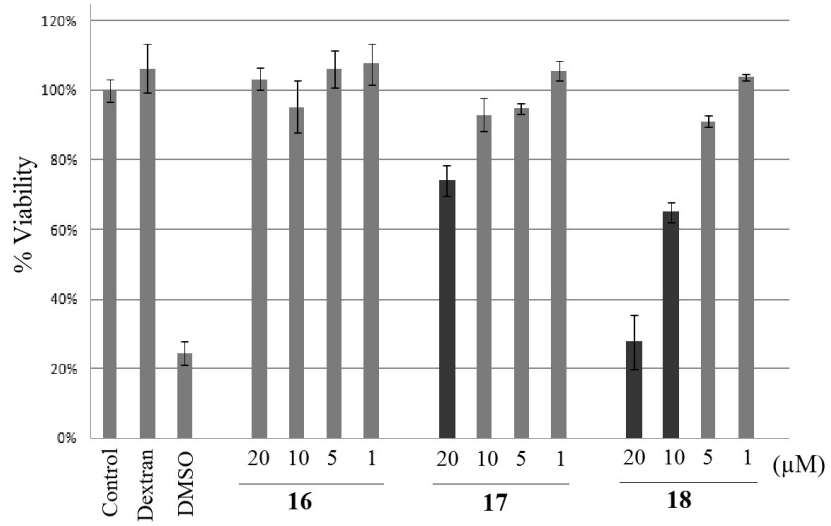


Figure 6. Cytotoxicity assay (MTT) of $[(FluN(Me))Gn(NMe_3)_m]^{m+}$ (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m =16 (**18**)) at different concentrations in PBMC after 48 h. Data are presented as percent of living cells compared with control cells as 100% of viable cells. Data are of three independent experiments.

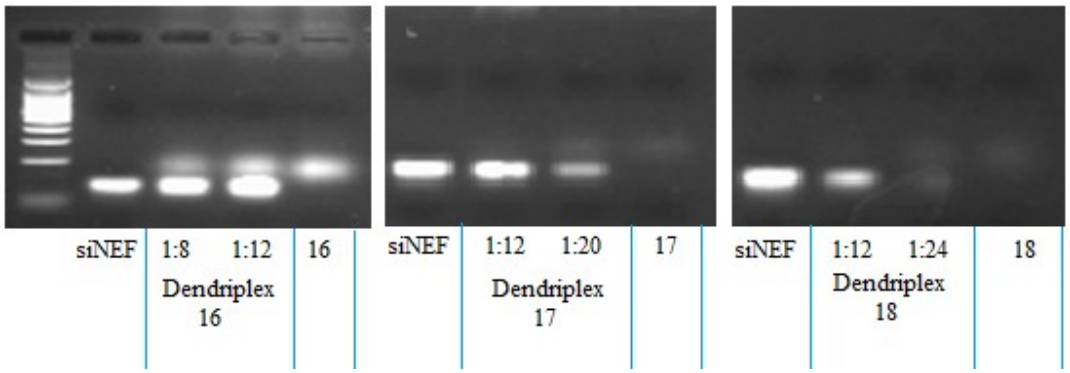


Figure 7. Dendriplex stability. Electrophoresis gel in agarose of dendriplexes $[(FluN(Me))Gn(NMe_3)_m]^{m+}$/siRNA Nef at different siRNA Nef:dendrimer charge ratios after 48 h (n = 2, m = 4 (**16**); n = 3, m = 8 (**17**); n = 4, m =16 (**18**)).

***Ex vivo* imaging: Analysis of biodistribution by IVIS system**

Taking into account the previous results, the labelled dendritic system used for distribution analysis was the second generation dendrimer $[G_2O_3(NMeFITC)(NMe_3)_{11}]^{11+}$ (**11**). Body distribution of this system was studied *in vivo* after systemic administration, using the dendriplex formed between fluorescein-

labelled dendrimer (**11**) and siRNA labelled with Cy5.5 (siRNA-Cy5.5) at a ratio **11**/siRNA Nef 3/1, and comparing with free dendrimer **11** and siRNA-Cy5.5. The fluorescence of dendrimers, siRNA, or dendriplex was analyzed using IVIS imaging system (IVIS Gamma Counter Packard 5005, Packard Instruments, Meriden, CT).

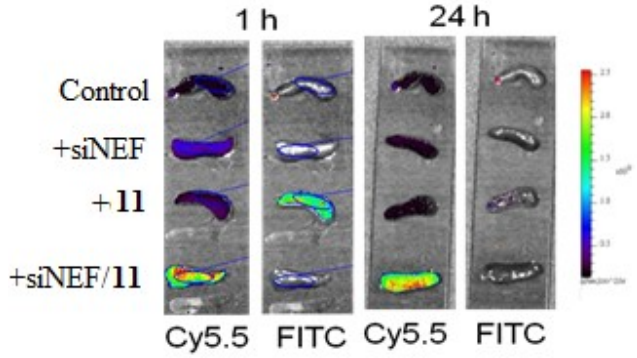


Figure 8. Distribution of dendrimer **11**, siRNA NEF, and dendriplex **11**/siRNA (15 mg/Kg) in spleen of BALB/c mice after 1 h and 24 h of administration. Analysis of biodistribution by IVIS system. The intensity of fluorescence was represented on composite light/fluorescent images by different colors with blue color reflecting the lowest fluorescence and red color – the highest intensity.

In the mouse spleen (Figure 8), which is the responsible of the reticuloendothelial system (RES) for the clearance of foreign materials, high accumulation of siRNA-Cy5.5 from the dendriplex was found at 1.h post injection (p. i.) that still remained after 24 h, whereas siRNA-Cy5.5 alone was not able to reach this organ. In accordance with retention of dendriplex by RES is the important accumulation observed in the liver (Figure S14, Supporting Information).

In the case of treatment with dendrimer **11** alone, its presence in the spleen and liver is showy because it is a second generation dendrimer and accumulation in these organs usually occurs for higher generation dendrimers as sixth generation PAMAM.[61] This fact is probably related with aggregation in aqueous medium that could favor longer circulation times.[62, 63]

Moreover, the results from these analyses also demonstrated that the **11**/siRNA-Cy5.5 nanoparticles distributed to and accumulated in the lymph nodes, being the amount of siRNA detected not as higher as in the organs mentioned before (Figure 9). At 24 hours it is important to note that the signal does not

disappear, confirming the presence of the dendriplex. When kidneys were analyzed, no bioluminescence was detected for the dendriplex at any time studied in this localization (Figure S15, Supporting Information). It is important to note that dendrimer alone also is well distributed in lymph nodes and in kidneys.

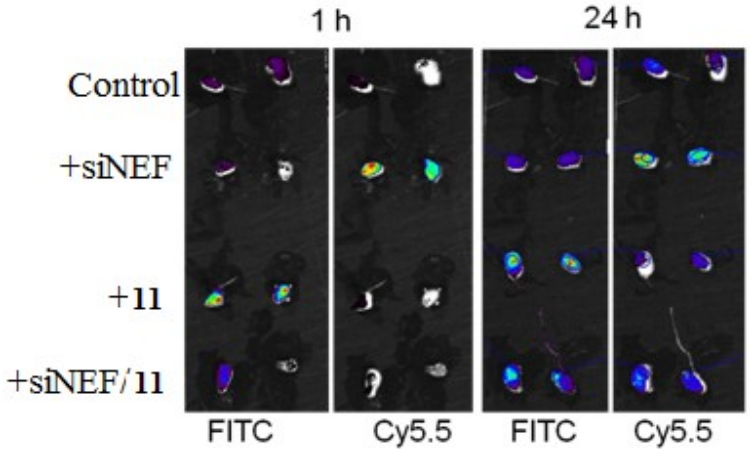


Figure 9. Distribution of dendrimer **11**, siRNA NEF, and dendriplex **11**/siRNA (15 mg/Kg) in lymph nodes of BALB/c mice after 1 h and 24 h of administration. Analysis of biodistribution by IVIS system. The intensity of fluorescence was represented on composite light/fluorescent images by different colors with blue color reflecting the lowest fluorescence and red color – the highest intensity.

Analysis in tissue sections of dendriplex distribution

Confocal microscopy was used for histological visualization of the transport and biodistribution of dendrimer. For this, the BALB/c mice were systemically injected with the dendrimer, siRNA or dendriplex as described above, and 1 h.p.i. sections of OCT-embedded tissues were prepared and analyzed by confocal microscopy. As the higher intensity of dendriplex was found in spleen, we then collected this organ for confocal analysis (Figure 10).

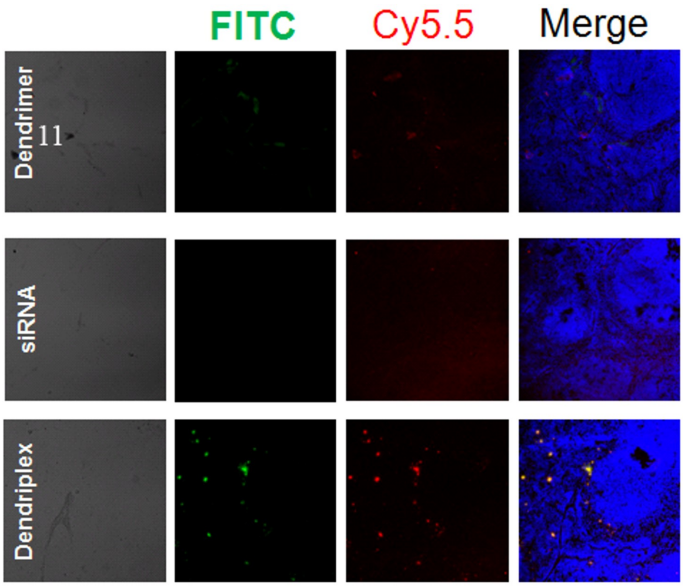


Figure 10. Confocal analysis of spleen of mice after different treatments at 1 h.p.i. Nuclei were stained with DAPI.

As shown in the figure, there was not Cy5.5 signal fluorescence in spleen of animal administered with fluorescein-dendrimer **11**, and indeed the signal in the fluorescein channel was very soft. In the same way, we did not detect Cy5.5 in spleen of animals injected with siRNA. However, it was easy to see both positive signals for FITC and Cy5.5 in spleen of animals administered with dendriplex, validating the dendrimer as siRNA carrier to this organ.

4. Conclusions

Stable fluorescein-labelled cationic carbosilane dendrimers and dendrons can be obtained following a random procedure for the first or a controlled process for the second. In the case of dendrimers, the use of thiol-ene addition proved to be very useful for the surface heterofunctionalization, whereas for the synthesis of the analogous fluorescein-labelled cationic carbosilane dendrons the use of compounds with a -NH_2 group at the focal point and –NMe_2 groups at the periphery was a suitable choice. All the cationic dendritic molecules were soluble in aqueous media, which is very important for biomedical applications.

The presence of a fluorescein moiety in dendrimers did not modify toxicity neither conditions for

dendriplex formation with respect to non-labelled dendrimers. Regarding dendrons, their topology hindered the formation of stable dendriplexes, and only a stable system was achieved for fourth generation dendron, with sixteen positive charges, at very high +/- ratio.

Biodistribution studies of a second generation labelled dendrimer **11** show that this dendrimer distribute either alone but more importantly complexed with the siRNA into the majority of tissues tested and remains relatively stable over 24 hours. This may prove its usefulness in the future for systemic *in vivo* delivery of agents (e.g., DNA or drugs) to numerous tissues.

4. Experimental Section

4.1. General Considerations. All reactions were carried out under inert atmosphere. 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 (1H), 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). Thiol-ene reactions were carried out employing a HPK 125 W mercury lamp from HeraeusNoblelight with maximum energy at 365 nm, in normal glassware under inert atmosphere.Compounds, $HS(CH_2)_2NH_2{\cdot}HCl$, 2,2'-dimethoxy-2-phenylacetophenone (DMPA), MeI, NEt_3, 5'-isocyanatefluorescein (Aldrich), $HS(CH_2)_2NMe_2{\cdot}HCl$ (Acros), and K_2CO_3 (Panreac) were obtained from commercial sources.Compounds GnO_3V_m (n = 1, m = 6; n = 2, m = 12; n = 3, m = 24)[55]and $NH_2Gn(NMe_2)_m$(n = 2, m = 4; n = 3, m = 8)[59] were synthesized as published.

4.2. Synthesis of compounds.

In this section the synthesis of first generation dendrimers and second generation dendrons is described. The procedures and data for all the new compounds described in this paper are collected in the Supporting

Information.

Synthesis of $[G_1O_3(NH_3Cl)V_5]$ (1).

$G_1O_3V_6$ (0.389 g, 0.70 mmol), cysteamine hydrochloride (0.075 g, 0.70 mmol) and 10 mol % of DMPA (0.018 g, 0.07 mmol) were combined in 3 ml of ½ THF/methanol solution. The reaction mixture was deoxygenated and irradiated for 0.5 h. The reaction mixture was directly used to obtain **4**. Compound **1** could also be aisled with yields around 87 % using a column chromatography in THF with Bio-Beads SX-1 (Bio-Rad) as stationary phase.

NMR data ($CDCl_3$): ^{1}H NMR: δ 0.08 and 0.12 (s, 9 H, Si*Me*), 0.67 (t, J_a = 8.3 Hz, 6 H, $OCH_2CH_2CH_2CH_2Si$), 0.90 (t, J_b = 8.5 Hz, 2 H, $SiCH_2CH_2S$), 1.46 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 1.76 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 2.55 (t, J_b = 8.5 Hz, 2 H, $SiCH_2CH_2S$), 2.89 (m, 2 H, $SCH_2CH_2NH_3$), 3.20 (m, 2 H, $SCH_2CH_2NH_3$), 3.87 (t, J_c = 6.2 Hz, 6 H, OCH_2), 5.70 and 6.07 (m, 18 H, SiC_2H_3 and $C_6H_3O_3$). ^{13}C NMR: δ -5.4 (Si*Me*), 13.6 ($OCH_2CH_2CH_2CH_2Si$), 14.5 ($SiCH_2CH_2S$), 20.2 ($OCH_2CH_2CH_2CH_2Si$), 27.2 ($SiCH_2CH_2S$), 28.8 ($SCH_2CH_2N^+$), 32.7 ($OCH_2CH_2CH_2CH_2Si$), 39.0 ($SCH_2CH_2N^+$), 67.4 (OCH_2), 93.6 ($C_6H_3O_3$; *C*-H), 132.8 ($SiCHCH_2$), 136.6 ($SiCHCH_2$), 160.8 ($C_6H_3O_3$; *C*-O).

Synthesis of $[G_1O_3(NH_3Cl)(NMe_2HCl)_5]$ (4).

To the reaction mixture of **1**, 2-(dimethylamino)ethanothiol hydrochloride (0.497 g, 3.51 mmol) and DMPA (0.090 g, 0.35 mmol) were added and the reaction mixture was irradiated for another 3 h and monitored by ^{1}H NMR. The final reaction mixture was concentrated by rotatory evaporation and redissolved in water. Afterward, it was purified by nanofiltration with a membrane of MWCO = 500. Volatiles are removed to get **1** as a white solid (0.660 g, 67 %).

NMR data (DMSO-d_6): ^{1}H NMR: δ 0.03 (s, 9 H, Si*Me*), 0.61 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 0.88 (m, 12 H, $SiCH_2CH_2S$), 1.40 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 1.69 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 2.61 (m, 12 H, $SiCH_2CH_2S$), 2.72 (s, 30 H, $SCH_2CH_2NMe_2H^+$), 2.90 (m, 12 H, $SCH_2CH_2N^+$), 3.19 (m, 12 H, $SCH_2CH_2N^+$), 3.90 (m, 6 H, OCH_2), 4.13 (bs, 3 H, $-NH_3^+$), 6.03 (s, 3 H, $C_6H_3O_3$), 10.85 (bs, 5 H, $-NMe_2H^+$). ^{13}C NMR: δ -4.8 (Si*Me*), 13.2 ($OCH_2CH_2CH_2CH_2Si$), 14.4 ($SiCH_2CH_2S$), 20.2

($OCH_2CH_2CH_2CH_2Si$), 25.0 ($SCH_2CH_2N^+$), 26.7 ($CH_2SC_2H_4NH_3^+$), 26.9 ($CH_2SC_2H_4NMe_2H^+$), 28.1 ($SCH_2CH_2NH_3^+$), 33.0 ($OCH_2CH_2CH_2CH_2Si$), 38.9 ($SCH_2CH_2NH_3^+$), 42.3 (-NMe_2H^+), 56.2 ($SCH_2CH_2NMe_2H^+$), 67.5 (OCH_2), 94.1 ($C_6H_3O_3$; *C*-H), 161.0 ($C_6H_3O_3$; *C*-O). ^{15}N NMR: δ -338.3 (-NMe_2H^+). ^{29}Si NMR: δ 3.1 (G_1–*Si*Me). Anal. Calcd. $C_{55}H_{122}Cl_6N_6O_3S_6Si_3$ (1404.96 g/mol): C, 47.02; H, 8.75; N, 5.98; S, 13.69; Found: C, 46.72; H, 8.44; N, 5.53; S, 13.17.

Synthesis of $[G_1O_3(NH_2)(NMe_2)_5]$.

To a H_2O/CH_2Cl_2 (1:1, 20 ml) mixture of **4** (0.310 g, 0.22 mmol), a NaOH solution was added drop by drop (0.063 g, 1.58 mmol). The reaction mixture was stirred for 15 minutes at room temperature, and finally the aqueous phase was removed. The organic phase was dried in Na_2SO_4 and finally filtered and evaporated in vacuo to afford $G_1O_3(NH_2)(NMe_2)_6$ as a pale yellow oil (0.261 g, 100%).

NMR data ($CDCl_3$): ^{1}H NMR: δ 0.02 (s, 9 H, Si*Me*), 0.58 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 0.91 (m, 12 H, $SiCH_2CH_2S$), 1.43 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 1.75 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 2.23 (s, 30 H, $SCH_2CH_2NMe_2$), 2.49 (m, 12 H, SCH_2CH_2N), 2.52 (m, 12 H, $SiCH_2CH_2S$), 2.59 (m, 10 H, $SCH_2CH_2NMe_2$), 2.82 (m, 2 H, $SCH_2CH_2NH_2$), 3.88 (m, 6 H, OCH_2), 6.03 (s, 3 H, $C_6H_3O_3$). ^{13}C NMR: δ -5.3 (Si*Me*), 13.4 ($OCH_2CH_2CH_2CH_2Si$), 14.6 ($SiCH_2CH_2S$), 20.4 ($OCH_2CH_2CH_2CH_2Si$), 27.2 ($CH_2SCH_2CH_2NH_2$), 27.7 ($SiCH_2CH_2S$), 29.9 ($SCH_2CH_2NMe_2$), 33.1 ($OCH_2CH_2CH_2CH_2Si$), 36.3 ($SCH_2CH_2NH_2$), 41.2 ($SCH_2CH_2NH_2$), 45.4 ($SiCH_2CH_2NMe_2$), 59.3 ($SCH_2CH_2NMe_2$), 67.4 (OCH_2), 93.8 ($C_6H_3O_3$; *C*-H), 160.9 ($C_6H_3O_3$; *C*-O). ^{15}N NMR: δ -352.1 (-NMe_2). ^{29}Si NMR: δ 2.4 (G_1–*Si*Me). Anal. Calcd. $C_{55}H_{116}N_6O_3S_6Si_3$ (1186.19 g/mol): C, 55.69; H, 9.86; N, 7.08; S, 16.22; Found: C, 55.26; H, 9.53; N, 6.97; S, 15.88.

Synthesis of $[G_1O_3(NMeFlu)(NMe_3)_5][I]_5$ (10).

Over a solution of **4** (0.225 g, 0.16 mmol) in EtOH/THF (8 ml), an excess of FITC (0.066 g, 0.17 mmol) and NEt_3 (0.22 ml, 1.58 mmol) were added and the mixture was stirred at room temperature in dark for 16 h. Later on volatiles were removed and the remaining oil was washed with water twice and filtered in THF to a new schlenck. An excess of MeI (0.05 ml, 0.80 mmol) was added and the resulting mixture was

stirred overnight. Afterward, volatiles were removed and remains were washed with 5 ml of EtOH twice and purified by nanofiltration with a membrane of MWCO = 500. The product was obtained as an orange solid (0.302 g, 82 %).

NMR data (DMSO-d_6): ^{1}H NMR: δ 0.05 (s, 9 H, Si*Me*), 0.63 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 0.88 (m, 12 H, $SiCH_2CH_2S$), 1.40 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 1.69 (m, 6 H, $OCH_2CH_2CH_2CH_2Si$), 2.42 (bs, 1 H, SC_2H_4NHCS), 2.64 (m, 12 H, $SiCH_2CH_2S$), 2.80 (s, 3 H, -NHC(S)N*Me*-), 2.90 (m, 12 H, $SCH_2CH_2NMe_3^+$), 3.10 (s, 45 H, $SCH_2CH_2NMe_3^+$), 3.54 (m, 12 H, $SCH_2CH_2NMe_3^+$), 3.89 (t, 6 H, OCH_2), 6.03 (s, 3 H, $C_6H_3O_3$), 6.55, 6.67 and 7.00 - 8.50 (m, 9 H, C_{Ar}-*H*). ^{13}C NMR: δ -5.7 (Si*Me*), 12.3 ($OCH_2CH_2CH_2CH_2Si$), 13.5 ($SiCH_2CH_2S$), 19.3 ($OCH_2CH_2CH_2CH_2Si$), 23.1 ($SCH_2CH_2NMe_3^+$), 26.3 ($SiCH_2CH_2S$), 32.0 ($OCH_2CH_2CH_2CH_2Si$), 41.8 (-NHC(S)N*Me*-), 51.7 ($SiCH_2CH_2NMe_3^+$), 64.0 ($SCH_2CH_2NMe_3^+$), 66.7 (OCH_2), 93.1 ($C_6H_3O_3$; *C*-H), 101.8, 109.3, 112.1, 128.6, 151.4 and 158.9 (C_{Ar}-H), 160.0 ($C_6H_3O_3$; *C*-O). ^{15}N NMR: δ -329.9 (-NMe_3^+). ^{29}Si NMR: δ 3.3 (G_1–*Si*Me).). IR (KBr, cm^{-1}): $\upsilon_{C=O}$ (1752). UV-Vis (H_2O): 226.2, 277.2 and 489.5. Anal. Calcd. $C_{82}H_{144}I_5N_7O_8Si_3$ (2299.29 g/mol): C, 42.83; H, 6.31; N, 4.26; S, 9.76; Found: C, 42.47; H, 6.11; N, 4.11; S, 9.13.

Synthesis of $[(FluN(Me)G_2(NMe_3)_4][I]_4$ (16).

Over a solution of $NH_2G_2(NMe_2)_4$ (0.150 g, 0.18 mmol) in EtOH/THF (8 ml) protected from light, an excess of FITC (0.090 g, 0.23 mmol) was added following the synthetic procedure described for **10**. The reaction mixture without further purification was solved in THF and an excess of MeI (0.06 ml, 0.96 mmol) was added to obtain **16** as an orange solid (0.221 g, 68 %).

NMR data (DMSO): ^{1}H NMR: δ -0.06 (s, 3 H, Si*Me*), 0.02 (s, 6 H, Si*Me*), 0.57 (m, 10 H, $SiCH_2CH_2CH_2Si$ and $NCH_2CH_2CH_2CH_2Si$), 0.85 (m, 8 H, $SiCH_2CH_2S$), 1.29 (m, 6 H, $NCH_2CH_2CH_2CH_2Si$ and $SiCH_2CH_2CH_2Si$), 1.60 (m, 2 H, $NCH_2CH_2CH_2CH_2Si$), 2.42 (bs, 1 H, -N(Me)C(S)N*H*-), 2.61 (m, 8 H, $SiCH_2CH_2S$), 2.88 (m, 8 H, -$SCH_2CH_2NMe_3^+$), 3.06 (s, 36 H, -$SCH_2CH_2NMe_3^+$), 3.28 (m, 2 H, -NCH_2), 3.49 (m, 8 H, -$SCH_2CH_2NMe_3^+$), 6.52, 6.61, and 7.05 - 8.30 30 (m, 9 H, C_{Ar}-*H*). ^{13}C NMR: δ -5.6 (Si*Me*), 12.7 ($NCH_2CH_2CH_2CH_2Si$), 13.6 ($SiCH_2CH_2S$), 17.2 – 19.8

($NCH_2CH_2CH_2CH_2Si$ and $SiCH_2CH_2CH_2Si$), 20.6 ($NCH_2CH_2CH_2CH_2Si$), 23.1 (-$SCH_2CH_2NMe_3^+$), 26.4 ($SiCH_2CH_2S$), 31.4 ($NCH_2CH_2CH_2CH_2Si$), 40.2 (-NCH_2), 44.1 (N*Me*CSNH-), 51.7 (-$SiCH_2CH_2NMe_3^+$), 63.9 ($SCH_2CH_2NMe_3^+$), 101.8, 109.8 and 128.8 (C_{Ar}-H). ^{15}N NMR: δ -329.9 (-NMe_3^+). ^{29}Si NMR: δ 1.8 (G_1–*Si*Me), 2.5 (G_2–*Si*Me). IR (KBr): $\upsilon_{C=O}$ (1751). UV-vis (H_2O): 504.5. ESI: (1278.18 g/mol) q=2 (765.2 $[M\text{-}2I^-]^{2+}$). Anal. Calc. $C_{62}H_{110}I_4N_6O_5S_5Si_3$ (1771.77 g/mol): C, 42.03; H, 6.26; N, 4.74; S, 9.05; Found: C, 42.51; H, 6.69; N, 4.34; S, 9.05.

4.3 Biomedical assays.

Primary cell cultures

Blood samples were obtained from healthy anonymous donors from the transfusion centers of Madrid (Spain), following national guidelines. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll-Hypaque density gradient (Rafer, Zaragoza, Spain) following the current procedures of Spanish HIV BioBank.[64] PBMC were cultured in RPMI-1640 medium (Gibco, Paisley, UK) containing 10% heat-inactivated FBS, 1% (2 mM) L-glutamine and antibiotic cocktail (1% ampicillin, 1% cloxacillin and 0.32% gentamicin; Sigma, St-Louis, MO, USA) at 37°C in a 5% CO_2 atmosphere. Prior to compounds treatment, PBMC were activated for 48 h with 1 μg/mL of phytohemagglutinin (PHA, Remel, Santa Fe, USA) and 60 U/mL of recombinant interleukin-2 (IL-2, Bachem, Bubendorf, Switzerland).

Dendriplex formation

Dendriplexes were prepared by mixing dendrimer and siRNA dissolved in sterile water or PBS depending on the future study at concentrations depending on the +/- charge ratios and molar concentrations desired. After the time indicated, depending on the experiment (2 h, 24 h, or 48 h) incubation at room temperature electrophoretic mobility of the mixture was visualized on a 2% (w/v) agarose gel at 90 V in a TAE buffer solution (40 mM Tris–HCl, 1% (v/v) acetic acid, and 1mM EDTA). The gel bands were quantified using Quantity One 1D Analysis Software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

MTT assay

PBMC were seeded in 96 well plates in OPTIMEM® Imedium containing 10 % FBS (1.5×10^5 cells in 190 µl/well) and submitted to the dendrimer at the indicated concentrations. 20 hours 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 hours, the supernatant was removed and the formed crystals 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.

Mice

BALB/c mice, 8 weeks old, were purchased from Charles River Labs (L'Arbresle Cedex, France) and they were maintained under specific pathogen-free conditions in the Animal Facility of the Centro de Biología Molecular Severo Ochoa (CBMSO-CSIC/HGUGM). All animals were housed 5 per cage and maintained on a constant 12 hours light-dark cycles. Food and sterile tap water were available ad lib. All mice handling were performed in accordance with the Spanish law of protection of animal life.

In vivo Imaging

Mice were anesthetized by inhalation of isoflurane and separately inoculated via retro-orbital injection: (1) 200 µL PBS; (2) naked Cy5.5-siRNA-NEF 2,7 µM in 200 µL PBS; (3) 2G-$(SNMe_3I)_{11}$-FITC 15 mg/kg in 200 µL PBS, and (4) dendriplex 15 mg/kg in 200 µL PBS. Mice were sacrificed 1 and 24 h post-treatment, and the biodistribution in dissected organs (spleen, lymph nodes, kidneys, and liver) was measured using a three-dimensional IVIS Gamma Counter Packard 5005 (Packard Instruments, Meriden, CT). These experiments were carried out independently three times. Images were captured under one or two channels as indicated: FITC for the dendrimer and Cy5.5 for siRNA NEF. Tissues were placed on omnisette tissue cassettes, embedded in optimal cutting temperature (OCT) compound, and frozen in a prechilled Dewar flask containing a liquid nitrogen/dry ice slurry for ~1 min until the OCT turned white and opaque. Subsequently, the specimens were cut into 10 µm histology slices with a cryostat, and placed on a glass slide, and stained with DAPI to label nuclei.

5. Acknowledgments.

This work has been supported by grants from CTQ2011-23245, CTQ-2014-54004-P (MINECO), and Consortium NANODENDMED ref S2011/BMD-2351 (CAM) to University of Alcalá. This work has been (partially) funded by the RD12/0017/0037 project (M. A. M.-F.) as part of the Plan Nacional R + D + I and cofinanced by ISCIII- Subdirección General de Evaluación y el Fondo Europeo de Desarrollo Regional (FEDER), RETIC PT13/0010/0028, Fondo de Investigacion Sanitaria (FIS) (grant number PI13/02016), "Fundación para la Investigación y la Prevención del Sida en España" (FIPSE) (M. A. M.-F.), Comunidad de Madrid (grant numbers S-2010/BMD-2351 and S-2010/BMD-2332], CYTED 214RT0482. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008-2011, IniciativaIngenio 2010, the Consolider Program, and CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund (R. G: and M. A. M.-F.). E.F.P. acknowledges Ministerio de Educación for a fellowship (AP2014-1470). We would like to thank Dra. Maribel Clemente Mayoral for her technical assistance and advice as cell culture technician (CA10/01274)

6. Supporting information.

Complete experimental description, NMR spectra of selected compounds, and additional distribution images.

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For Table of Contents:

Fluorescein Labelled Cationic Carbosilane Dendritic Systems for Biologic Studies

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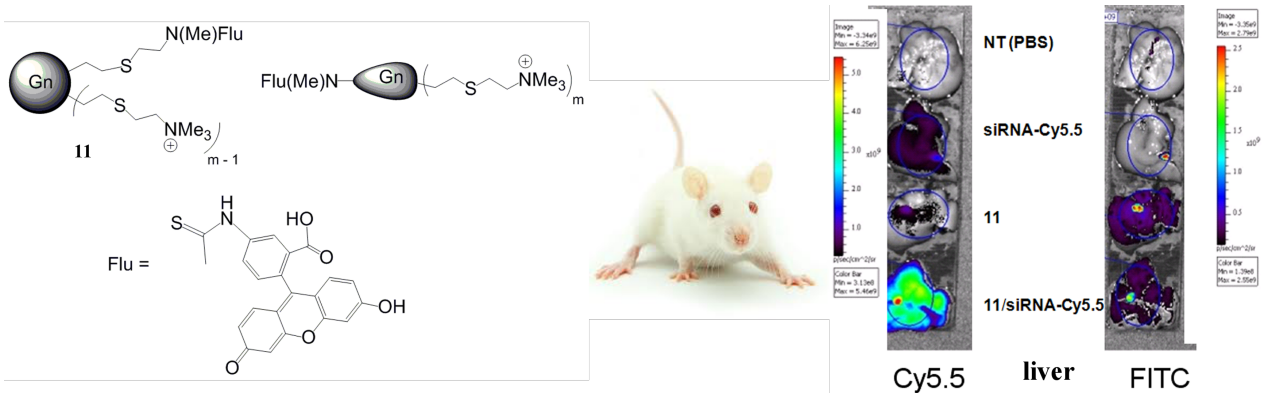
Fluorescein-cationic carbosilane dendrimers and dendrons have been synthesized and formation of dendriplexes with siRNA Nef have been studied. The biodistribution assays in a model of BALB/c mice showed the importance for siRNA of dendriplex formation.



Keywords: carbosilane dendrimers and dendrons, thiol-ene, biodistribution, gene therapy.