Alkyl sulfonyl derivatized PAMAM-G2 dendrimers as nonviral gene delivery vectors with improved transfection efficiencies†

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Amphiphilic dendrimer-based gene delivery vectors bearing peripheral alkyl sulfonyl hydrophobic tails were constructed using low-generation PAMAM-G2 as the core and functionalized by means of the aza-Michael type addition of its primary amino groups to vinylsulfone derivatives as an efficient tool for surface engineering. While the unmodified PAMAM-G2 was unable to efficiently transfect eukaryotic cells, functionalized PAMAM-G2 dendrimers were able to bind DNA at low N/P ratios, protect DNA from digestion with DNase I and showed high transfection efficiencies and low cytotoxicity. Dendrimers with a C18 alkyl chain produced transfection efficiencies up to 3.1 fold higher than LipofectAMINE™ 2000 in CHO-k1 cells. The dendriplexes based in functionalized PAMAM-G2 also showed the ability to retain their transfection properties in the presence of serum and the ability to transfect different eukaryotic cell lines such as Neuro-2A and RAW 264.7. Taking advantage of the vinylsulfone chemistry, fluorescent PAMAM-G2 derivatives of these vectors were prepared as molecular probes to determine cellular uptake and internalization through a clathrin-independent mechanism.

Introduction

The potential of gene therapy for treating genetic disorders has led to prolific research activity on the design of safe and efficient gene carrier vectors that compact and protect oligonucleotides and DNA against degradation by serum nucleases. Nonviral vector systems[1] are an attractive alternative to viral carriers,[2] retroviruses and adenoviruses, to overcome the fundamental problems associated with these systems despite the high delivering efficiency that they exhibit: toxicity, immunogenicity and limitations related with scale-up production.[3] Although synthetic vectors have inherently reduced transfection efficiencies due to the extra- and intracellular barriers that they find for their cellular uptake,[1f] these nonviral vectors can easily be structurally modified to allow systematic study of the factors implicated in transfection efficiency.

The design of cationic compounds including lipids, polymers, dendrimers and peptides that interact in a non-covalent and strong manner with the negatively charged phosphate backbone of DNA has been demonstrated to be an useful strategy for developing efficient synthetic transfecting agents. In this context, hyperbranched dendrimers[4] have proved to have greater potential as gene delivery vectors compared to traditional polymeric systems as a result of their unique characteristics: multivalent-functionalized terminal surface and well-defined three-dimensional architectures, molecular weights and sizes.[5] In particular, poly(amidoamine) (PAMAM) dendrimers that have easily cationized primary amino termini and tertiary internal amino groups have become a standard tool in the laboratory due to their easy synthesis and commercial availability.[6] The PAMAM–DNA complexes, termed dendriplexes and formed by the interaction with the primary amine groups,[7] are positively charged supramolecular systems that have the ability to interact with the negatively charged membrane of cells allowing their entrance through endosomal uptake without inducing significant cytotoxicity and immunogenicity.[8] Once internalized the buffering capacity of the unprotonated amino groups permits them to act as a proton sponge in the acidic environment of endosomes, enhancing in this way the release of DNA into the cytoplasm through the inhibition of pH-dependent endosomal nucleases.[9]

The influence of the dendrimer generation on the efficiency of transfection (also denoted as the dendritic effect) was identified early and medium sized dendrimers (G5–G10) are generally the most efficient ones.[10] Three different strategies have been developed to improve the performance of PAMAMs as nucleic acid carriers: (a) enhancing the flexibility of their branches, (b) rendering the dendrimers amphiphilic and (c) making them biocompatible.

The thermal degradation of high generation PAMAM dendrimers produces fractured PAMAM-based vectors with a greater flexibility that are commercialized under the name Superfect™. In addition, surface engineering by chemical modification involving functionalization at the dendrimer's periphery has been extensively investigated in an effort to improve not only the complex formation, interaction with the plasma membrane and endosomal release but also to reduce the cytotoxicity and to incorporate cell-targeting capabilities into the PAMAM-based dendriplexes to promote cell-specific uptake.[6f,8b,11] Conjugation of the primary amino groups with poly(ethylenglycol), amino-acids, peptides, proteins, sugars, cyclodextrins, lipids and glucocorticoids

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† Electronic supplementary information (ESI) available: ^{1}H- and ^{13}C-NMR spectra for compounds **4a–d**, **5a–d**, **6a–d**, **8a–b**, **8d**, **9a–d**, **10d**, **11d** and **13**, gel electrophoresis shift assays, DNase I protection experiments and hydrodynamic diameter and ζ potential values for PAMAM-G2 derivatives and their complexes with DNA. See DOI: 10.1039/c0ob00355g

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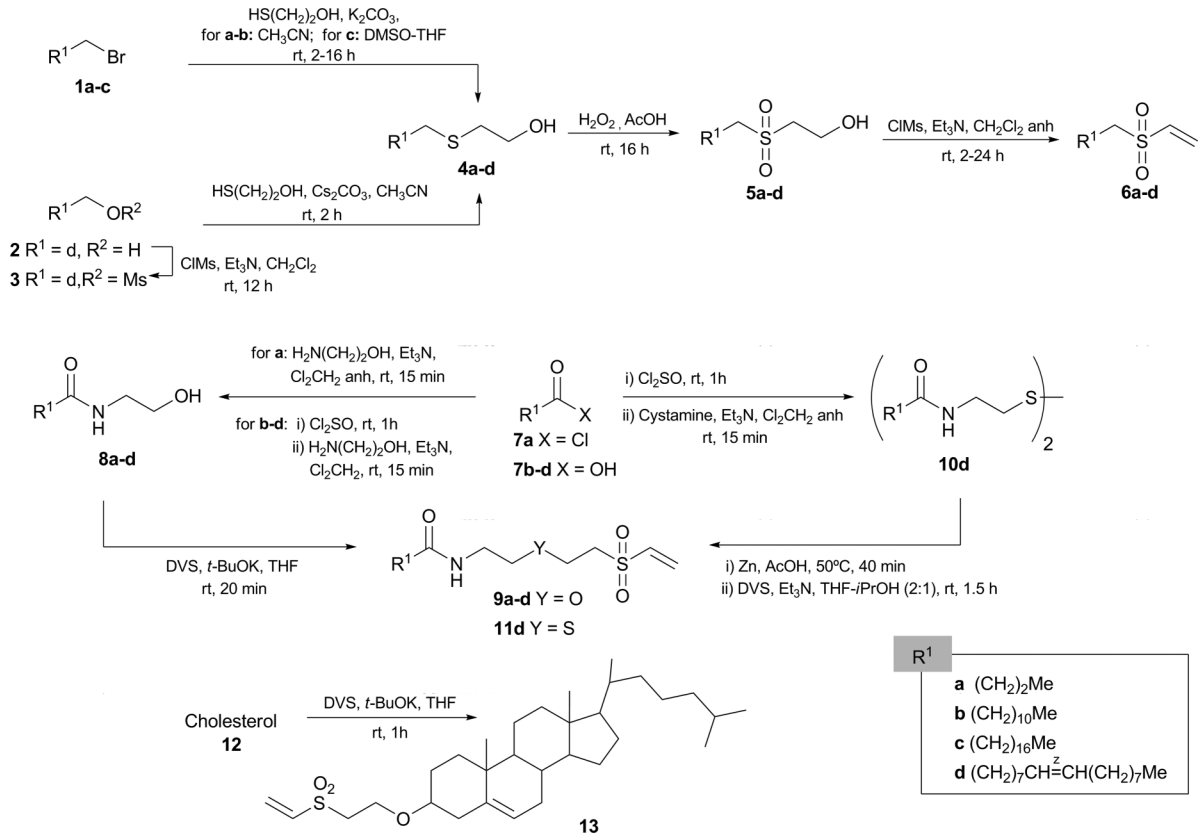


Scheme 1 Synthesis of vinylsulfone reagents for the functionalization of PAMAM-G2.

has been described in the literature with differing results on their transfection properties.

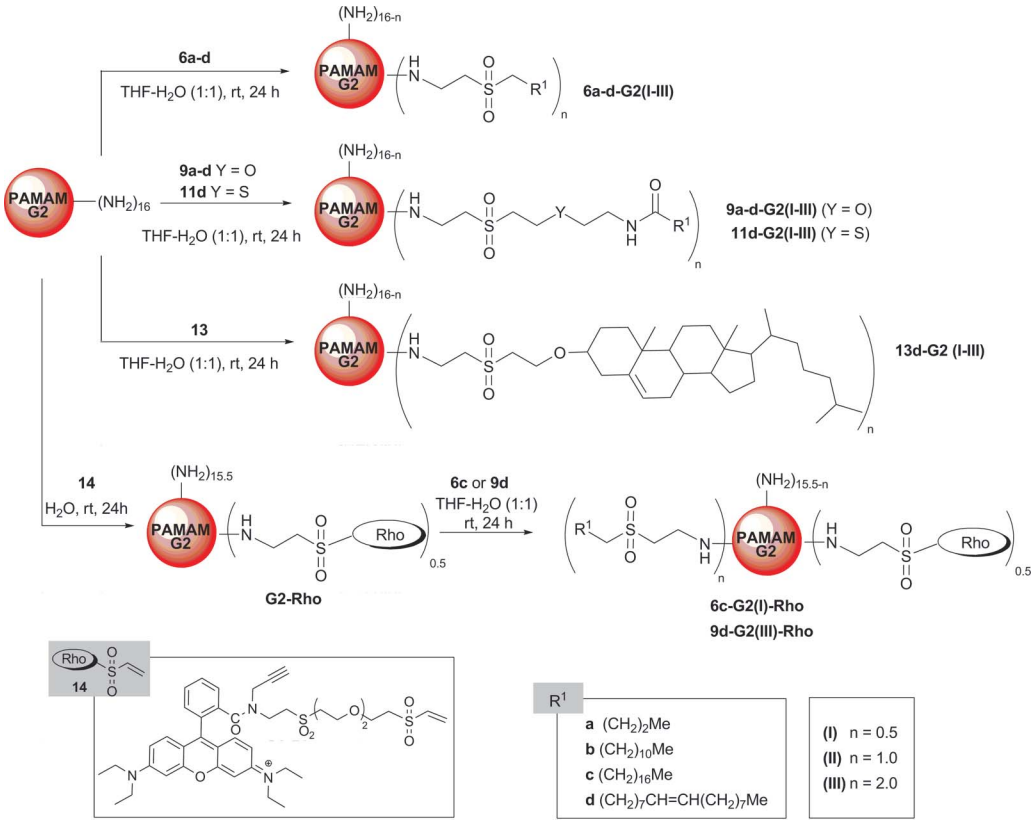
Several studies have demonstrated the benefits of incorporating lipophilic motifs to render the dendrimer amphiphilic in synthetic gene delivery systems.[1f] With this concern, lipid derivatized PAMAMs dendritic vectors have been engineered with topologies in which the dendritic hydrophilic moiety was present on one side of the vector while the hydrophobic moiety, mainly alkyl chains of different length, was installed on the opposite side.[12] Alternatively, PAMAM dendrimers having a hydrophilic interior and a hydrophobic corona prepared by randomly grafting alkyl tails at the dendrimer periphery have also been reported.[13] In both cases, the influence of parameters such as dendritic generation, length of the alkyl chain and extent of functionalization on the transfection efficiency and cytotoxicity has been evaluated. Michael addition of acrylate derivatives as well as acylation of activated esters fatty derivatives have been the chemical tools used for the construction of such hybrid dendritic–lipidic transfection agents.[11–13]

Herein we report on the synthesis and transfection efficiency properties of novel amphiphilic dendrimer-based gene delivery vectors bearing peripheral hydrophobic tails that are constructed using low-generation PAMAM-G2 as the core and functionalized by means of the aza-Michael type addition of its primary amino groups to vinylsulfone derivatives containing hydrophobic alkyl chains as an efficient conjugation strategy. Despite the poor transfection properties usually exhibited by native PAMAM-G2 dendriplexes, this cationic polymer was chosen over other higher PAMAM generations for the preparation of lipid conjugates on the basis of (a) its non-toxicity, attributed to the limited surface charges of this compound,[14] and (b) its easy synthesis with respect to high generation PAMAMs that usually requires tedious and low yielding procedures. The incorporation of hydrophobic alkyl chains of different length was anticipated to be a structural factor that would positively contribute to improving synergistically the cellular uptake and internalization of the corresponding dendriplexes.

Results and discussion

Chemical synthesis of functionalized PAMAM-G2 dendrimers

With a view to obtain PAMAM-G2 amphiphilic derivatives to be used as nonviral vectors with improved transfection efficiencies and diminished cytotoxicity, we selected a set of compounds containing hydrophobic chains for conjugation with the amino terminal groups of that dendrimer (Scheme 1). To evaluate the influence of the hydrophobic chain length on the process of transfection, commercially available reagents containing linear alkyl chains with four, twelve and eighteen carbon atoms were selected, namely, the bromide derivatives **1a–c**, oleic alcohol ((*Z*)-9-octadecen-1-ol) **2**, butyric chloride **7a**, and lauric acid (docedanoic acid) **7b**, stearic acid (octadecanoic acid) **7c** and oleic acid (*cis*-9-octadecenoic acid) **7d**. The aza-Michael type addition of vinylsulfone derivatives of such hydrophobic compounds was thought to be an optimal ligation tool for their coupling with the peripheral amine groups as this reaction usually proceeds with



Scheme 2 Synthesis of amphiphilic PAMAM-G2 dendrimers as nonviral vectors.

high efficiency by simple mixing of the reagents in an adequate solvent without the formation of any undesired by-product.[15]

The different functionality of compounds **1a–c**, **2** and **7a–b** (bromide, alcohol and carboxylic acid, respectively) allows the design of simple chemical strategies for the preparation of their corresponding vinylsulfone derivatives, and also the introduction of a structural variability in the linker that will join the hydrophobic alkyl chain and the dendrimer core whose influence on the transfection properties will be also evaluated. Thus, nucleophilic displacement on the bromide derivatives **1a–c** and the mesylate derivative **3** of oleic alcohol **2** by the sulfur atom of 2-thioethanol gave access to the hydroxyethyl thioethers **4a–d** in high yields. Oxidation with peroxyacetic acid and subsequent dehydration of the corresponding sulfone derivatives **5a–d** *via* their mesylate derivatives led easily to the vinylsulfones **6a–d** in which the original functionality has been replaced by the vinyl sulfonyl group (Scheme 1). Alternatively, a strategy was envisaged starting from the fatty acids **7a–d** which enables the introduction of a longer vinylsulfone linker for the hydrophobic chains. Thus, reaction with 2-amino ethanol of the fatty acid chloride derivatives, using the commercial reagent **7a** or obtained *in situ* by treatment with thionyl chloride in the case of the acids **7b–d**, gave the corresponding 2-hydroxylethyl amides **8a–d**. Reaction of these compounds with divinylsulfone (DVS) gave the ether vinylsulfones **9a–d** in moderate yields (Scheme 1). Additionally, in the case of oleic acid the homologous thioether vinylsulfone **11d** was also prepared in order to evaluate a possible influence of the nature of the heteroatom of the linker on the transfection properties. This compound was accessible by reaction of oleic acid chloride with cystamine, which gave the disulfide **10d** in high yield. Reduction of this compound with powdered Zn followed by Michael addition to DVS afforded the thioether derivative **11d** in a one-step procedure (Scheme 1).

Considering that cholesterol has often been exploited[1f] as a hydrophobic motif in synthetic transfection agents including PAMAM-based vectors[16] to give them a lipidic character, the preparation of a vinylsulfone derivative of this steroid was also envisaged in order to further synthesize the corresponding cholesterol–PAMAM-G2 conjugate and evaluate if this structural modification allows an optimization on gene delivery. Simple treatment of commercial cholesterol **12** with DVS affords the corresponding vinylsulfone derivative **13** in moderate yield (Scheme 1).

With the vinylsulfone derivatives (**6a–d**, **9a–d**, **11d** and **13**) in hand, the synthesis of the novel gene delivery vectors was performed by the simple addition of a THF solution of these reagents to an aqueous solution of PAMAM-G2. To evaluate the effect of the extent of functionalization, three different vinylsulfone : PAMAM-G2 molar ratios (I = 0.5, II = 1.0 and III = 2) were used in each case, giving rise to the corresponding conjugates [**6a–d-G2(I–III)**, **9a–d-G2(I–III)**, **11d-G2(I–III)** and **13-G2(I–III)**] after elimination of the reaction solvent (Scheme 2).

In addition, to perform studies aimed at the observation of the intracellular events in the transfection of the PAMAM-G2 based dendrimers and at the elucidation of their cellular uptake and internalization mechanisms, the synthesis of fluorescent PAMAM-G2 probes was also designed to be used in confocal microscopy studies. The vinylsulfone rhodamine derivative **14** (Scheme 2), previously reported by us,[17] allows the easy incorporation of this

fluorescent probe in native PAMAM-G2 to give the **PAMAM-G2-Rho** dendrimer that will be later functionalized with hydrophobic motifs on the basis of the results obtained in transfection efficiency studies (see next Sections).

^{1}H NMR spectroscopic data of the new amphiphilic PAMAM-G2 derivatives confirm successful grafting of the hydrophobic or the rhodamine vinyl sulfone reagents at the periphery of the PAMAM-G2 as evidenced by the absence of the characteristic vinyl sulfone proton peaks and the presence of new signals ascribed to the alkyl chains and the fluorescent residue (see ESI Fig S1†).

Gene delivery capabilities of amphiphilic PAMAM-G2 derivatives

The capabilities of the novel amphiphilic PAMAMs to neutralize, bind and compact plasmid DNA (pDNA) were first studied due to their importance for efficient gene delivery. Plasmid pEGFP-N3 [coding for the enhanced Green Fluorescent Protein (eGFP)] was selected for the preparation of the DNA complexes and transfection assays. The PAMAMs conjugates were mixed with pDNA at several N/P ratios (0.5 to 50) for the formation of the corresponding dendriplexes. Gel electrophoresis shift assays were performed as they reflect the capability of the PAMAM-G2 derivatives to compact pDNA.[18] Formation of the corresponding complexes results in pDNA lacking migration in the gel due to polycation binding and charge-neutralization. The agarose gel shift assays performed revealed that binding of the majority of the PAMAM-G2 derivatives to pDNA occurs at N/P ratios of 0.5–1 for the functionalized compounds at the three molar ratios (I = 0.5, II = 1.0 and III = 2). (Fig. 1 and ESI Fig. S2†).

The capability of protecting pDNA from endonucleases degradation was next evaluated as this is a key point to achieve a successful transfection since unprotected pDNA are rapidly degraded by the nucleases presented in the culture media serum.[1e] The results obtained with the PAMAM-G2 amphiphilic derivatives (Fig. 2 and ESI Fig. S3†) showed that these derivatives have the capability to efficiently protect pDNA from endonuclease degradation while unmodified PAMAM-G2 failed to protect pDNA from digestion.

In the next phase, cellular uptake of the novel dendriplexes was studied in CHO-k1 cells, a common standard cell line used for a variety of transfection agents. Plasmid pEGFP-N3 that encodes for the green fluorescent protein allows us to evaluate the uptake by measuring the fluorescence of the cells transfected with the complexes that have been grown 48 h after transfection to allow the expression of eGFP. Cellular uptake has been studied in the absence of serum at N/P ratios of 2.5–50 (Fig. 3A). 100% value has been assigned to the transfection level obtained when LipofectAMINE™ 2000 (LP2000), a commercial reagent that has been described as a high efficiency vector for the transfection of many cell types, is used. The results obtained in these assays clearly indicated that unmodified PAMAM-G2 have a very poor transfection capability (less than 5% of the LP2000 value) which is in accordance with previous reported observations[10] and with our own results: (a) no retardation of pDNA in gel electrophoresis (Fig. 1A) and (b) absence of protection towards DNase I degradation (Fig. 2A). These facts corroborate the benefits obtained by the incorporation of a hydrophobic moiety to promote binding of the dendrimer to the hydrophobic DNA bases. In addition, it is observed that the incorporation of the moderately hydrophobic moiety of rhodamine is enough to promote DNA binding to the PAMAM-G2 (Fig. 1C) although it fails to protect DNA from DNase I degradation (Fig. 2C).

From the assembly of the results obtained some conclusions can be extracted concerning the influence of the chemical structure of the engineered PAMAM-G2 vectors on their transfection efficiency. PAMAM-G2 derivatives functionalized with a butyl chain, **6a-G2** and **9a-G2**, are not able to transfect the CHO-k1 cells (data not shown) despite the enhanced pDNA complexing abilities and protection from DNase I digestion that they exhibited. Among the assayed compounds, the minimum length of the hydrophobic chain that enables the PAMAM-G2 derivatives to efficiently transfect CHO-k1 cells is 12 carbons (compounds **6b-G2** and **9b-G2**). PAMAM-G2 derivative **6b-G2(I)** exhibited a remarkably high transfection level ($p < 0.001$ compared to the LP2000 value) at N/P ratios between 5 and 20 while the efficiency of **9b-G2(I)** to deliver pDNA is lower but similar to LP2000 at a N/P

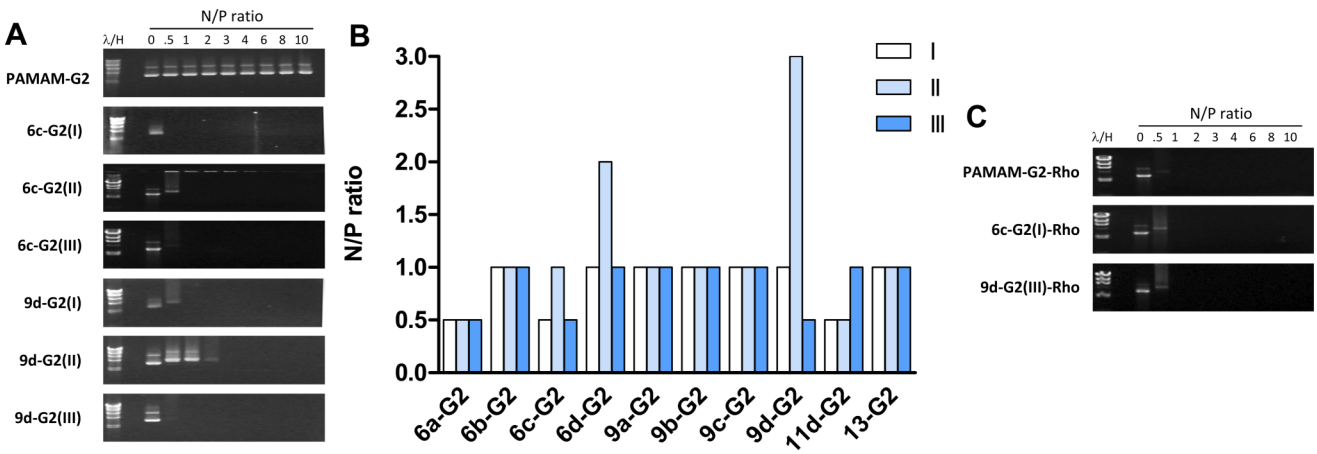


Fig. 1 *Gel Electrophoresis Shift Assay.* **A**.- Gel shift assays showing PAMAM-G2 or selected PAMAM-G2 derivatives–pDNA binding at N/P ratios between 0 (pEGFP-N3 alone) and 10. λ/H lane corresponds to λ/*Hind*III molecular weight marker. The absence of plasmid band in the wells correlates with the inhibition of the plasmid DNA electrophoretic mobility. I, II and III correspond to the three different vinylsulfone : PAMAM-G2 molar ratios (I = 0.5, II = 1.0 and III = 2) used **B**.- Minimum N/P ratio needed to completely inhibit electrophoretic mobility in the shift assay. **C**.- Rhodamine labeled PAMAM-G2 or PAMAM-G2 derivatives gel shift assays.

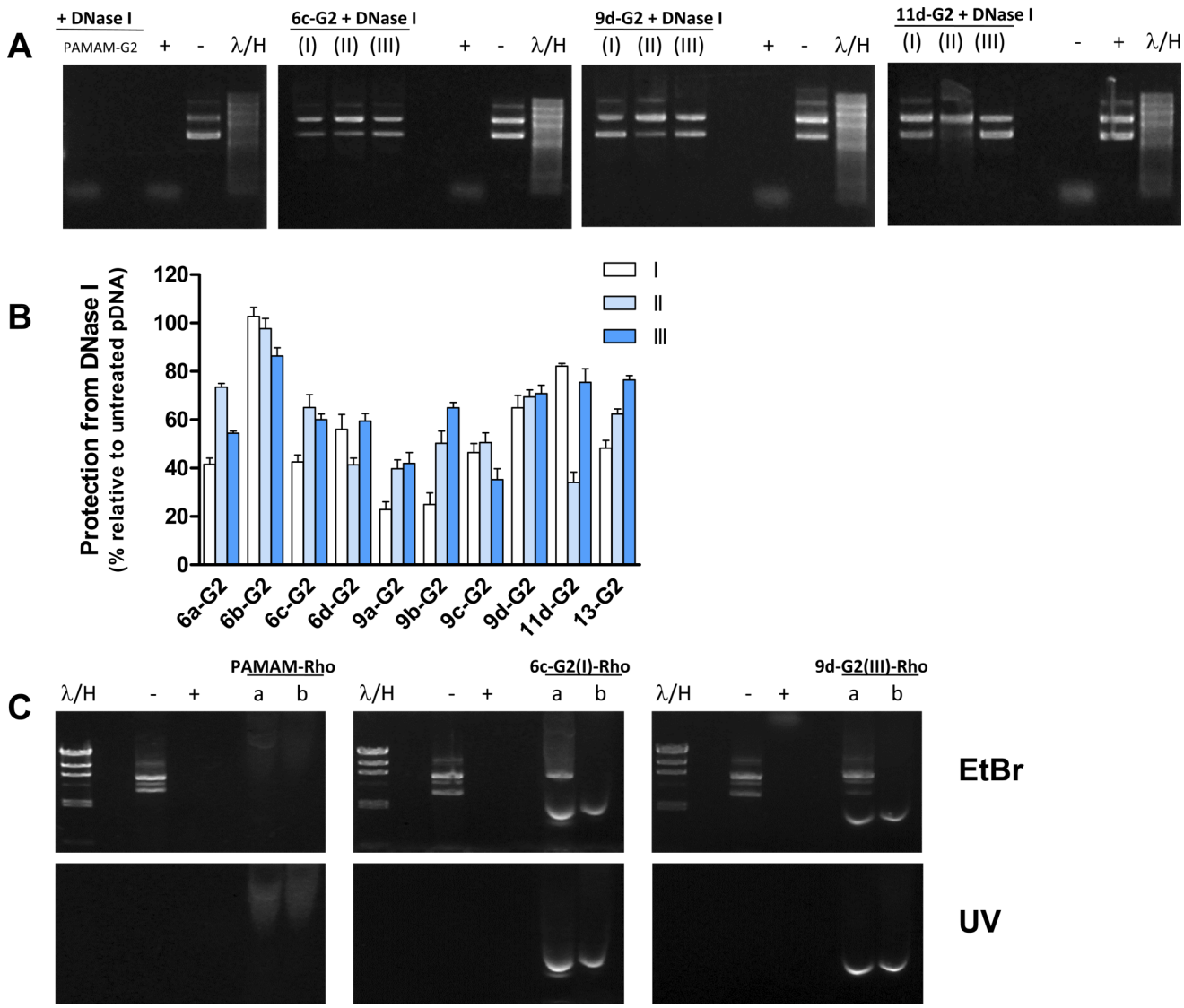


Fig. 2 *DNase I protection experiments.* **A**.- Representative agarose electrophoresis of samples corresponding to pEGFP-N3 incubated in the absence (–) or presence (+) of DNase I as controls. pEGFP-N3 samples that have been complexed with the PAMAM-G2 derivatives before the DNase I treatment (as described in the experimental section) have been run in parallel. **B**.- Quantitation of the relative intensity (untreated pEGFP-N3 value equal to 100) of the sum of relaxed and supercoiled electrophoretic plasmid bands corresponding to the pEGFP-N3 samples complexed with PAMAM-G2 derivatives and treated with DNase I. Results are expressed as means ± SEM (n = 4). I, II and III correspond to the three different vinylsulfone : PAMAM-G2 molar ratios (I = 0.5, II = 1.0 and III = 2) used. **C**.- Samples of PAMAM-G2 derivatives labeled with Rhodamine have been processed as above and the DNase I protection has been assayed by gel electrophoresis. (a) dendrimer–pDNA complexes, (b) labeled dendrimer alone. Since the dendrimers are labeled with fluorescence, the gels have been photographed before (UV) and after ethidium bromide staining (EtBr). Notice that due to the interaction of the free labeled dendrimer with the SDS used in the sample preparation, the free labeled dendrimers have a net negative charge in the electrophoresis.

ratio of 20 (Fig. 3). Both compounds showed enhanced pDNA complexing abilities (ESI Fig. S2†) although, as expected from its transfection efficiency, **9b-G2(I)** partially failed to protect pDNA from DNase I degradation (ESI Fig. S3†). Amphiphilic dendrimer **6c-G2(I)** bearing a C18 hydrophobic chain produced the highest levels of transfection in CHO-k1 (approximately between 3.1 fold ($p < 0.001$) more efficiently than LP2000), a capacity that is complemented with the formation of stable complexes warranting full pDNA protection from the environment (Fig. 2). However, compound **9c-G2** which has an aliphatic chain of the same length presented very poor transfection levels that correlate well with the observed susceptibility to endonucleases of the relaxed pDNA band in the DNase I protection assay (ESI Fig. S3†). Oleic-containing PAMAM-G2 derivatives **6d-G2(I)**, **9d-G2(III)** and **11d-G2(III)**, which have a double bond in the C18 aliphatic chain, exhibit higher levels of transfection than LP2000 (1.6 to 2.7 fold higher than LP2000, $p < 0.01$) (Fig. 3). These dendrimers also presented high complexing abilities (Fig. 1 and ESI Fig. S2†) and fully protected pDNA from DNases (Fig. 2, and ESI Fig. S3†). Overall, it can be stated that although the inclusion of a hydrophobic moiety is enough to obtain a tight binding of the PAMAM-G2 dendrimers to the pDNA, an alkyl chain length of 18 carbons suits better for optimal DNase I protection and transfection efficiency.

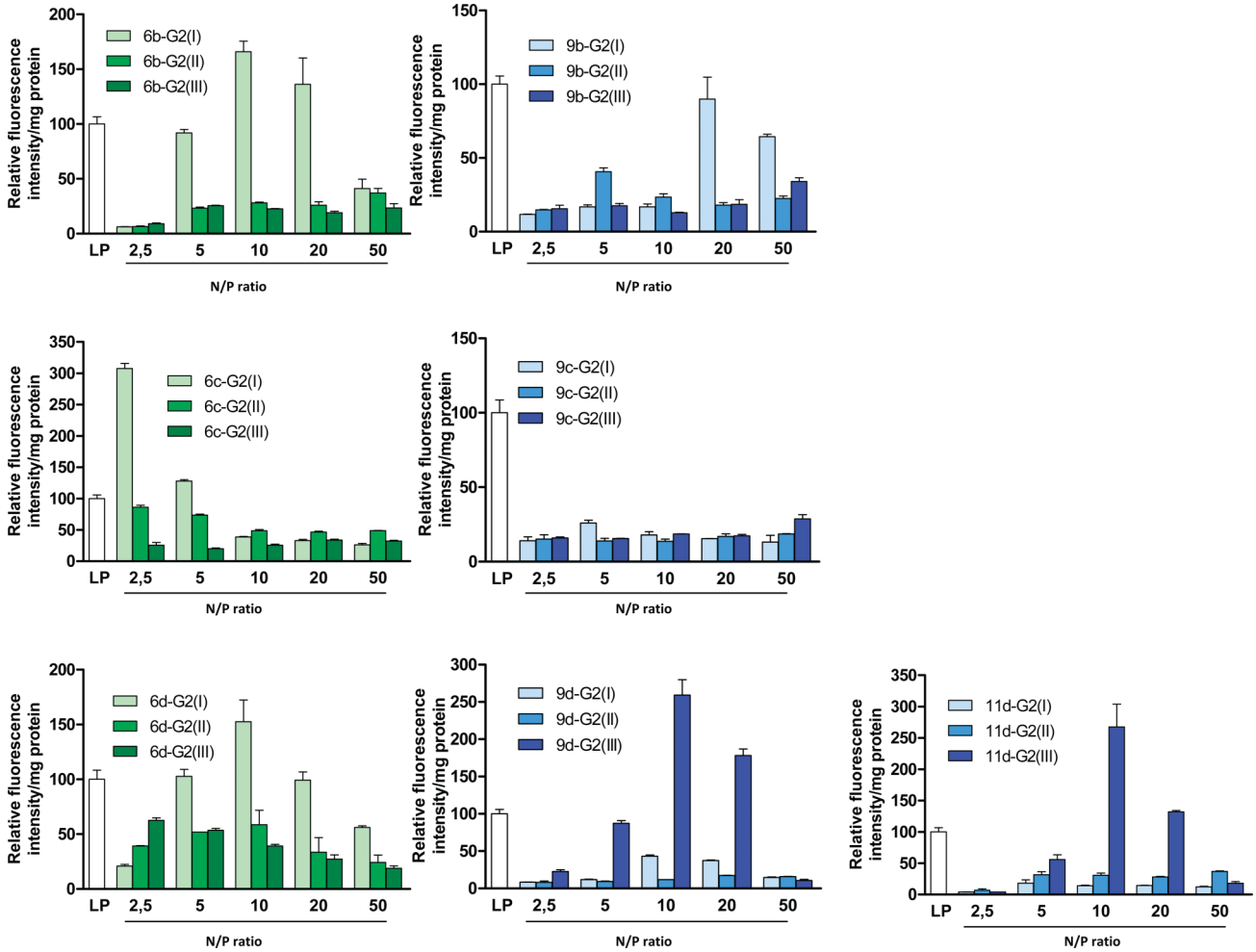


Fig. 3 *In vitro gene transfection efficiency of the PAMAM-G2 based dendrimer complexes in CHO-k1 cells.* CHO-k1 cells were transfected with dendriplexes using pEGFP-N3 plasmid as described in the Experimental section. For each condition, DNA was mixed with the PAMAM-G2 derivatives at N/P ratios of 2.5 to 50. As a positive control (LP), a transfection was performed using LP2000. Fluorescence was assayed 48 h after transfection. The ratio fluorescence/proteins for each compound is shown. The eGFP fluorescence value for the LP2000 transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 8).

Concerning the extension of the functionality by hydrophobic chains, a comparison of dendrimers from series **6** and **9** indicates that the optimal transfection is related to the number of alkyl chains grafted per dendrimer. Compounds from **6** series are very good transfection reagents with only 0.5 hydrophobic chain grafted per PAMAM-G2 (I = 0.5) while the functionalized dendrimers of series **9** needed two alkyl chains grafted (III = 2) to be efficient at transfecting pDNA. The reasons for this different behavior are not clear.

On the other hand, the nature of the heteroatom of the ether linkage, oxygen for compound **9d** and sulfur for compound **11d**, seems to have no influence on the transfecting abilities of these compounds since both exhibited similar efficiencies (Fig. 3).

Chemical functionalization of PAMAM-G2 with cholesterol, compounds **13-G2(I–III)**, does not improve the abilities of the corresponding dendriplexes as for these compounds high protection of pDNA (Fig. 2B and ESI Figs. S2 and S3†) but very low transfection efficiency (data not shown) are observed. This behavior could be tentatively ascribed to a strong binding of the pDNA to those PAMAM-G2 derivatives that makes difficult the release of the pDNA free into the nucleus.

Taken together, the results obtained allows us to conclude that from the set of the amphiphilic PAMAM-G2 derivatives prepared the best transfecting agents are the dendrimers **6c-G2(I)** and **9d-G2(III)**.

In the next stage of our study, dynamic light scattering and ζ-potential measurements were carried out for the more efficient dendrimers at the best N/P ratios, to determine the size and electrokinetic properties of the formed complexes (ESI Table S1†). As expected, ζ-potential values decreased when the functionalized dendrimers were complexed with pDNA. However, no relationship between particle size and transfection efficiency can be deduced since the best transfecting agents **6c-G2(I)** and **9d-G2(III)** have hydrodynamic diameters of 524 ± 18 and 226 ± 3 nm, respectively.

Once the influence of the structure on the transfection efficiencies had been studied for the amphiphilic PAMAM-G2-based vectors, the cytotoxicity of their pDNA complexes was evaluated by determining the percentage of cell viability with respect to unexposed cells using a MTT assay (Fig. 4). Cell viability was

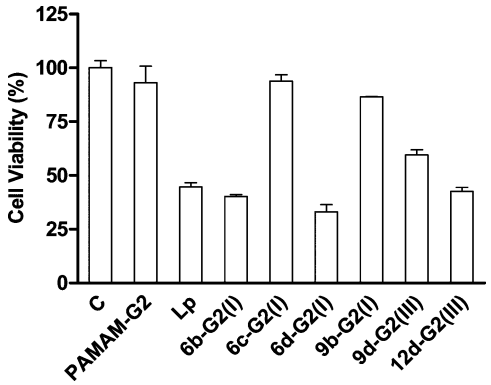


Fig. 4 *Cytotoxicity of the complexes.* Cytotoxicity of the complexes formed by the functionalized dendrimers and pDNA was evaluated in CHO-k1 cells 24 h after transfection by determining the percentage of cell viability using a MTT assay. The dendrimer derivatives to be assayed were those that showed a higher transfection efficiency. The complexes were assayed for cytotoxicity using the most efficient N/P ratio for transfection (**6b-G2(I)**: 10; **6c-G2(I)**: 2,5; **6d-G2(I)**: 10; **9b-G2(I)**: 20; **9d-G2(III)**: 10; **11d-G2(III)**: 10). Results are reported as % viability based on the untreated control cells normalized to 100% viable.

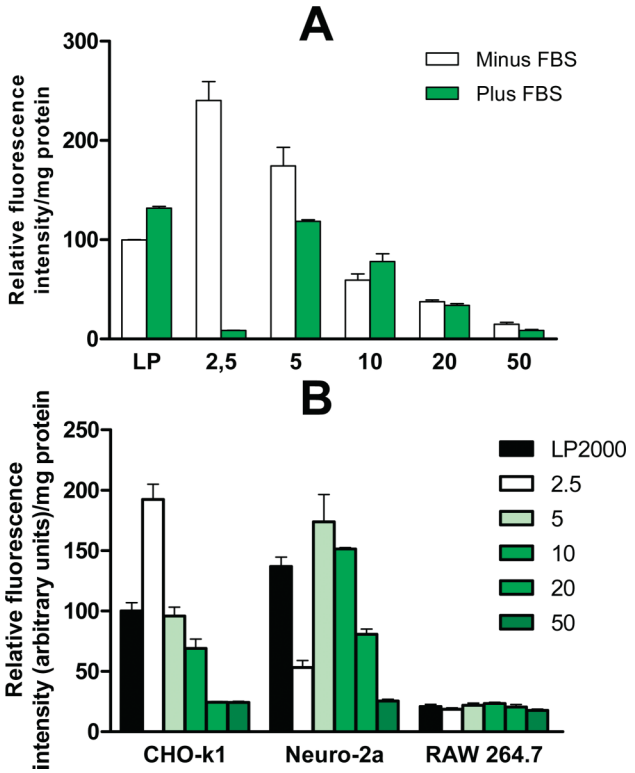


Fig. 5 *Factors that modulate the transfection efficiency of PAMAM-G2 derivatives complexes.* **A**.- Effects of serum in the transfection media. LP2000 (as a positive control) or **6c-G2(I)** dendrimer (N/P ratios 2.5–50) have been complexed with pEGFP-N3 plasmid and then used for transfection of CHO-k1 cells in the absence or presence of 10% fetal bovine serum in the transfection media. Fluorescence was assayed 48 h after transfection. The ratio fluorescence/proteins for each condition is shown. The eGFP fluorescence value for the LP2000 transfection carried out in the absence of fetal bovine serum was normalized to 100%. Results are expressed as means ± SEM (n = 7). **B.-** Cell type. CHO-k1, Neuro-2a or RAW 264.7 cells were transfected using **6c-G2(I)** dendrimer complexed with pEGFP-N3 plasmid as described in the Experimental section. For each condition, DNA was mixed with the PAMAM-G2 derivative at N/P ratios from 2.5 to 50. For each cell line a transfection was performed using LP2000 as a positive control. Fluorescence was assayed 48 h after transfection. The ratio fluorescence/protein for each condition is shown. The eGFP fluorescence value for the LP2000 transfection in CHO-k1 cells was normalized to 100%. Results are expressed as means ± SEM (n = 8).

studied in CHO-k1 cells as a function of the functionalized PAMAM-G2 derivative and the N/P ratio at which the compound showed its higher transfection levels. Unmodified PAMAM-G2 complexed with pDNA showed itself to be not toxic as expected while lipoplexes with LP2000 are not exempt of toxicity as they reduced by 50% the cell viability ($p < 0.001$). For the PAMAM-G2 derivatives the most efficient transfecting compounds, **6c-G2(I)** and **9d-G2(III)**, displayed a high level of cell viability

The effect of serum in the media on the transfection efficiency of the pDNA was evaluated on the PAMAM-G2 derivative **6c-G2(I)**, which was chosen considering that it is one of the best at protecting pDNA against DNases and for its high transfection efficiency at very low N/P ratios (Fig. 5A). In the presence of serum, the transfection levels of this vector were similar to those obtained with LP2000 at 5–10 N/P ratios. The increase in N/P ratio needed for transfection due to the presence of serum has been reported previously for cationic liposomes.[19]

To assess the pDNA transfection capabilities in other cell lines, the PAMAM-G2 derivative **6c-G2(I)** has also been used in assays using neuroblast cells (Neuro-2a) and a cell line derived from macrophages (RAW 264.7) which usually exhibits low transfection levels.[20] A 100% value was assigned to the transfection levels observed when LP2000 in CHO-k1 cells was used. Compound **6c-G2(I)** has the capability to transfect these three cell lines in a similar or better way than LP2000 (Fig. 5B).

Cellular uptake and internalization mechanism studies for amphiphilic PAMAM-G2-based vectors

Nonviral gene complexes can enter into mammalian cells through different endocytic pathways.[21] Therefore, for a novel vector it is important to characterize its cellular uptake because this determines how the gene carrier is processed intracellularly and therefore influences its transfection efficiency. Most of the reports suggest that endocytosis is the preferred route of cellular uptake mechanism for nonviral gene carriers. Two main endocytic pathways, clathrin-dependent and clathrin-independent, are implicated in the uptake of DNA complexes, dependent on cell type, the nature of the carrier and particle size. The clathrin-dependent pathway is preferred for microspheres up to 200 nm in size.[22] The clathrin independent route includes uptake from lipid rafts in caveolae or *via* flotillin-dependent pathway.[23] To determine the pathway of the cellular uptake of the functionalized PAMAM-G2 dendrimers two chemical inhibitors of endocytosis were used: chlorpromazine and genistein as inhibitors of clathrin-dependent and -independent endocytosis, respectively.[21] Fig. 6 shows that chlorpromazine significantly increased ($p < 0.001$) the efficiency of transfection using LP2000, **6c-G2(I)** or **9d-G2(III)** while it did not modify the capability of transfection of compounds **6b-G2(I)**, **6d-G2(I)**, **9d-G2(III)** y **11d-G2(III)**. Conversely, preincubation with genistein decreased the transfection levels of the majority of compounds, including LP2000. Therefore, from these observations

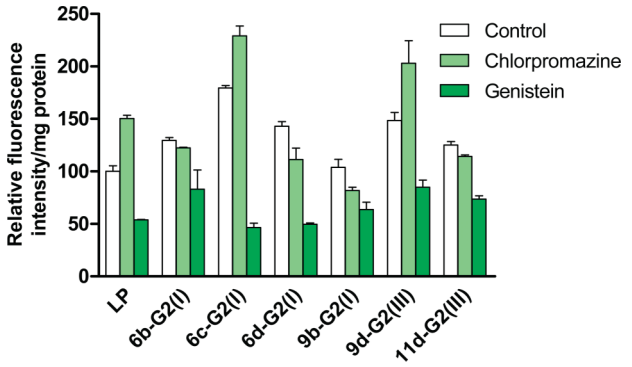


Fig. 6 *Effects of inhibitors on cellular transfection of PAMAM-G2 derivatives complexes.* CHO-k1 cells were pretreated with chlorpromazine (14 μM) or genistein (200 μM) for 30 min before transfection with dendrimer complexes at their most efficient N/P ratio (**6b-G2(I)**: 10; **6c-G2(I)**: 2,5; **6d-G2(I)**: 10; **9b-G2(I)**: 20; **9d-G2(III)**: 10; **11d-G2(III)**: 10). As a control (LP), a transfection was performed using LP2000. Fluorescence was assayed 48 h after transfection. The ratio fluorescence/proteins for each compound is shown. The eGFP fluorescence value for the LP2000 transfection was normalized to 100% in each experiment. Results are expressed as means ± SEM (n = 6).

it can be concluded that amphiphilic PAMAM-G2 derivatives use mainly a clathrin independent pathway.

Finally, the internalization process of the dendriplexes was studied by confocal microscopy using Cy5-labeled pDNA and rhodamine-labeled dendrimer **6c-G2(III)-Rho** and **9d-G2(III)-Rho**. These compounds were prepared following the vinylsulfone-based chemical strategy indicated in Scheme 2 that implies the functionalization of **PAMAM-G2-Rho** with the stearic and oleic vinylsulfone derivatives **6c** and **9d**, respectively. For compound **9d-G2(III)-Rho** confocal images are shown. The transfected cells have been identified by the expression of eGFP, since the experiments have been performed using pEGFP-N3 plasmid. Images were collected 24 h post-transfection to allow eGFP expression. Fig. 7A corresponding to visible differential interface contrast (Nomarki) images and fluorescence images shows the distribution of fluorescence in the transfected and non-transfected cells. In all cells, red fluorescence corresponding to the labeled dendrimer can be located in the cytosol of the cells, close to the nucleus. Almost all blue fluorescence corresponding to the labeled-pDNA is associated with the red labeled-dendrimers (purple particles in the overlayed images). Only in the transfected cells, free blue fluorescence corresponding to the pDNA released from the dendrimers can be located inside the nucleus (Fig. 7B). No free blue fluorescence is located in the nucleus of the non-transfected cells. Similar results have been obtained for the rhodamine-labeled **6c-G2(III)-Rho** (data not shown). These results confirm that the rate limiting step in hydrophobic PAMAM-G2-based vectors transfection is the release of the pDNA from the endosomes to the nucleus, since all the cells are able to uptake the dendrimer complex, but only in a subset is the pDNA released from the internal reservoirs to the nucleus. Fig. 7C shows CHO-k1 cells that have been pre-incubated with chlorpromazine prior to transfection with rhodamine-labeled **9d-G2(III)-Rho** complexed with Cy5-labeled pEGFP-N3 plasmid. In these cells the uptake of dendrimers to the perinuclear space is greatly reduced and shows an apical distribution. However, the transfection efficiency (the blue-fluorescence associated with the free pDNA released to the nucleus) is increased. These results, together with the transfection efficiency values measured in the presence of endocytosis inhibitors, confirm that the clathrin-independent pathway is the productive endocytic pathway used by these functionalized PAMAM-G2 derivatives.

Conclusions

In summary, PAMAM-G2-based amphiphilic dendrimers are prepared in a straightforward and efficient manner by using the aza-Michael type addition of vinylsulfone derivatives as an optimal tool for the grafting of hydrophobic alkyl chains to the peripheral amino groups of those dendrimers. This chemical strategy is general and allows the facile functionalization of any amino-containing polymer for the preparation of synthetic gene carriers. The transfection properties are dependent on the chemical structure of the hydrophobic chain and on the extension of the functionalization of the dendrimers surface. Some of these engineered PAMAM-G2 dendrimers are useful vectors with improved transfection properties when compared to unmodified PAMAM-G2 and commercial transfection reagents such as LipofectAMINE™: higher transfection efficiencies, lower cytotoxicity, the capability to maintain their transfection efficiency in the presence of serum and the ability to transfect different eukaryotic cell lines. The flexibility of the vinylsulfone based chemistry also allowed the fluorescent labeling of these PAMAM derivatives for the preparation of probes to monitor the endocytic pathway used for successful transfection by the dendrimers and to gain insights in the cellular uptake and internalization mechanisms of these vectors.

Experimental

General experimental procedures

Generation 2 PAMAM dendrimer (PAMAM-G2) was purchased from Aldrich. Commercially available reagents **1a–c**, **2**, **7a–d**, **12** and solvents were used as purchased without further purification. Compound **3** was obtained from oleyl alcohol following a procedure described in the literature.[24] TLCs were performed on Merck Silica Gel 60 F254 aluminium sheets. Reagent used for developing plates include potassium permanganate (1% w/v), ninhydrin (0.3% w/v) in ethanol and UV light when applicable. Flash column chromatography was performed on Silica Gel Merck (230–400 mesh, ASTM). Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected. Optical rotations were recorded on a Perkin-Elmer 141 polarimeter at room temperature. IR spectra were recorded on a Satellite Mattson FTIR. ^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Varian Direct Drive (300, 400 and 500 MHz) spectrometer. Chemical shifts are given in ppm and referenced to internal $CDCl_3$. *J* values are given in Hz. FAB mass spectra were recorded on a Fissons VG Autospec-Q spectrometer, using *m*-nitrobenzyl alcohol or thioglycerol as matrix. NALDI-TOF mass spectra were recorded on an Autoflex Brucker spectrometer using NaI as matrix. Electrospray Ionization (ESI) mass spectra were recorded on a LCT Premier Spectrometer. LipofectAMINE™ 2000 (LP2000) was purchased form Invitrogen (Carlsbad, CA, USA). pEGFP-N3 plasmid (Genbank U57609) was obtained from Clontech Laboratories (Palo Alto, CA). This 4729 bp plasmid

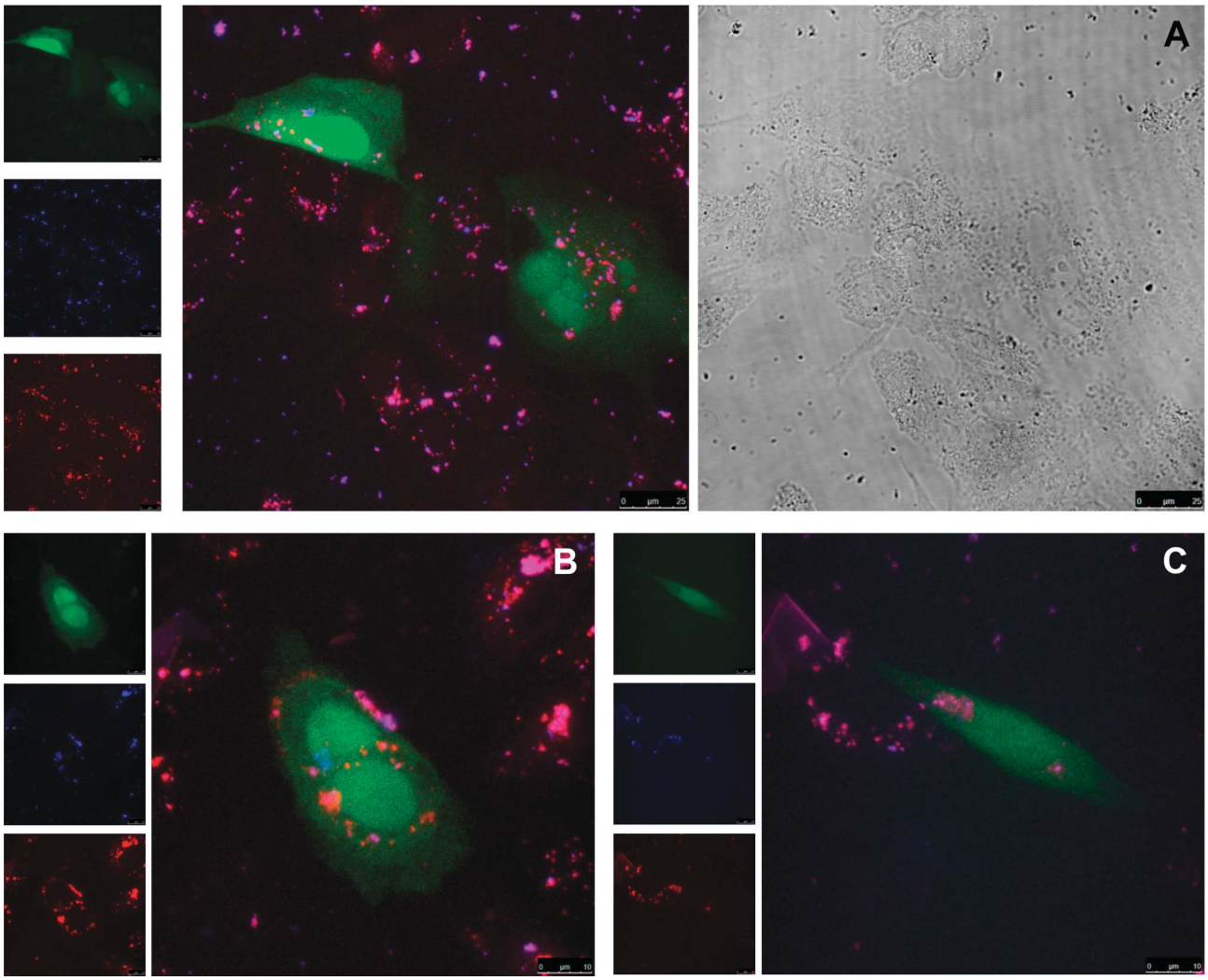


Fig. 7 *Confocal microscopy.* CHO-k1 cells were seeded onto coverslips and transfected with dendrimer complexes based on Rhodamine labeled-**9d-G2** (**III**) PAMAM derivative and Cy5 labeled pEGFP-N3 plasmid DNA at an N/P ratio of 10. 24 h after transfection, cells were fixed and confocal microscopy was performed to detect eGFP protein in the transfected cells (green), Rhodamine labeled-**9d-G2** (**III**) (red) or Cy5 labeled pEGFP DNA (blue). A merged image as well as images for each independent channel are shown. **A** and **B**.- CHO-k1 transfected cells at two magnifications. **C**.- CHO-k1 cells preincubated with chlorpromazine prior to transfection.

encodes for an enhanced red-shifted variant of wild-type GFP (eGFP). Plasmid was purified from transformed bacteria using standard methods.[25] DNA concentration was measured by a fluorimetric method using the Hoechst 33258 dye.[26] For confocal microscopy experiments, DNA was covalently labeled for 30 min with Cy5 dye using Mirus *label*IT® Tracker Intracellular Nucleic Acid Labeling Kit Cy™5 (Madison, WI, USA) at a DNA : label ratio 0.5 : 1 according to the manufacturer's instructions. Unbound label was removed by ethanol precipitation.

Synthesis of vinyl sulfone reagents for the functionalization of PAMAM-G2

General procedure for the synthesis of thioether 4a–d. To a solution of 2-thioethanol (70 mg, 0.9 mmol for **4a–c**; 155 mg, 2.0 mmol for **4d**) in deoxygenated acetonitrile (10 mL) (for **4a,b,d**) or DMSO-THF 1 : 1 (for **4c**) was added the bromo derivative **1a–c** or the mesyl derivative **3** (1.0 mmol) and K_2CO_3 (193 mg, 1.4 mmol) for **4a–c** or Cs_2CO_3 (650 mg 2.0 mmol) for **4d**. The solution was magnetically stirred at room temperature until TLC showed complete disappearance of starting materials (2 h for **4a,d**, 16 h for **4b–c**). After filtration, the solvent was evaporated under reduced pressure and the crude was purified by column chromatography. In the case of compound **4c**, filtration was followed by partial evaporation of the solvent under reduced pressure, addition of H_2O (30 mL) and extraction with CH_2Cl_2 (2 × 30 mL). The combined organic extracts were then dried (Na_2SO_4) and the solvent removed under reduced pressure to give a crude that was purified by column chromatography.

Compound 4a. Column chromatography (hexane–ether 1 : 1) of the crude gave **4a** as a liquid (127 mg, 95%), previously described in the literature.[27] ν_{max}(film)/cm^{-1}: 3372, 2958, 2873, 1465, 1275 and 1046; **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 3.73 (t, 2 H, J = 5.9 Hz), 2.74 (t, 2 H, J = 5.9 Hz), 2.54 (t, 2 H, J = 7.4 Hz), 2.32 (br s, 1 H), 1.59 (m, 2 H), 1.43 (m, 2 H), 0.93 (t, 3 H, J = 7.3 Hz); **^{13}C-NMR**

($CDCl_3$, 75 MHz): δ 60.3, 35.4, 31.9, 31.8, 22.0, 13.7; HRMS (*m/z*) (NALDI-TOF) calcd. for $C_6H_{13}S$ [M - OH]$^+$: 117.0738; found: 117.0736.

Compound 4b. Column chromatography (hexane–ether 2 : 1) of the crude gave **4b** as a syrup (214 mg, 87%). ν_{max}(film)/cm^{-1}: 3315, 2918, 2849, 1461, 1044 and 997; **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 3.72 (t, 2 H, *J* = 6.0 Hz), 2.73 (t, 2 H, *J* = 5.8 Hz), 2.52 (t, 2 H, *J* = 7.4 Hz), 2.43 (s, 1 H), 1.66–1.52 (m, 2 H), 1.45–1.15 (m, 18 H), 0.89 (t, 3 H, *J* = 6.6 Hz); **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 60.5, 35.5, 32.2, 31.9, 30.0, 29.9, 29.9, 29.8, 29.8, 29.6, 29.5, 29.1, 22.9, 14.3. HRMS (*m/z*) (ESI): calcd. for $C_{14}H_{31}OS$ [M + H]$^+$: 247.2096; found: 247.2098.

Compound 4c. Column chromatography (hexane–ether 1 : 1) of the crude gave **4c** as a solid (304 mg, 92%). M.P. 60–61 °C; ν_{max}(film)/cm^{-1}: 3213, 2918, 2849, 1460, 1063 and 719; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 3.71 (t, 2 H, *J* = 6.0 Hz), 2.72 (t, 2 H, *J* = 5.6 Hz), 2.51 (t, 2 H, *J* = 7.4 Hz), 2.11 (s, 1 H), 1.58 (m, 2 H), 1.41–1.20 (m, 30 H), 0.87 (t, 3 H, *J* = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 60.3, 35.5, 32.0, 31.8, 29.9, 29.8, 29.8, 29.7, 29.7, 29.6, 29.4, 29.3, 29.0, 22.8, 14.2.

Compound 4d. Column chromatography (hexane–ether 2 : 1) of the crude gave **4d** as a syrup (308 mg, 94%). ν_{max}(film)/cm^{-1}: 3371, 3003, 2923, 2852, 1461 and 1045; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 5.36–5.28 (m, 2 H), 3.69 (t, 2 H, *J* = 6.0 Hz), 2.70 (t, 2 H, *J* = 5.6 Hz), 2.49 (t, 2 H, *J* = 7.4 Hz), 2.35 (s, 1 H), 1.99 (dd, 4 H, *J* = 12.4 and 6.5 Hz), 1.60–1.52 (m, 2 H), 1.41–1.25 (m, 22 H), 0.86 (t, 3 H, *J* = 6.8 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 130.1, 129.9, 60.4, 35.4, 32.1, 31.8, 29.9, 29.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.0, 27.4, 27.3, 22.8, 14.3; HRMS (*m/z*) (ESI) calcd. for $C_{20}H_{41}OS$ [M + H]$^+$: 329.2878; found: 329.2865.

General procedure for the synthesis of sulfones 5a–d. To a solution of the corresponding thioether derivative **4a–d** (1 mmol) in AcOH (5 mL) was added 33% H_2O_2 (2 mL). The solution was kept in the dark for 16 h. Evaporation of the solvent under reduced pressure gave a crude that was purified by column chromatography.

Compound 5a. Column chromatography (ether) gave **5a** as a solid (144 mg, 87%). M.P. 42–43 °C; ν_{max}(film)/cm^{-1}: 3450, 2961, 2875, 1462, 1272, 1122 and 1065; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 4.12 (t, 2 H, *J* = 4.8 Hz), 3.21 (t, 2 H, *J* = 5.0 Hz), 3.09 (t, 2 H, *J* = 7.9 Hz), 2.62 (br s, 1 H), 1.84 (m, 2 H), 1.49 (m, 2 H), 0.97 (t, 3 H, *J* = 7.3 Hz); **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 56.4, 54.9, 54.4, 23.8, 21.7, 13.6. HRMS (*m/z*) (ESI) calcd. for $C_6H_{15}O_3S$ [M + H]$^+$: 167.0742; found: 167.0743.

Compound 5b. Column chromatography (ether) gave **5b** as a solid (230 mg, 83%). M.P. 81–82 °C; ν_{max}(film)/cm^{-1}: 3392, 2914, 2844, 1259 and 1116; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 4.14 (t, 2 H, *J* = 5.1 Hz), 3.20 (t, 2 H, *J* = 5.1 Hz), 3.07 (t, 2 H, *J* = 8.0 Hz), 1.80–1.70 (m, 2 H), 1.48–1.40 (m, 2 H), 1.38–1.20 (m, 16 H), 0.88 (t, 3 H, *J* = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 56.4, 54.8, 54.6, 31.9, 29.6, 29.5, 29.3, 29.2, 29.0, 28.4, 22.7, 21.8, 14.1. HRMS (*m/z*) (ESI) calcd. for $C_{14}H_{31}O_3S$ [M + H]$^+$: 279.1994; found: 279.1989.

Compound 5c. Column chromatography (EtOAc) gave **5c** as a solid (308 mg, 85%). M.P. 93–94 °C; ν_{max}(film)/cm^{-1}: 3368, 2913, 2847, 1594, 1470, 1256, 1133 and 1059; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 4.13 (t, 2 H, *J* = 5.0 Hz), 3.19 (t, 2 H, *J* = 5.0 Hz), 3.07 (t, 2 H, *J* = 8.0 Hz), 1.85 (m, 2 H), 1.50–1.18 (m, 30 H), 0.88 (t, 3 H, *J* = 6.6 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 56.5, 54.9, 54.7, 32.0, 29.8, 29.7, 29.6, 29.5, 29.4, 29.2, 28.6, 22.8, 22.0, 14.2. HRMS (*m/z*) (FAB+) calcd. for $C_{20}H_{42}O_3SNa$ [M + Na]$^+$: 385.2752; found: 385.2755.

Compound 5d. Column chromatography (hexane–EtOAc 1 : 1) gave **5d** as a syrup (245 mg, 68%). ν_{max}(film)/cm^{-1}: 3384, 2918, 2847, 1460, 1323, and 1121; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 5.30–5.26 (m, 2 H), 4.06 (t, 2 H, *J* = 5.2 Hz), 3.13 (t, 2 H, *J* = 5.2 Hz), 3.00 (t, 2 H, *J* = 8.1 Hz), 2.47 (br s, 1 H), 1.94 (dd, 4 H, *J* = 11.9 and 6.1 Hz), 1.83–1.73 (m, 2 H), 1.42–1.32 (m, 2 H), 1.30–1.12 (m, 20 H), 0.81 (t, 3 H, *J* = 6.8 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 130.3, 129.9, 56.6, 55.0, 54.8, 32.1, 30.0, 29.9, 29.7, 29.5, 29.5, 29.4, 29.3, 29.2, 28.7, 27.4, 27.3, 22.9, 22.0, 14.3; HRMS (*m/z*) (ESI) calcd. for $C_{20}H_{41}O_3S$ [M + H]$^+$: 361.2776; found: 361.2775.

General procedure for the synthesis of vinyl sulfones 6a–d. A solution of the corresponding sulfone **5a–d** (1 mmol) in anhydrous CH_2Cl_2 (20 mL) was cooled by means of an ice bath. Et_3N (0.41 mL, 3 mmol for **5a–c**; 0.82 mL, 6 mmol for **5d**) and mesyl chloride (0.12 mL, 1.5 mmol for **5a–c**; 0.24 mL, 3.0 mmol for **5d**) were then added and the solution was left to warm to room temperature until TLC showed complete disappearance of starting materials (2 h for **4a–b**, 24 h for **4c**, 6 h for **4d**). The solvent was removed under reduced pressure and the resulting crude purified by column chromatography.

Compound 6a. Column chromatography (hexane–ether 1 : 1) gave **6a** as a syrup (136 mg, 92%). ν_{max}(film)/cm^{-1}: 2919, 1673, 1463, 1384, 1312, 1130, 978 and 808; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.64 (dd, 1 H, *J* = 16.6 and 10.1 Hz, CH =), 6.44 (d, 1 H, *J* = 16.4 Hz, = CH_2*trans*), 6.17 (d, 1 H, *J* = 10.2 Hz, = CH_2*cis*), 2.99 (t, 2 H, *J* = 8.2 Hz), 1.80–1.72 (m, 2 H), 1.46 (m, 2 H), 0.95 (t, 3 H, *J* = 7.4 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 136.1, 130.4, 54.0, 24.3, 21.6, 13.5. HRMS (*m/z*) (ESI) calcd. for $C_6H_{13}O_2S$ [M + H]$^+$: 149.0636; found: 149.0640.

Compound 6b. Column chromatography (hexane–ether 1 : 1) gave **6b** as a syrup (218 mg, 84%). ν_{max}(film)/cm^{-1}: 2925, 1719, 1647, 1459, 1307, 1131, 1069, 973 and 666; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.60 (dd, 1 H, *J* = 16.6 and 9.9 Hz, CH =), 6.37 (d, 1 H, *J* = 16.6 Hz, = CH_2*trans*), 6.11 (d, 1 H, *J* = 9.9 Hz, = CH_2*cis*), 2.92 (t, 2 H, *J* = 8.1 Hz), 1.75–1.67 (m, 2 H), 1.36–1.18 (m, 18 H), 0.83 (t, 3 H, *J* = 6.8 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 136.1, 130.4, 54.3, 31.9, 29.6, 29.5, 29.3, 29.2, 29.0, 28.3, 22.7, 22.3, 14.1. HRMS (*m/z*) (ESI) calcd. for $C_{14}H_{29}O_2S$ [M + H]$^+$: 261.1888; found: 261.1879.

Compound 6c. Column chromatography (hexane–ether 1 : 1) gave **6c** as a solid (279 mg, 81%). M.P. 61–62 °C; ν_{max}(film)/cm^{-1}: 3062, 2915, 2847, 1462, 1284, 1124, 908 and 730; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.62 (dd, 1 H, *J* = 16.6 and 9.8 Hz, CH =), 6.43 (d, 1 H, *J* = 16.6 Hz, = CH_2*trans*), 6.15 (d, 1 H, *J* = 9.8 Hz, = CH_2*cis*), 2.96 (t, 2 H, *J* = 8.0 Hz), 1.77 (m, 2 H), 1.46–1.20 (m, 30 H), 0.88 (t, 3 H, *J* = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 136.3, 130.4, 54.4, 32.0, 29.8, 29.7, 29.7, 29.7, 29.6, 29.4, 29.3, 29.1, 28.5, 22.8, 22.4, 14.2. HRMS (*m/z*) (FAB+) calcd. for $C_{20}H_{40}O_2SNa$ [M + Na]$^+$: 367.2647; found: 367.2644.

Compound 6d. Column chromatography (hexane–EtOAc 5 : 1) gave **6d** as a liquid (274 mg, 80%). ν_{max}(film)/cm^{-1}: 3003,

2922, 2852, 1613, 1463, 1314, 1132 and 973; **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ 6.56 (dd, 1 H, J = 16.7 and 9.7 Hz), 6.37 (d, 1 H, J = 16.7 Hz), 6.09 (d, 1 H, J = 9.7 Hz), 5.32–5.23 (m, 2 H), 2.90 (t, 2 H, J = 8.1 Hz), 1.96–1.92 (m, 4 H), 1.74–1.67 (m, 2 H), 1.38–1.16 (m, 22 H), 0.81 (t, 3 H, J = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 125 MHz): δ 136.1, 130.3, 130.0, 129.6, 54.3, 31.9, 29.7, 29.6, 29.5, 29.3, 29.3, 29.1, 29.0, 28.3, 27.2, 27.1, 22.7, 22.3, 14.1; HRMS (*m/z*) (ESI) calcd. for $C_{20}H_{39}O_2S$ $[M + H]^+$: 343.2671; found: 343.2664.

Synthesis of 2-hydroxylethyl amide 8a. A solution of butyric acid chloride (10 mmol) in anhydrous Cl_2CH_2 (30 mL) was added dropwise to a solution of 2-amino ethanol (9 mL, 15 mmol) and Et_3N (2.8 mL, 20 mmol) in anhydrous Cl_2CH_2 (30 mL) The reaction mixture was kept at room temperature (15 min) and then evaporated under reduced pressure to give a crude that was purified by column chromatography (EtOAc) yielding **8a**, previously described in the literature,[28] as a syrup (1.18 g, 90%). ν_{max}(film)/cm^{-1}: 3294, 2940, 2873, 1649, 1558 and 535; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 4.41 (br s, 1 H, NH), 3.56 (t, 2 H, J = 4.7 Hz), 3.26 (q, 2 H, J = 5.3 Hz), 2.08 (t, 2 H, J = 7.5 Hz), 1.53 (m, 2 H), 0.83 (t, 3 H, J = 7.4 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 174.5, 61.4, 42.2, 38.3, 19.1, 13.6.

General procedure for the synthesis of 2-hydroxylethyl amides 8b–d. A solution of the corresponding acid **7b–d** (10 mmol) in Cl_2SO (11 mL for **8b–c**; 22 mL for **8d**) was magnetically stirred for 1 h at room temperature. Evaporation and coevaporation with anhydrous toluene (3 × 15 mL) under reduced pressure gave a crude that was dissolved in anhydrous Cl_2CH_2 (30 mL) for **8b,d** or anhydrous THF (50 ml) for **8c**. This solution was added dropwise to a solution of 2-amino ethanol (0.9 mL,15 mmol) and Et_3N (2.8 mL, 20 mmol) in anhydrous Cl_2CH_2 (30 mL) for **8b,d** or anhydrous THF (50 ml) for **8c**. The reaction mixture was kept at room temperature (15 min) and then evaporated under reduced pressure to give a crude that was purified by column chromatography.

Compound 8b. Column chromatography (EtOAc) gave **8b** as a solid (2.25 g, 93%). M.P. 89–91 °C (lit.[29] 90–92 °C); ν_{max}(film)/cm^{-1}: 3292, 2984, 2917, 1740, 1640, 1373, 1240 and 1046;**^{1}-NMR** ($CDCl_3$, 400 MHz): δ 6.18 (br s, 1 H), 3.69 (t, 2 H, J = 4.9 Hz), 3.39 (q, 2 H, J = 5.1 Hz), 2.20 (t, 2 H, J = 7.6 Hz), 1.60 (m, 2 H), 1.24 (m, 16 H), 0.86 (t, 3 H, J = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 174.6, 62.3, 42.4, 36.7, 31.9, 29.6, 29.6, 29.5, 29.3, 29.3, 29.3, 25.7, 22.6, 14.1.

Compound 8c. Column chromatography (Cl_2CH_2–MeOH 15 : 1 → 5 : 1) gave **8c** as a solid (3.0 g, 91%). M.P. 106–108 °C. ν_{max}(film)/cm^{-1}: 3294, 2917, 2848,1639, 1549, 1460 and 1214. Spectroscopic data identical to those previously reported in literature.[30]

Compound 8d. Column chromatography (EtOAc–hexane 2 : 1 → EtOAc) gave **8d** as a solid (2.88 g, 88%). M.P. 59–61 °C (lit.[31] 62–63 °C); ν_{max}(KBr)/cm^{-1}: 3301, 1642, 1561, 1464, 1265, 1211, 1057, and 1035; ^{1}H-NMR data identical to those reported in literature;[29] **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 174.7, 130.1, 129.8, 62.4, 42.5, 36.7, 32.0, 29.8, 29.8, 29.6, 29.4, 29.4, 29.3, 29.3, 29.2, 27.3, 27.2, 25.8, 22.7, 14.2.

General procedure for the synthesis of vinyl sulfones 9a–d. To a solution of the corresponding 2-hydroxyethyl amide **8a–d** (1 mmol) in THF (50 mL) was added DVS (0.21 mL, 2 mmol) and *t*-BuOK (11 mg, 0.10 mmol). The reaction mixture was magnetically stirred (20 min) at room temperature. Evaporation of the solvent under reduced pressure gave a crude that was purified by column chromatography.

Compound 9a. Column chromatography (EtOAc–hexane 2 : 1 → EtOAc) gave **9a** as a syrup (150 mg, 60%). ν_{max}(film)/cm^{-1}: 3380, 2963, 2874, 1649, 1544, 1313, 1126 and 756; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.67 (dd, 1 H, J = 16.6 and 9.9 Hz), 6.35 (d, 1 H, J = 16.6 Hz), 6.29 (br s, 1 H), 6.07 (d, 1 H, J = 9.9 Hz), 3.82 (t, 2 H, J = 5.5 Hz), 3.49 (t, 2 H, J = 5.1 Hz), 3.37 (q, 2 H, J = 5.2 Hz), 3.20 (t, 2 H, J = 5.5 Hz), 2.09 (t, 2 H, J = 7.5 Hz), 1.58 (m, 2 H), 0.86 (t, 3 H, J = 7.4 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 173.3, 137.5, 129.4, 69.8, 63.8, 54.5, 38.6, 38.4, 19.0, 13.7. HRMS (*m/z*) (NALDI-TOF) calcd. for $C_{10}H_{19}NO_4SNa$ $[M + Na]^+$: 272.0932; found: 272.0934.

Compound 9b. Column chromatography (EtOAc–hexane 2 : 1 → EtOAc) gave **9b** as a solid (191 mg, 53%). M.P. 64–66 °C; ν_{max}(KBr)/cm^{-1}: 3306, 3061, 2914, 2848, 1636, 1551, 1460, 1380, 1295, 1248, and 1123; **^{1}H-NMR** ($CDCl_3$, 500 MHz): δ 6.70 (dd, 1 H, J = 16.6 and 9.9 Hz), 6.43 (d, 1 H, J = 16.6), 6.12 (d, 1 H, J = 9.9 Hz), 3.89 (t, 2 H, J = 5.5 Hz), 3.55 (t, 2 H, J = 5.1 Hz), 3.44 (q, 2 H, J = 5.2 Hz), 3.24 (t, 2 H, J = 5.5 Hz), 2.17 (t, 2 H, J = 7.6 Hz), 1.61 (m, 2 H), 1.24 (br s, 16 H), 0.87 (t, 3 H, J = 6.8 Hz); **^{13}C-NMR** ($CDCl_3$, 125 MHz): δ 173.4, 137.6, 129.4, 70.0, 63.8, 54.6, 38.7, 36.7, 29.6, 29.6, 29.5, 29.3, 29.3, 25.7, 22.6, 14.1. HRMS (*m/z*) (NALDI-TOF) calcd. for $C_{18}H_{35}O_4SNa$ $[M + Na]^+$: 384.2184; found: 384.2189.

Compound 9c. Column chromatography (EtOAc) gave **9c** as a solid (249 mg, 56%). M.P. 89–91 °C; ν_{max}(KBr)/cm^{-1}: 3309, 2917, 2847, 1636, 1552, 1460, 1380, 1294, 1254, 1123, and 1089; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.70 (dd, 1 H, J = 16.6 and 9.9 Hz), 6.44 (d, 1 H, J = 16.6 Hz), 6.13 (d, 1 H, J = 9.9 Hz), 6.1 (br s, 1 H), 3.89 (t, 2 H, J = 5.5 Hz), 3.56 (t, 2 H, J = 5.0 Hz), 3.45 (q, 2 H, J = 5.2 Hz), 3.25 (t, 2 H, J = 5.5 Hz), 2.17 (t, 2 H, J = 7.7 Hz), 1.62 (m, 2 H), 1.24 (m, 28 H), 0.87 (t, 3 H, J = 6.7 Hz); **^{13}C-NMR** ($CDCl_3$, 100 MHz): δ 173.4, 137.6, 129.4, 70.0, 63.8, 54.6, 38.7, 36.7, 31.9, 29.7, 29.6, 29.6, 29.6, 29.5, 29.4, 29.3, 29.3, 25.7, 22.7, 14.1. HRMS (*m/z*) (NALDI-TOF) calcd. for $C_{24}H_{47}NO_4SNa$ $[M + Na]^+$: 468.3123; found: 468.3124.

Compound 9d. Column chromatography (EtOAc–hexane 2 : 1 → EtOAc) gave **9d** as a syrup (226 mg, 51%). ν_{max}(film)/cm^{-1}: 3303, 1646, 1541, 1460, 1379, 1312, 1249, and 1124; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.71(dd, 1 H, J = 16.4 and 9.7 Hz), 6.45 (d, 1 H, J = 16.4 Hz), 6.14 (d, 1 H, J = 9.6 Hz), 6.12 (br s, 1 H), 5.35 (m, 2 H), 3.91 (t, 2 H, J = 5.4 Hz), 3.57 (t, 2 H, J = 5.0 Hz), 3.46 (q, 2 H, J = 5.2 Hz), 3.26 (t, 2 H, J = 5.4 Hz), 2.18 (t, 2 H, J = 7.7 Hz), 2.01 (m, 4 H), 1.62 (m, 2 H), 1.28 (several m, 20 H), 0.88 (m, 3 H); **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 173.5, 137.6, 130.0, 129.8, 129.5, 70.0, 63.9, 54.6, 38.8, 36.7, 32.0, 29.8, 29.8, 29.6, 29.4, 29.4, 29.2, 27.3, 27.3, 25.8, 22.8, 14.2; HRMS (*m/z*) (FAB+) calcd. for $C_{24}H_{45}NO_4SNa$ $[M + Na]^+$: 466.2967; found: 466.2968.

Compound 10d. A solution of oleic acid **7d** (850 mg, 3 mmol) in Cl_2SO (10 mL) was magnetically stirred for 1 h at room temperature. Evaporation and coevaporation with anhydrous toluene (3 × 15 mL) under reduced pressure gave a crude that was

dissolved in anhydrous Cl_2CH_2 (30 mL). This solution was added dropwise to a solution of cystamine dihydrochloride (260 mg, 1.15 mmol) and Et_3N (0.98 mL, 6.9 mmol) in anhydrous Cl_2CH_2 (30 mL). The reaction mixture was kept at room temperature (15 min) and then evaporated under reduced pressure to give a crude that was purified by column chromatography (ether–hexane 2 : 1 → ether) yielding **10d** as a solid (720 mg, 92%). M.P. 83–85 °C; ν_{max}(KBr)/cm^{-1}: 3320, 1632, 1536, 1464, 1419, 1261, and 1192; **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 6.28 (br s, 2 H), 5.34 (m, 4 H), 3.57 (q, 4 H, J = 6.3 Hz), 2.82 (t, 4 H, J = 6.4 Hz), 2.20 (t, 4 H, J = 7.5 Hz), 2.00 (m, 8 H), 1.63 (m, 4 H),1.28 (several m, 40 H), 0.88 (m, 6 H); **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 173.9, 130.2,129.9, 38.6, 38.1, 36.8, 32.1, 30.0, 29.9, 29.8, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 27.4, 25.9, 22.9, 14.3; HRMS (*m*/*z*) (FAB+) calcd. for $C_{40}H_{76}N_2O_2S_2Na$ [M + Na]$^+$: 703.5246; found: 703.5250.

Compound 11d. To a solution of **10d** (960 mg, 1.4 mmol) in AcOH (20 mL) was added powdered Zn (1.1 g, 17 mmol). The reaction mixture was magnetically stirred at 50 °C for 40 min. After cooling and filtration over zeolite, Cl_2CH_2 (70 mL) was added and the resulting organic solution was washed with water (2 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL) and additional water (30 mL). The organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to give a crude that was dissolved in previously deoxygenated THF : isopropanol (2 : 1, 25 mL) by bubbling Ar for 5 min. DVS (809 μL, 5.65 mmol) and Et_3N (40 μL, 0.28 mmol) were added and the new reaction mixture was magnetically stirred at room temperature under an Ar atmosphere for 1.5 h. Evaporation of the solvent gave a crude that was purified by column chromatography (ether) yielding **11d** as a solid (890 mg, 69%). M.P. 85–87 °C; ν_{max}(KBr)/cm^{-1}: 3319, 1632, 1536, 1463, 1418, 1261, and 1191; **^{1}H-NMR** ($CDCl_3$, 300 MHz): δ 6.70 (dd, 1H, J = 16.6 and 9.7 Hz), 6.48 (d, 1 H, J = 16.6 Hz), 6.23 (d, 1 H, J = 9.7 Hz), 5.85 (br s, 1 H), 5.36 (m, 2H), 3.45 (q, 2 H, J = 6.4 Hz), 3.26 (m, 2 H), 2.91 (m, 2 H), 2.71 (t, 2H, J = 6.5 Hz), 2.18 (t, 2 H, J = 7.6 Hz), 2.00 (m, 4 H), 1.62 (m, 2 H), 1.28 (several m, 20 H), 0.88 (m, 3 H); **^{13}C-NMR** ($CDCl_3$, 75 MHz): δ 173.5, 136.3, 131.5, 130.2, 129.9, 54.5, 38.5, 36.9, 32.8, 32.7, 32.2, 32.1, 30.0, 29.9, 29.7, 29.5, 29.5, 29.4, 27.4, 27.4, 25.9, 23.9, 22.9, 14.3; HRMS (*m*/*z*) (FAB+) calcd. for $C_{24}H_{45}NO_3S_2Na$ [M + Na]$^+$: 482.2738; found: 482.2734.

Compound 13. To a solution of cholesterol **12** (300 mg, 0.77 mmol) in THF (20 mL) was added DVS (0.12 mL, 1.16 mmol) and *t*-BuOK (9 mg, 0.077 mmol). The reaction mixture was magnetically stirred at room temperature for 1 h. Amberlita IR 120H was then added and the magnetic stirring continued for additional 30 min. After filtration, the solvent was evaporated under reduced pressure. TLC of the crude showed the presence of cholesterol. Ac_2O (8 mL) and pyridine (4 mL) were added to the resulting crude and the new reaction mixture was kept at room temperature for 16 h. Acetylation of the crude reaction allowed the separation of compound **13**. Evaporation under reduced pressure gave a crude that was purified by column chromatography (ether–hexane 1 : 2) yielding **13** as a solid (204 mg, 52%). M.P. 133–135 °C; $[\alpha]_D$ −19 (*c* 1, chloroform); ν_{max}(KBr)/cm^{-1}: 3409, 1461, 1373, 1319, 1115, and 1052; **^{1}H-NMR** ($CDCl_3$, 400 MHz): δ 6.75 (dd, 1 H, J = 16.7 and 9.9 Hz), 6.40 (d, 1 H, J = 16.7 Hz), 6.07 (d, 1 H, J = 9.9 Hz), 5.35 (br s, 1 H), 3.88 (t, 2 H, J = 5.6 Hz), 3.23 (t, 2 H, J = 5.6 Hz), 3.19 (m, 1 H), 2.35–1.84 (several m, 7 H), 1.56–0.95 (several m, 21 H), 0.99 (s, 3 H), 0.92 (d, 3 H, J = 6.4 Hz), 0.86 (d, 6 H, J = 6.6 Hz), 0.67 (s, 3 H); **^{13}C-NMR** ($CDCl_3$, 125 MHz): δ 140.2, 138.0, 128.5, 122.1, 79.8, 61.5, 56.7, 56.1, 55.4, 50.1, 42.3, 39.7, 39.5, 38.8, 37.0, 36.8, 36.2, 35.8, 31.9, 31.8, 28.2, 28.1, 28.0, 24.3, 23.8, 22.9, 22.5, 21.0, 19.3, 18.7, 11.8; HRMS (*m*/*z*) (FAB+) calcd. for $C_{31}H_{52}O_3SNa$ [M + Na]$^+$: 527.3535; found: 527.3535.

Synthesis of amphiphilic PAMAM-G2 dendrimers

General procedure. To a solution of PAMAM-G2 (0.3 mM, 1 mg mL^{-1}) in milliQ-water (15 mL) was added the vinyl sulfone reagents **6a–d**, **9a–d**, **11d** and **13** (0.15 mM for **I**, 0.3 mM for **II** and 0.6 mM for **III**) in THF (15 mL). The solution was magnetically stirred at room temperature for 1 day. Then the solvent (THF) was evaporated under reduced pressure and the water was freeze-dried. The products were directly used in the transfection assays.

Synthesis of fluorescent PAMAM-G2. To a solution of PAMAM-G2 (0.6 mM, 2 mg mL^{-1}) in milliQ-water (7.5 mL) was added the vinylsulfone rhodamine B **14** [17] (0.3 mM) in milliQ-water (7.5 mL). The solution was magnetically stirred at room temperature for 1 day and then water was freeze-dried. The product (**G2-Rho**) was directly used in the transfection assays.

Synthesis of fluorescent amphiphilic PAMAM-G2 derivatives. To a solution of PAMAM-G2 (0.6 mM, 2 mg mL^{-1}) in milliQ-water (7.5 mL) was added the vinylsulfone rhodamine B **14** (0.3 mM) in milliQ-water (7.5 mL). The solution was magnetically stirred at room temperature for 1 day. Then compound **6c** (0.15 mM) or **9d** (0.6 mM) were added in THF (15 mL). The reaction mixture was again magnetically stirred at room temperature for 1 day. The solvent (THF) was evaporated under reduced pressure and the water was freeze-dried. The products (**6c-G2(I)-Rho** and **9d-G2(III)-Rho**) were directly used in the transfection assays.

Preparation of amphiphilic PAMAM-G2/Plasmid pEGFP-N3 complexes

Amphiphilic PAMAM-G2/pDNA complexes were prepared at several N/P ratios where N = number of primary amines in the conjugate and P = number of phosphate groups in the pDNA backbone. Plasmid pEGFP-N3 was used for the preparation of the DNA complexes and for transfection assays. The quantities of functionalized PAMAM-G2 dendrimers used were calculated according to the desired DNA concentration of 0.1 mg mL^{-1}, the N/P ratio, the molecular weight and the number of positive charges in the selected PAMAM derivative. Experiments were performed for N/P 0.5 to 50. The desired amount of functionalized PAMAM-G2 dendrimer was added from a 20 mg mL^{-1} stock solution (1 : 2 DMSO–H_2O). The solution was previously diluted so that the desired N/P ratio was reached by mixing with an equal volume of the plasmid solution. In this way, the DMSO proportion was identical in all experiments (16.7% v/v). The preparation was vortexed for a few seconds and incubated for 20 min.

Gel electrophoresis shift assay

The binding capability of PAMAM derivatives for DNA was analyzed by gel electrophoresis. PAMAM-G2 derivatives were prepared in a DMSO–H_2O (1 : 2) solution while DNA was dissolved in NaCl 150 mM. pEGFP-N3 DNA (10 μL at 0.1

μg μL^{-1}) was mixed with an equal volume of PAMAM-G2 derivatives using N/P ratios 0–10 and incubated for 30 min at room temperature before the addition of loading buffer (2 μL). An aliquot (5 μL) of each sample was subjected to agarose gel electrophoresis (0.8% w/v) in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Electrophoresis was carried out at 7 V cm^{-1} and gels were stained after electrophoresis with ethidium bromide.

DNase protection assays

pEGFP-N3 DNA (10 μL at 0.1 μg μl^{-1}) was mixed with 5 μl of PAMAM-G2 derivatives to give a final N/P ratio of 5 and incubated for 30 min at room temperature. To the mixture, 10 μL of a solution of DNase I (0.05 mg mL^{-1} in Tris HCl 50 mM pH 8, 2000 U/mg) were added and incubated for 1 h at 37 °C. After the digestion, 2 μL of a 10% SDS solution were added and the samples incubated for 15 min at 65 °C before the addition of loading buffer (4 μL). Finally, an aliquot (20 μL) of each sample was subjected to agarose gel electrophoresis (0.8% w/v) in TAE buffer. Quantification of the band intensity was performed with the NIH Image Software.[32] A value of 100 has been assigned to the intensity of the band corresponding to the control undigested DNA.

Cell culture and DNA transfection assays

Wild type Chinese Hamster Ovary (CHO-k1; ATCC no. CCL-61), RAW 264.7 (ECACC No. 91062702) and Neuro-2a (ATCC No. CCL-131) cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine plus 100 U/mL penicillin, and 0.1 μg mL^{-1} streptomycin. All cell lines were maintained at 37 °C in a humidified incubator containing CO_2 (5%) and air (95%). Prior to transfection, cells were seeded in 24 well plates at a density of 2.25×10^4 cells cm^{-2} and incubated for 24 h to reach a cell confluence of 80–90%. For transfection experiments, pEGFP-N3 plasmid (0.5 μg cm^{-2}) was mixed with the corresponding PAMAM-G2 derivative at N/P ratios of 2.5, 5, 10, 20 and 50 at room temperature for 20 min in a final volume of 20 μL. The mixture was diluted to 0.5 mL with DMEM without serum or supplemented with 10% serum and added to each well. Cells were incubated with the DNA–PAMAM derivatives mixtures for 5 h, then the transfection media was removed and cells were further grown in DMEM media plus 10% FBS for an additional period of 48 h. Untransfected cells and naked pEGFP-N3 were used as negative controls. Transfections using unmodified PAMAM-G2 were always included as a control. LP2000 was also used as a positive control in gene delivery experiments. LP2000 polyplexes were prepared using 2 μl of LP2000 and 1.0 μg of DNA, according to the manufacturer's instructions. For the assay of the effects of dendrimers internalizations, cells were preincubated for 30 min with chlorpromazine (Sigma), 14 μM or genistein (Sigma), 200 μM prior to the adition of the dendriplexes.

Particle size and ζ potential measurements

The particle diameter was measured at 633 nm on a dynamic light scattering instrument (MALVERN 4700C) at room temperature with a detection angle of 60°. Zeta potential measurements were performed using a ZetaPALS instrument (Brookhaven, USA). Zeta potentials were calculated using the Smoluchowsky model, each data point was taken as an average over five independent sample measurements. Solutions of dendriplexes were prepared with a pDNA concentration of 0.1 mg mL^{-1} at different N/P ratios (the optimum for transfection in each case) in the same way as commented before. Measurements were taken on these solutions before and after addition of DNA.

Cytotoxicity of the complexes

Cytotoxicity of the complexes formed by the functionalized dendrimers and pDNA was evaluated 24 h after transfection. Cytotoxicity was assayed by determining the percentage of cell viability (with respect to unexposed cells) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) method,[33] that correlate the cellular metabolic activity and the number of viable cells in culture. Results are reported as % viability based on the untreated control cells normalized to 100% viable.

Fluorescence and protein assay

Transfected cells were washed three times with PBS and 600 μl of 0.5% Triton X-100 in PBS was added. Plates were shaken for 10 min at room temperature and the lysate was recovered and the eGFP fluorescence was quantified in a Shimadzu RF-5301PC fluorimeter using an excitation of 480 nm (5 nm) and 510 nm (10 nm) emission wavelengths. Protein concentration was measured using the Bio-Rad Protein Assay (Hercules, CA, USA). In the transfection experiments, fluorescence in the positive control using LP2000 polyplexes was assigned a value of 100 and the fluorescence results obtained in the transfections with the PAMAM dendrimers were normalized to this value.

Confocal microscopy

CHO-k1 cells were seeded onto 12-mm coverslips in 24-well plates at a density of 11250 cells cm^{-2} 24 h before transfection. Polyplexes were prepared as described before, but using Cy5 labeled pEGFP-N3 as plasmid DNA and rhodamine-labeled PAMAM derivatives. Control experiments were carried out using either unlabeled pEGFP-N3 plasmid or unlabeled PAMAM derivatives. Transfection was carried out using 1 μg Cy5-labeled pEGFP-N3 and the rhodamine labeled compound **9d-G2(III)-Rho** at a N/P ratio of 10. 5 h after transfection, the medium was changed to complete medium and cells were grown for an additional period of 24 h to allow expression of eGFP in the transfected cells. Cells were fixed with 2% paraformaldehyde in PBS for 15 min at room temperature and coverslips were mounted on glass slides using Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA). Confocal microscopy was performed on a Leica DMI6000 confocal microscope. To prevent cross-talk of eGFP, rhodamine or Cy5 chromophores, distinct excitation laser lanes and non-overlapping detection channels were selected for their detection: eGFP was excited using the 488 nm line of a krypton/argon laser and the emitted fluorescence was detected with a 504–562 nm channel. Rhodamine was detected using the He/Ne 543 nm excitation line and a 574–626 nm channel and Cy5 was excited using a He/Ne 633 nm laser and a 651–688 nm chanel. Under these conditions, no signal overspill among the individual fluorescence channels was observed. For color analysis, images were collected separately in single channel mode using a

sequential acquisition mode. All samples were exposed to laser for a time interval not >5 min to avoid photobleaching. The laser was set to the lowest power able to produce a fluorescent signal. Maximum voltage of photomultipliers was used to decrease the required laser power as much as possible. A pinhole of 1 Airy unit was used. Images were acquired at a resolution of 1024 × 1024. Series were acquired in the xyz mode. Data was processed using Leica AF software package.

Statistical analysis

Results are expressed as mean ± SEM for the number of experiment indicated. The statistical significance of variations was evaluated using ONE-WAY Analysis of Variance (ANOVA). When significant effect was found, post hoc comparisons of the means were done using the *t* adjusted Tukey's test. A p value <0.05 was considered significant.

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