Chapter 4

Fluorescently labeled arginino-calix[4]arenes for cell uptake investigation

4.1 Introduction

Despite the large number of reports about the successful pDNA delivery using non-viral vectors, especially in vitro, the precise mechanism of their internalization is controversial. Not surprisingly, for systems whose function is a consequence of numerous variables multiple mechanisms might be involved.[1]

The generally agreed first step of the translocation mechanism involving arginine-rich CPPs or their analogues is the association of the vectors with the membrane. Each positively charged guanidinium head groups thanks to their rigid planar array forms strong bidentate hydrogen bonds with the negatively charged units of many cell membrane constituents such as phospholipids, fatty acids, proteins, or with heparan sulfate proteoglycans. The accumulation of these molecules on cell surface is so relevant that it has been assumed it can promote the alteration and following crossing of the membrane thus allowing a direct membrane translocation by arginine-rich CPPs and their NAP conjugates.[2]

Endocytosis is considered to be the dominant cellular uptake pathway for DNA delivery using cationic lipids or polymers and CPPs.[3] It is certainly the most investigated, in particular in the forms of clathrin-mediated and caveolin-mediated endocytosis,[4] which start with the formation of concave structures of plasmatic membranes, and macropinocytosis, which involves the enclosing of actin-containing membrane protrusion to form vescicles called macropinosomes.[5] In both cases, the efficient internalization is again induced by the high cationic charge density that facilitates the formation of vesicular compartments (endosomes).[6]

Even though many endocytic uptake studies threw light on the first steps of the transfection process, the exact mechanism of DNA release from the complex with the vector, DNA escape from the endosome and DNA entry into the nucleus, in order to be expressed, remain still rather unknown.[7]

Despite the transfection and cell viability studies performed so far for macrocyclic delivery systems, no definitive information on the cellular uptake process and cellular fate for calixarenes have been collected. The uptake of a fluorescently labeled cationic calix[4]arene was recently investigated.[8] This compound bearing a NBD moiety was rapidly taken up into the CHO cells and localized over time into lysosomes. Despite the high concentration used (10 mM) no cytotoxic effects were observed. However, it was not tested in transfection experiments.

In order to disclose the mechanism through which the complexes **3**:pDNA are internalized into RD-4 cells, some transfection experiments were performed in presence of inhibitors of the principal uptake pathways. No effects on transfection efficiency were evidenced by treatment with wortmannin, an inhibitor of macropinocytosis, or with cythochalasin D, an inhibitor of caveole-mediate endocytosis. On the contrary, the percentage of transfected cells was partially reduced by using chlorpromazine, an inhibitor of clathrin-mediate endocytosis, which was recently found out as one of the uptake mechanisms for cyclodextrin based vector.[9] However, on the basis of these results, it is not possible to draw conclusions also due to the toxicity observed during the tests.

Therefore, another important structural modification respect to our best vector **3** was the introduction of a fluorophore unit addressed to follow the macrocycle and its complex with DNA into the cell by fluorescence microscopy techniques and then to better understand the mechanism determining its high transfection efficiency.

As described in Chapter 3, the groups at the upper rim of calixarene **3** could not be modified without losing most of its transfection efficiency. We thus decided to carry out a chemical modification of the macrocyclic core at the lower rim in order to prepare an asymmetrically functionalized derivative incorporating a single fluorescent unit (**Fig 4.1**). This strategy should on one hand avoid fluorescence quenching and, on the other hand, preserve the amphiphilic features of the vector, important for biological properties.

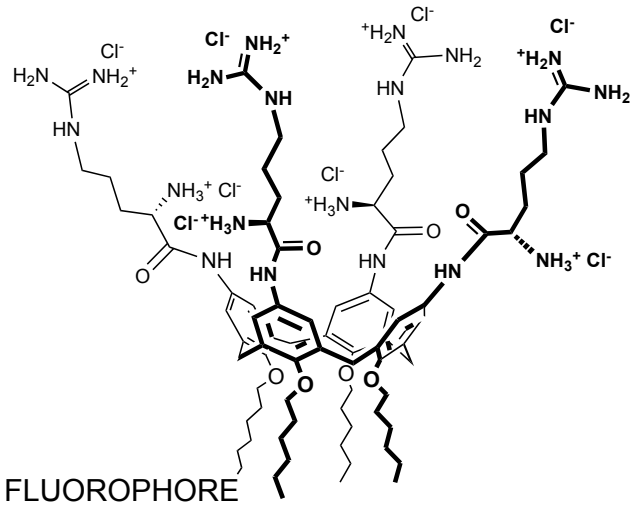
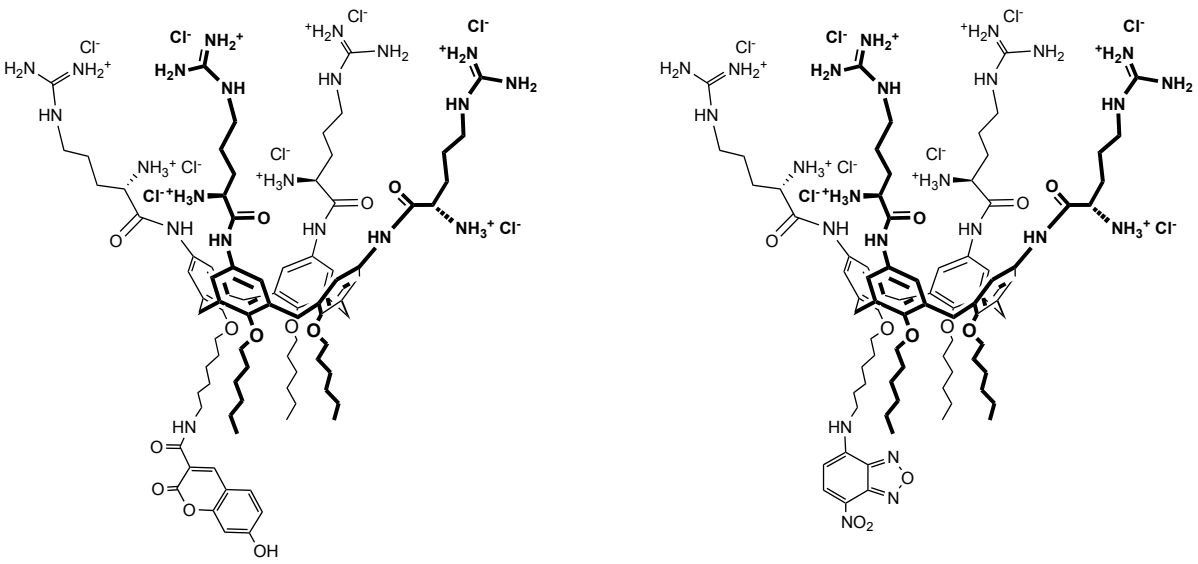


Fig 4.1 A general structure of an analogue of vector **3** bearing a fluorophore at the lower.

Initially, the coumarin was chosen as fluorophore (see vector **51**) because its emission in the blue region can be clearly distinguished from that of the Green Fluorescent Protein, expressed in cells upon the transfection experiments.

As an alternative, the nitrobenzofurazane moiety (NBD) connected to the vector through a nitrogen atom was used as fluorescent probe in a second analogue (**53**). It emits in the green part of the visible spectrum but for its detection during the transfection experiments we considered to use in this case the plasmid of the Red Fluorescent Protein. The NBD derivative offers chemical stability and strong fluorescence intensity over a wide range of conditions (pH and ionic strength) and as mentioned above, it already demonstrated to work when installed on a calixarene derivative.[8a] Possible quenching processes are in fact difficult to foreseen when a fluorophore is introduced in a molecule, but in this case could reasonably be ruled out.



51 **53**

Fig 4.2 Structural formulas of coumarin- (**51**) and NBD-labeled (**53**) calixarenes.

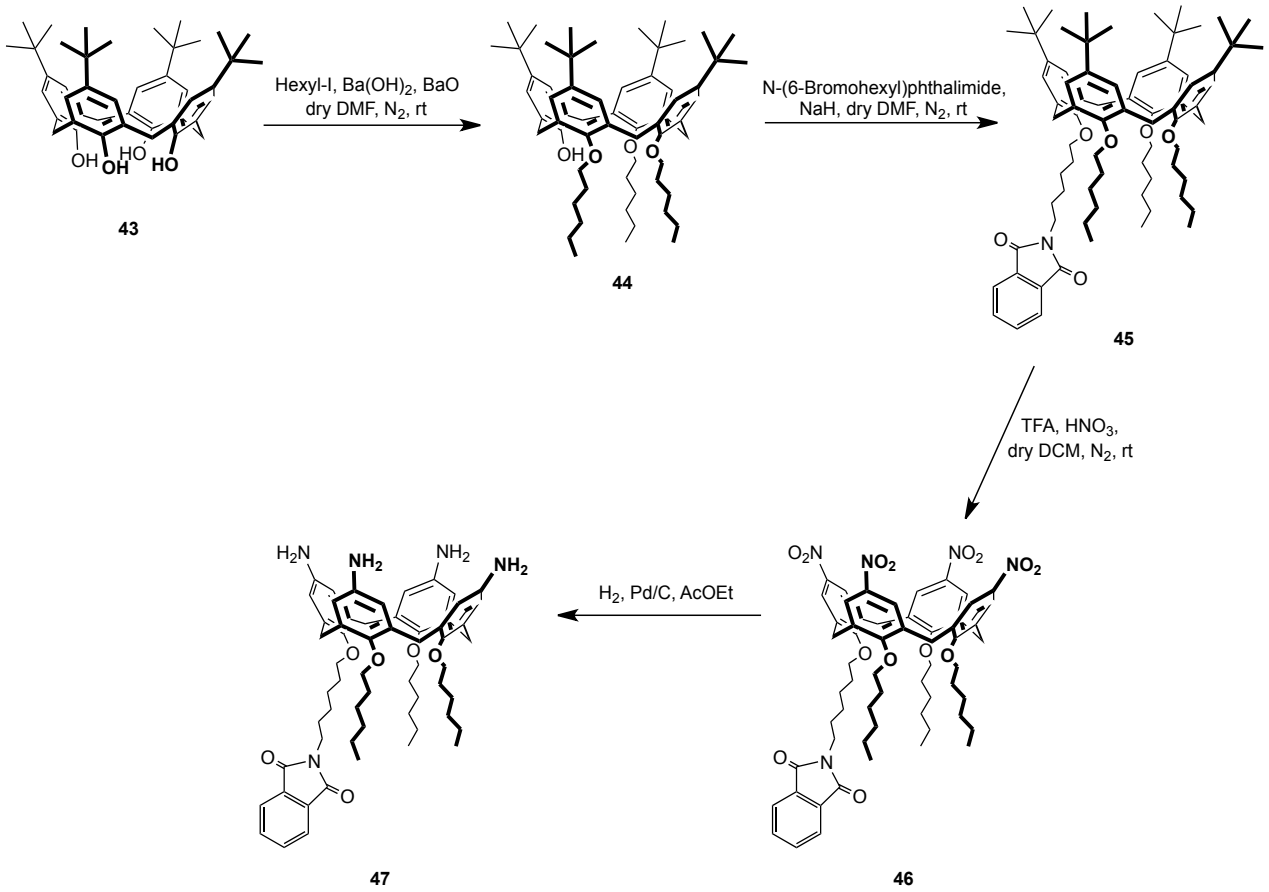
4.2 Results and discussion

4.2.1 Synthesis

For the synthesis of the two labeled derivatives **51** and **53** it was necessary to introduce at the lower rim a chain terminating with an active unit available for the coupling with the fluorophore. This unit was conveniently considered to be for both compounds an amine, being commercially available the coumarin with the carboxy group for an easy condensation and the 4-chloro-7-nitrobenzofurazane for a well-established nucleophilic aromatic substitution.

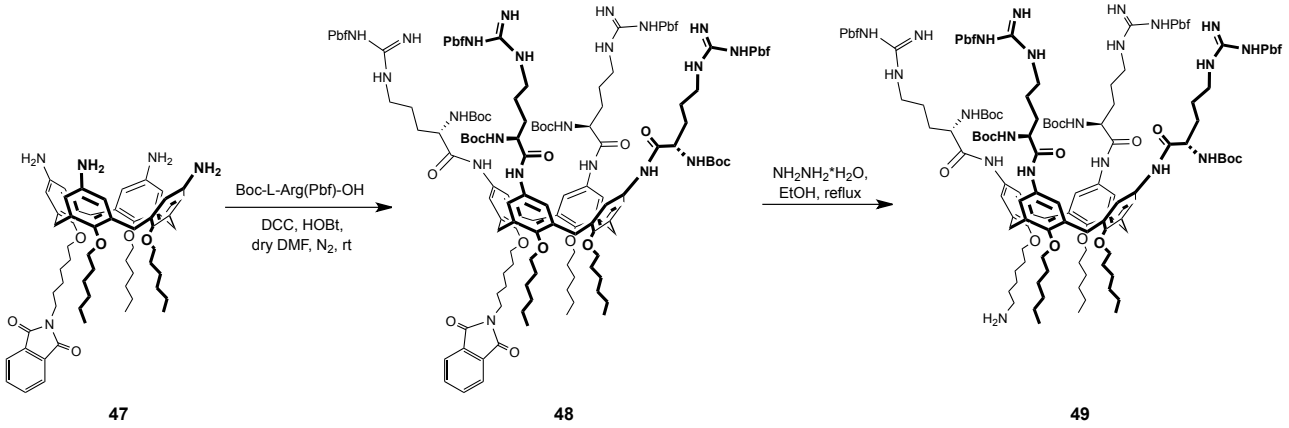
The simpler and more direct way of functionalizing all the four phenolic hydroxyl groups with a protected amine containing chains was discarded because the total absence of the lipophilic hexyl chains could completely modify the amphiphilicity of the vector and therefore the transfection properties of the system.

We started then with the introduction of three hexyl chains at the lower rim of *p*-tertbutylcalix[4]arene **43** (**Scheme 1**) exploiting the $Ba(OH)_2$/BaO couple as base in conditions known to afford the desired selectivity. To **44**[10] it was linked the fourth chain terminating with a protected amine in the form of phtalimide (**45**). NaH was necessary as base for the complete deprotonation of the last hydroxy group of the calixarene scaffold.



Scheme 1: synthesis of calixarene **47**.

Subsequent *ipso*-nitration to compound **46** and reduction gave the upper rim tetra-amino calixarene **47**. The reduction of nitro groups at the upper rim of calixarenes had been performed on similar compounds in the previous chapters by using hydrazine in the presence of Pd as catalyst. In the case of compound **46**, however, the presence of the protective group at the lower rim precluded the use of this method. The transformation into amines was, thus, carried out in different conditions. A first attempt was performed using $SnCl_2$, but the product isolation resulted rather problematic because of the large amount of inorganic salts formed during the workup, which drastically lowered the yield (10%). The reduction step was then carried out by catalytic hydrogenation, which allowed to isolate **47** by simple filtration with a significantly higher yield (60%).

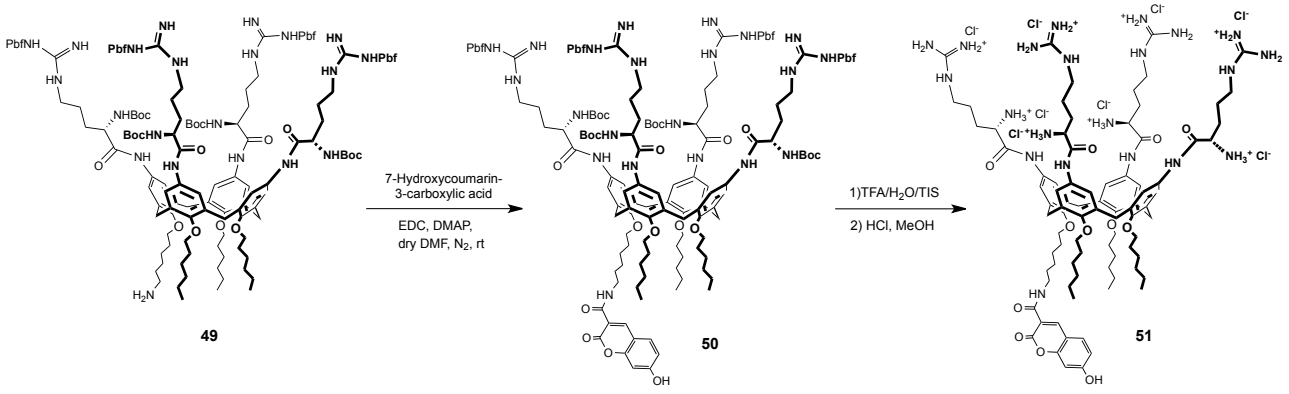


Scheme 2: synthesis of the difunctional intermediate **49**.

Compound **47** was then coupled with the four units of protected L-arginine (**Scheme 2**). The reaction was carried out once again by using Boc-L-Arg(Pbf)-OH, being its protecting groups easily removed in acidic conditions and stable if treated with hydrazine as requested for the removal of phthalimide.

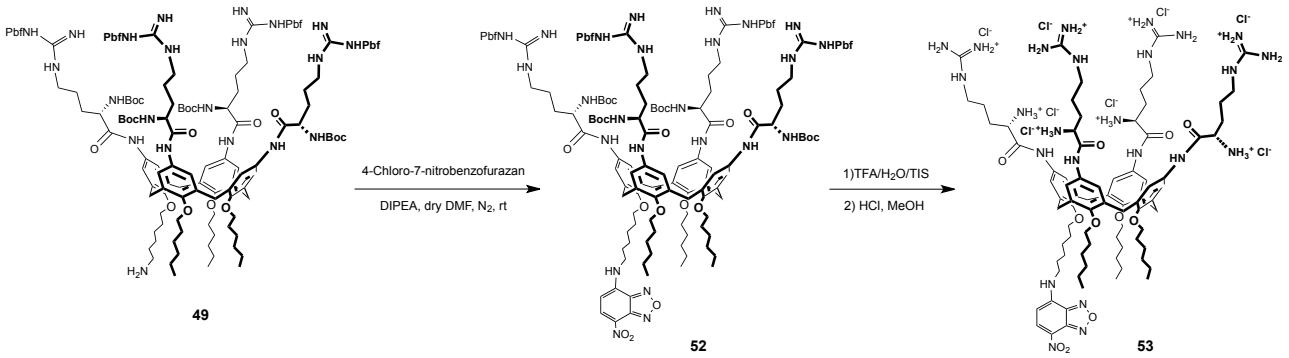
The following step was in fact the deprotection from phthalimido group, leading to compound **49**, which was covalently labeled with coumarin or NBD units giving derivatives **50** (**Scheme 3**) and **52** (**Scheme 4**), respectively.

First, for the conjugation with the carboxy-coumarin, the formation of the amide bond was obtained with the use of EDC as coupling reagent. The resulting coumarin labeled calixarene **50** was subsequently deprotected with a TFA solution in presence of TIS (triisopropylsilane) as carbocation scavenger and then isolated as the corresponding octahydrochloride salt **51**.



Scheme 3: synthesis of the coumarin-labeled calixarene **51**.

In parallel, calixarene **49** was also reacted in a nucleophilic substitution with 4-chloro-7-nitro-benzofurazane (NBD-Chloride) leading, after arginine deprotection and counterion exchange, to the second fluorescent calixarene **53** (**Scheme 4**).



Scheme 4: synthesis of the NBD-labeled calixarene **53**.

The ^{1}H-NMR spectra of labeled derivatives **51** (**Fig. 4.3**) and **53** (**Fig 4.4**) showed several signals for the aromatic protons, at around 7 ppm, as a consequence of the difference between the two aromatic protons of the same phenolic ring induced by resonance and chirality of the substituent at the upper rim and by the presence of the fluorophore unit at the lower rim. In the case of derivative **53** (**Fig 4.4**) the effect due to the functionalization at the lower rim results particularly marked. Even seven singlets well distinguishable for the eight different aromatic protons were in fact observed. Moreover, two superimposed doublets for the axial protons of the calixarene methylene bridge and other two for the equatorial ones are clearly visible as consequence of the presence of the NBD unit.

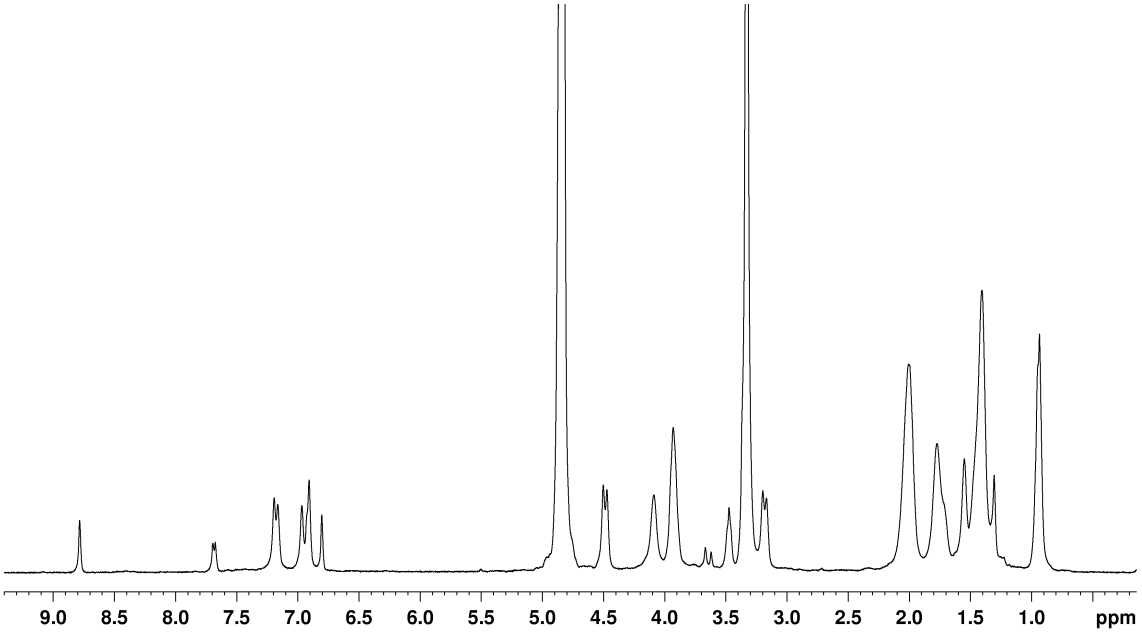


Fig 4.3 ^{1}H-NMR spectrum (CD_3OD, 400 MHz) of **51**.

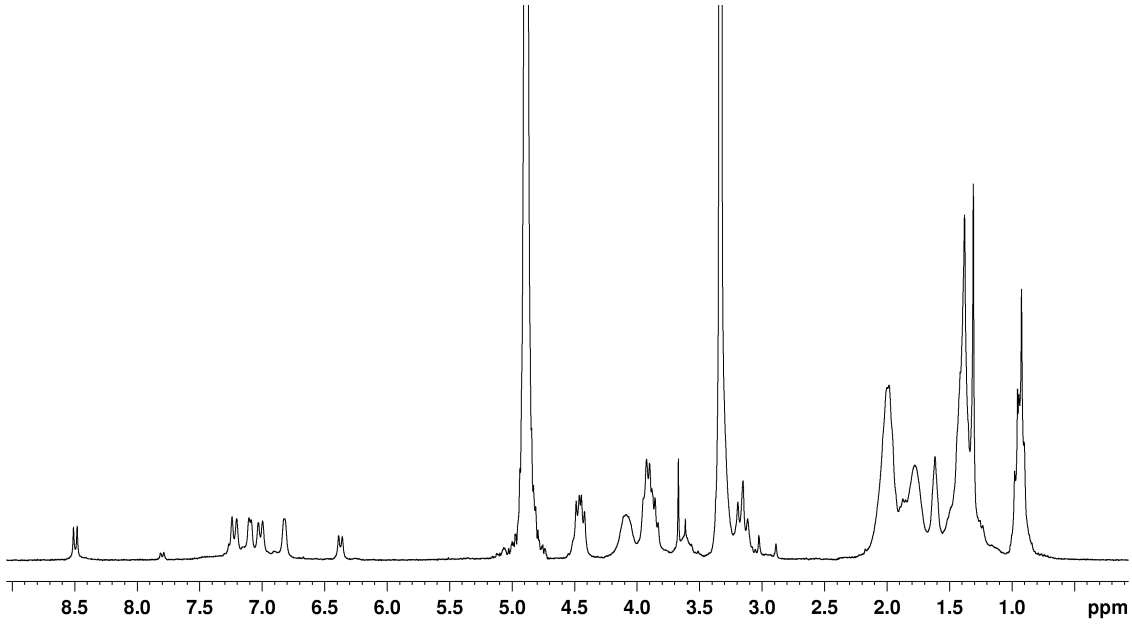


Fig 4.4 ^{1}H-NMR spectrum ($CDCl_3/CD_3OD$ 1:1, 300 MHz) of **53**.

Unfortunately, compounds **51** and **53** are not soluble in pure water. So they were studied in DMSO/H_2O (1:9) solutions. The absorption and emission spectra were determined in this polar medium. The wavelength of their maximum of absorption was used to excite the molecules and to obtain the corresponding fluorescence spectra (**Fig. 4.5**).

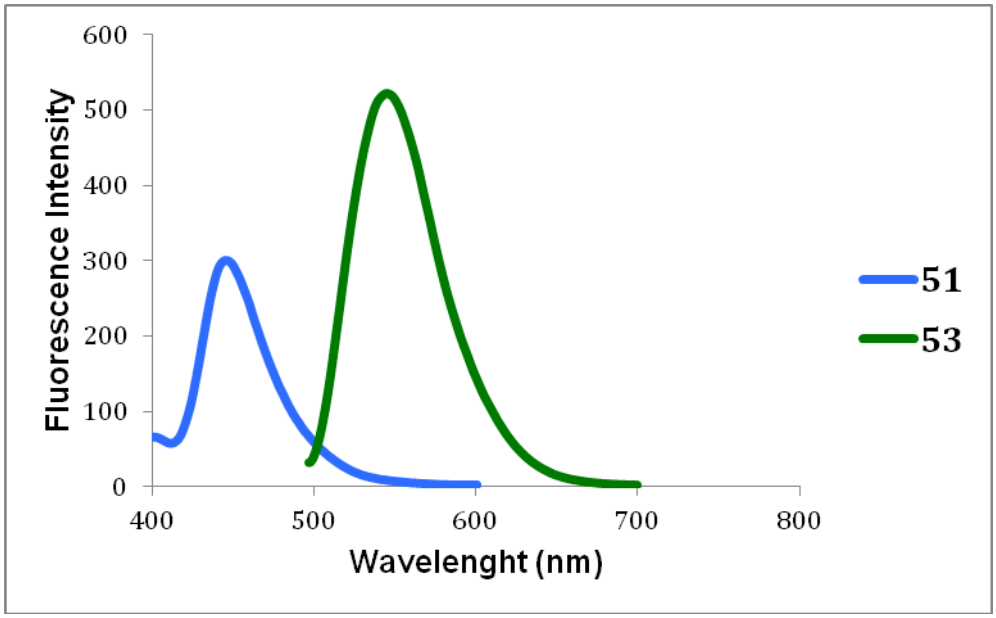


Fig 4.5 Fluorescence spectra of **51** (excitation at 353 nm in DMSO/H_2O 1:9) and **53** (excitation at 481 nm in DMSO/H_2O 1:9).

For coumarin-labeled calixarene **51**, spectrum was recorded at concentration $1 \cdot 10^{-6}$ M and the emission maximum was found at 445 nm (blue). NBD-labeled calixarene **53**, at $6.83 \cdot 10^{-6}$ M, emits at a maximum of 545 nm (green).

The preliminary important result was, therefore, that as designed, the two fluorophores installed on this specific calixarene derivative do not lose their fluorescent properties, allowing in principle, from this point of view, their use to study by fluorescence the fate of the two synthesized probes **51** and **53** and their complexes with DNA inside the cells.

4.2.2 Transfection experiments

After characterization, the two fluorescent calixarenes **51** and **53** were tested in transfection using the same conditions reported in Chapters 2 and 3 for the studies with the other vectors.

Unfortunately, compound **51** bearing the coumarin moiety was unable to give cell transfection on RD-4 cells, even in presence of the helper lipid. In fact, compound **51** with pEGFP-C1 plasmid formed big blue aggregates, clearly visible by fluorescence microscopy, remaining outside of the cells (**Fig 4.6**). From images, the main part of these aggregates seemed to adhere to the outer cell membrane.

The presence of this fluorophore at the lower rim is then definitely detrimental for the gene delivery properties of the arginino-calix[4]arenes.

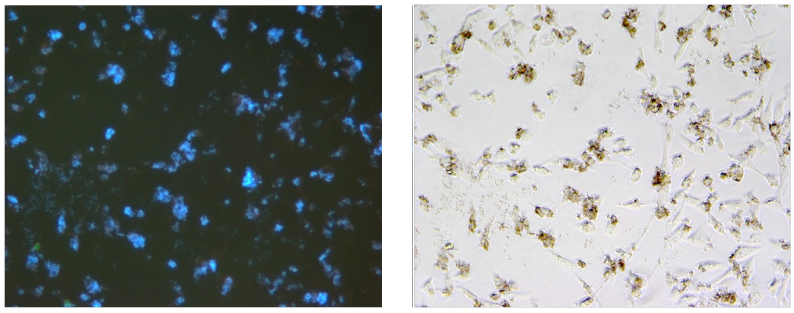


Fig 4.6 Aggregates of **51** with pEGFP-C1 plasmid outside RD-4 cells. The image on the left was recorded with the fluorescence microscope, the right to the Optical Microscope.

However, when it was mixed with calixarene **3,** at concentration 9 and 1 μM respectively, good levels of transfection were observed, comparable to those found with the vector **3** alone at 10μM concentration (**Fig 4.7**). In particular a 66% of transfected cells was reached without DOPE and 37% in presence of the helper lipid. It is important to highlight that, in this experiment, no transfection was observed using calixarene **3** at 1μM concentration, indicating, then, that compound **51,** in mixture with **3**, actively participates in complexation of DNA and in its delivery into cells. This result and the following hypothesis appear rather surprising if compared with the behavior of **51** alone.

On the whole, the use of coumarin containing compound **51** in formulation with vector **3** could represent, then, a possibility to investigate the uptake way featuring these transfection processes.

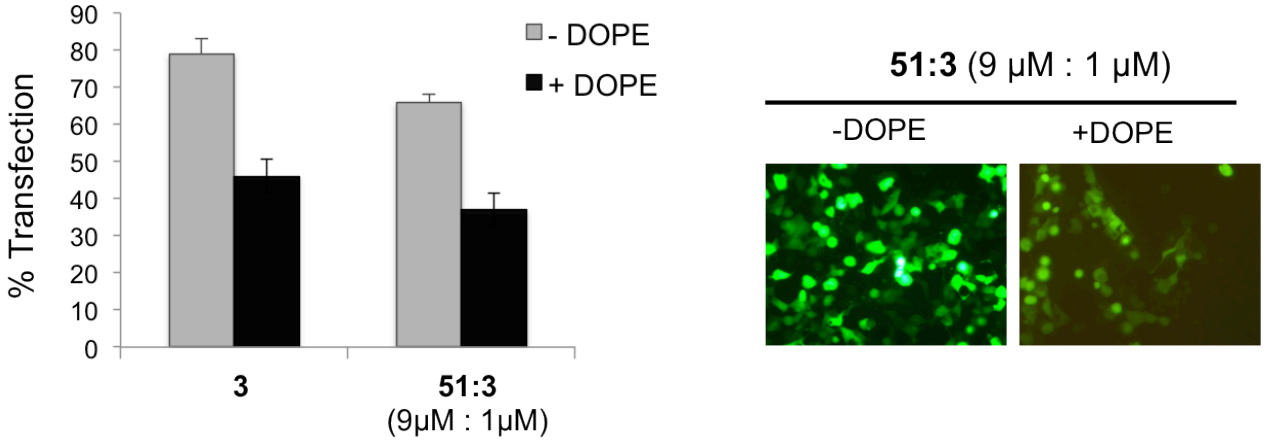


Fig 4.7 Transfection experiments performed with 1nM pEGFP-C1 plasmid and mixture of **51** (9 μM) with **3** (1 μM), on RD-4 cells. Left image: transfection efficiency as percentage of transfected cells Right images: fluorescence microscopy images of the transfected cells, in absence and in presence of DOPE.

However, moving to compound **53,** we found more advantageous results. In the case of this second probe, because of its green fluorescence, transfection studies were conducted using the pmCherry plasmid (4722 bp) that contains the gene coding for the Red Fluorescent Protein (RFP).

Before of these tests, thanks to fluorescence microscopy, it could be seen that the calixarene **53** was able to complex the pDNA giving green fluorescent aggregates of small dimensions, whose fluorescence intensity increased in the presence of DOPE (**Fig 4.8**). The interaction with plasmid filaments, moreover, did not quench the fluorescence of the carrier.

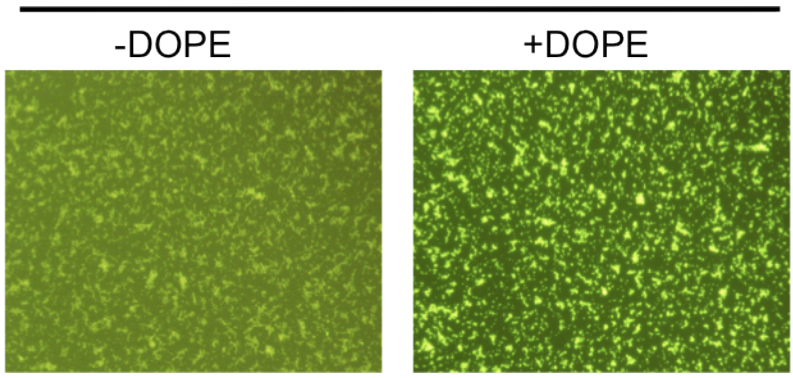


Fig 4.8 Aggregates of **53** with pmCherry plasmid in presence and in absence of DOPE. The images were recorded with fluorescence microscope.

Moreover, it was also revealed that vector **53**, in absence of DNA, due to its amphiphilic features, could self-associate in fluorescent aggregates (**Fig 4.9**). When DOPE was present in the calixarene sample (again in absence of DNA), fluorescence resulted even increased. These aggregates were able to enter RD-4 cells, confirming the excellent cell penetration properties of arginino-derivatives.

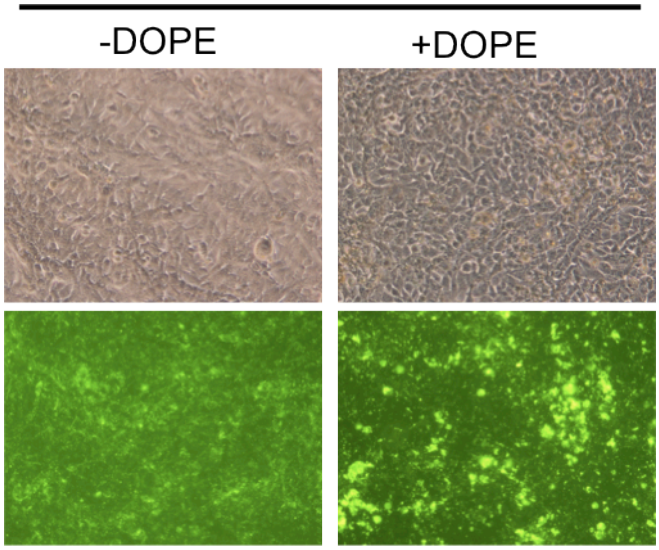


Fig 4.9 Aggregates of **53**, alone or with DOPE, tested on RD-4 cells. Upper row: phase contrast images; lower row: fluorescence microscopy images.

Much more interestingly for our aim, NBD-conjugate **53**, unlike its coumarin analogue **51**, showed quite good transfection ability, especially in the absence of DOPE with a transfection efficiency of 47%. After 48h, about the half of cells became in fact red as result of the expression of Red Fluorescent Protein and were clearly visible by fluorescent microscopy (**Fig 4.10 b**). Analysis with a green filter was also possible, detecting green fluorescence inside the cells (**Fig 4.10 c**) and providing the first confirmation that calixarene **53** was easily taken up into cells. Cell viability was not estimated by a MTT assay but, from a qualitative analysis by phase contrast microscopy, no high levels of toxicity were observed for **53**.

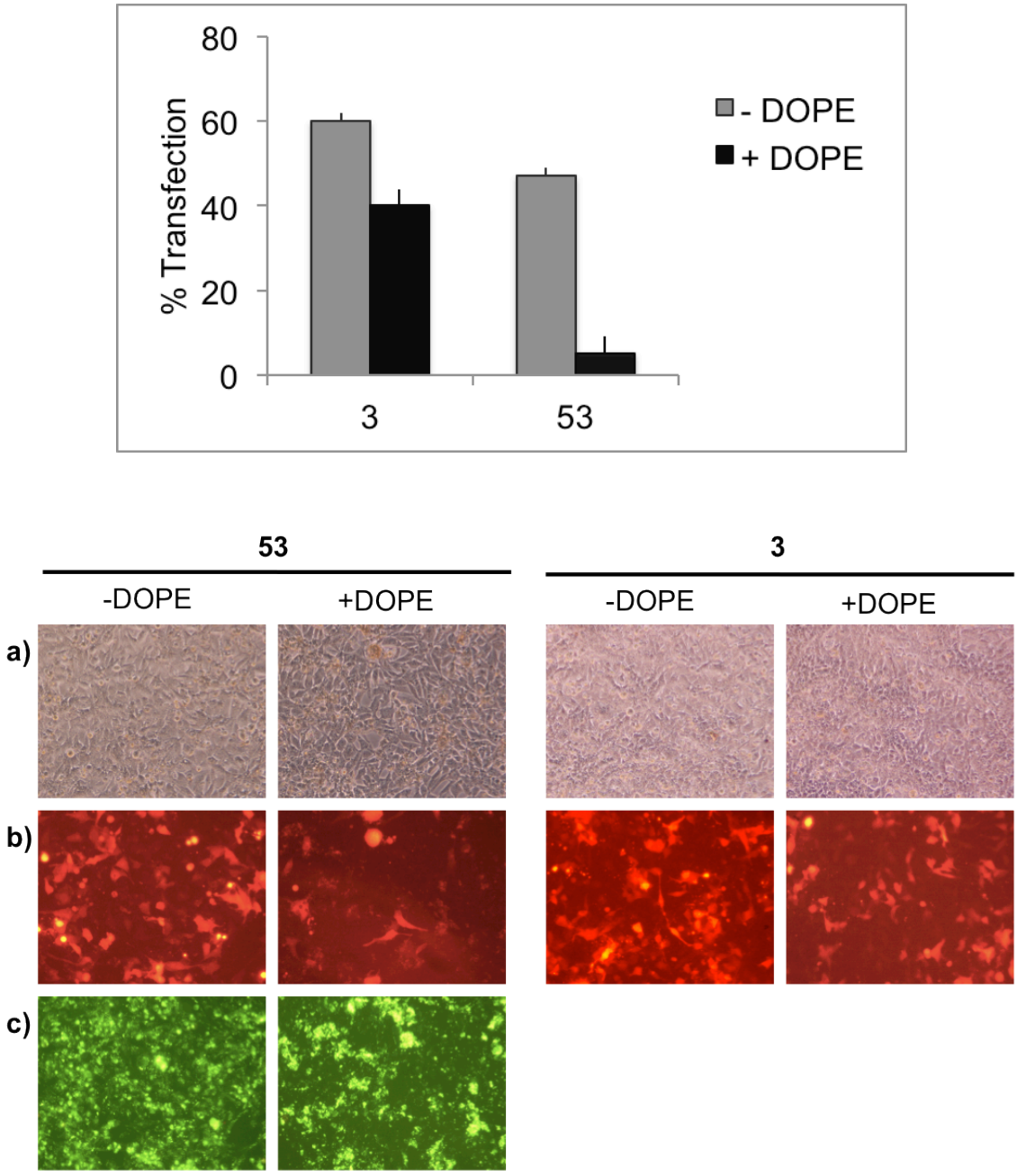


Fig 4.10 Transfection experiments performed with 1nM pmCherry plasmid, NBD-calixarene **53** and calixarene **3**, alone and in presence of DOPE (1/2 molar ratio, 10/20 μM), to RD-4 cells. Upper image: in vitro transfection efficiency as percentage of transfected cells upon treatment with ligands **53** and **3**. Lower images: phase contrast images and fluorescence microscopy images of the transfected cells.

The transfection percentage reached by **53** was even more interesting when compared to that of the carrier **3** that with this different plasmid showed percentage of transfection around 60%.

The presence of the NBD unit, although negatively alters the transfection properties, is not so effective as coumarin, making thus compound **53** a useful vector for the study of the pathway and fate of carrier itself and of its complexes with DNA in live on fixed cells. On the basis of these encouraging results transfection experiments using the NBD-labelled vector will be performed and followed by confocal microscopy.

4.3 Conclusions

Taking as reference the compound **3**, the best calixarene based non-viral vectors achieved so far, two new fluorescent calix[4]arenes were designed in order to answer to three major questions: i) how the upper rim arginino calixarene based vectors enter the cells, ii) how they accompany and release the pDNA into the nucleus and iii) how they are processed by the cells.

Thanks to a selective functionalization of the calixarene lower rim, an alkyl chain terming with an amino group was introduced. This strategy allowed the linkage of fluorescent probes resulting in the synthesis of two novel analogues of **3**, the ligand **51** and **53** bearing a coumarin and NBD unit, respectively.

Calixarene **51** resulted able to form blue fluorescent aggregates with DNA but did not give transfection when used alone. However, when it used in a mixture with **3** in ratio 9/1, an efficiency of transfection comparable to that of **3** alone was found.

Calixarene **53**, on the contrary, not only self-aggregated in species able to enter into the cells alone, but more importantly, it showed good transfection abilities and low cytotoxicity.

On one hand these results suggest that it thus represents an interesting system, when used alone, for the visualization of its localization and processing within cells. On the other hand, as hoped when designed, this compound could successfully be used as probe for transfection experiments to study and understand the mechanism/pathway these macrocyclic based vectors follow during the gene delivery process.

Moreover, it could for the first time be proved that the calixarene vectors enter the cells accompanying the plasmid DNA during the transfection processes.

4.4 Experimental section

General Information. All moisture sensitive reactions were carried out under nitrogen atmosphere, using previously oven-dried glassware. All dry solvents were prepared according to standard procedures, distilled before use and stored over 3 or 4 Å molecular sieves. Most of the solvents and reagents were obtained from commercial sources and used without further purification. Analytical TLC were performed using prepared plates of silica gel (Merck 60 F-254 on aluminum) and then, according to the functional groups present on the molecules, revealed with UV light or using staining reagents: $FeCl_3$ (1% in H_2O/CH_3OH 1:1), ninhydrin (5% in EtOH), basic solution of $KMnO_4$ (0.75% in H_2O). Reverse phase TLC were performed by using silica gel 60 RP-18 F-254 on aluminum sheets. Merck silica gel 60 (70-230 mesh) was used for flash chromatography and for preparative TLC plates. ^1H NMR and ^{13}C-NMR spectra were recorded on Bruker AV300 and Bruker AV400 spectrometers (observation of ^1H nucleus at 300 MHz and 400 MHz respectively, and of ^{13}C nucleus at 75 MHz and 100 MHz respectively). All chemical shifts are reported in part per million (ppm) using the residual peak of the deuterated solvent, which values are referred to tetramethylsilane (TMS, $\delta_{TMS} = 0$), as internal standard. All ^{13}C NMR spectra were performed with proton decoupling. Electrospray ionization (ESI) mass analyses were performed with a Waters spectrometer. Melting points were determined on an Electrothermal apparatus in closed capillaries. UV and Fluorimetric experiments were performed on Perkin Elmer UV-Vis Lambda BIO 20 spectrophotometer and LS55 Perkin Elmer fluorimeter, respectively.

Synthesis of 5,11,17,23-Tetra-tert-butyl-25,26,27-trihexyloxy-28-hydroxy-calix[4]arene (44)

5,11,17,23-Tetra-tert-butyl-25,26,27,28-tetrahydroxycalix[4]arene (5.00 g, 7.70 mmol), $Ba(OH)_2$ (8.50 g, 26.95 mmol), BaO (7.95 g, 51.84 mmol) and hexyl-I (10.53 mL, 71.15 mmol) were dissolved in DMF dry (100 mL). After 1 h the reaction was quenched with water (100 mL). The mixture was stirred for 30 min. and then extracted with AcOEt (3x500 mL). The organic layers ware washed with water (3x500 mL), dried over sodium sulfate and the solvent removed under reduced pressure. The residue was precipitated from cold DCM/MeOH to yield **44** as a white powder (4.86 g, 70%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.13 (s, 2H, Ar*H*), 7.04 (s, 2H, Ar*H*), 6.55-6.48 (m, 4H, Ar*H*), 5.72 (s, 1H, O*H*), 4.37 (d, *J* = 12.8 Hz, 2H, ArCH_{ax}Ar), 4.33 (d, *J* = 13.0, 2H, ArCH_{ax}Ar), 3.90 (t, *J* = 8.0 Hz, 2H, OCH_2), 3.79 (t, *J* = 7.6 Hz, 4H, OCH_2), 3.24 (d, *J* = 12.8 Hz, 2H, ArCH_{eq}Ar), 3.18 (d, *J* = 13.0 Hz, 2H, ArCH_{eq}Ar), 2.35-2.22 (m, 2H, OCH_2CH_2), 2.05-1.80 (m, 4H, OCH_2CH_2), 1.60-1.26 (m, 27H, $O(CH_2)_2CH_2CH_2CH_2$ and *t*-Bu), 0.98-0.85 (m, 9H, CH_2CH_3), 0.83 (s, 18H, *t*-Bu).

^{13}C NMR (100 MHz, $CDCl_3$) δ 151.9, 150.8, 145.4, 145.0, 141.3, 136.1, 132.3, 131.9, 129.4, 125.6, 125.0, 124.8, 124.7, 76.4, 74.9, 34.1, 33.9, 33.7, 32.3, 32.0, 31.8, 31.7, 31.4, 31.2, 31.1, 30.3, 29.4, 26.0, 25.9, 23.2, 22.7, 14.2.

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{62}H_{92}NO_4$; 923.5, found 924.2.

Synthesis of 5,11,17,23-Tetra-tert-butyl-25,26,27-trihexyloxy-28-phthalimidohexyloxy-calix[4]arene (45)

To a solution of **44** (2.00 g, 2.22 mmol) in dry DMF (50 mL) under N_2 atmosphere NaH (60%, 0.18 g, 4.44 mmol) was added. After 1 h, *N*- 6(bromohexyl)phthalimide (97%, 2.13 g, 6.65 mmol) was added and the solution stirred for 7h. The reaction was quenched with HCl 1 M (50 mL) and extracted with AcOEt (50 mL). The organic phase was again washed with HCl 1 M (2x50 mL) and H_2O (2x50 mL), dried over sodium sulfate and the solvent removed under reduced pressure. The crude was purified by flash chromatography (eluent hexane/DCM 65:35). The pure product **45** was obtained as white solid (1.27 g, yield 51%).

Mp: 76-78 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.84-7.80 (m, 2H, ArH_{Pht}), 7.74-7.67 (m, 2H, ArH_{Pht}), 6.79 (s, 4H, ArH_{calix}), 6.74 (s, 2H, ArH_{calix}), 6.73 (s, 2H, ArH_{calix}), 4.40 (d, *J* = 12.4 Hz, 2H, ArCH_{ax}Ar), 4.38 (d, *J* = 12.4 Hz, 2H, ArCH_{ax}Ar), 3.88-3.77 (m, 8H, OCH_2), 3.70 (t, *J* = 7.2 Hz, 2H, CH_2N), 3.10 (d, *J* = 12.4 Hz, 4H, ArCH_{eq}Ar), 2.07-1.92 (m, 8H, OCH_2CH_2), 1.79-1.66 (m, 2H, CH_2CH_2N), 1.50-1.29 (m, 22H, $O(CH_2)_2CH_2CH_2$ and CH_2CH_3), 1.08 (s, 18H, *t*-Bu), 1.04 (s, 18H, *t*-Bu), 0.96-0.85 (m, 9H, CH_2CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 153.8, 153.7, 153.6, 144.1, 144.1, 134.0, 133.8, 133.7, 132.2, 124.9, 124.8, 123.2, 75.5, 75.4, 75.2, 38.0, 33.8, 32.2, 31.5, 31.1, 30.4, 30.3, 30.2, 28.9, 27.1, 26.0, 25.9, 22.9, 14.2.

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{76}H_{107}N_1O_6$; 1152.6, found 1153.0, $[M + K]^+$ calcd 1169.3, found 1170.0.

Synthesis of 5,11,17,23-Tetra-nitro-25,26,27-trihexyloxy-28-phthalimidohexyloxy-calix[4]arene (46)

To a solution of **45** (0.14 mg, 0.12 mmol) in dry DCM (2.4 mL), under N_2 atmosphere at 0°C, TFA (0.13 mL, 3.24 mmol) and nitric acid (65%, 0.19 mL, 2.81 mmol) were added. The solution was stirred for 2 days. The reaction was quenched with H_2O (10 mL) and extracted with DCM (2 x 250 mL). The organic layers were combined and dried over sodium sulfate. The solvent removed under reduced pressure. The residue was precipitated from MeOH to yield **46** as a pale yellow powder (75.6 mg, 58%).

Mp: 91-93 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.86-7.80 (m, 2H, Ar*H* $_{Pht}$), 7.78-7.69 (m, 2H, Ar*H* $_{Pht}$), 7.63 (s, 4H, Ar*H* $_{calix}$), 7.50 (s, 2H, Ar*H* $_{calix}$), 7.49 (s, 2H, Ar*H* $_{calix}$), 4.49 (d, *J* = 14.0 Hz, 2H, ArC*H*$_{ax}$Ar), 4.48 (d, *J* = 14.0 Hz, 2H, ArC*H*$_{ax}$Ar), 4.05-3.88 (m, 8H, OC*H*$_2$), 3.68 (t, *J* = 7.2 Hz, 2H, C*H*$_2$N), 3.38 (d, J = 14.0 Hz, 4H, ArC*H*$_{eq}$Ar), 1.95-1.77 (m, 8H, OCH_2CH_2), 1.76-1.62 (m, 2H, CH_2CH_2N), 1.50-1.20 (m, 22H, $OCH_2CH_2CH_2CH_2$ e CH_2CH_3), 0.97-0.80 (m, 9H, CH_2CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 168.4, 161.8, 161.5, 161.4, 142.9, 135.6, 135.2, 134.0, 132.1, 124.2, 123.9, 123.2, 76.2, 75.9, 37.7, 31.8, 31.2, 30.1, 29.9, 28.6, 26.7, 25.7, 25.6, 25.5, 22.7, 14.0.

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{60}H_{71}N_5O_{14}$; 1108.4, found 1108.9.

Synthesis of 5,11,17,23-Tetra-amino-25,26,27-trihexyloxy-28-phthalimidohexyloxy-calix[4]arene (47)

Calix[4]arene **46** (0.24 g, 0.21 mmol) was dissolved in AcOEt (22 mL), and a catalytic amount of Pd/C (10%) was added. Hydrogenation was carried out at 2 bar in a Parr reactor for 29 h. Then the catalyst was filtrated and the solvent removed under reduced pressure. The pure product was isolated as yellow solid (99.4 mg, yield 49%).

Mp: 81-83 °C. ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$= 19:1) δ 7.82-7.72 (m, 2H, Ar*H* $_{Pht}$), 7.71-7.62 (m, 2H, Ar*H* $_{Pht}$), 6.66 (bs, 4H, Ar*H* $_{calix}$), 6.58 (bs, 4H, Ar*H* $_{calix}$), 4.29 (d, *J* = 11.7 Hz, 4H, ArC*H*$_{ax}$Ar), 3.85-3.63 (m, 8H, OC*H*$_2$), 3.63 (t, *J* = 7.1 Hz, 2H, C*H*$_2$N), 3.19 (bs, 8H, N*H*$_2$), 3.07 (d, *J* = 11.7 Hz, 4H, ArC*H*$_{eq}$Ar), 1.90-1.70 (m, 8H, OCH_2CH_2), 1.69-1.57 (m, 2H, CH_2CH_2N), 1.42-1.10 (m, 22H, $OCH_2CH_2CH_2CH_2$ e CH_2CH_3), 0.90-0.70 (m, 9H, CH_2CH_3).

^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$= 19:1) δ 168.5, 155.4, 155.2, 154.9, 136.2, 136.0, 135.9, 134.0, 132.0, 123.2, 122.1, 75.5, 75.3, 37.8, 31.9, 30.5, 30.1, 30.0, 29.6, 28.7, 26.9, 25.8, 25.7, 22.7, 14.0.

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{60}H_{79}N_5O_6$; 988.5, found 989.1.

Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Arg(Pbf))amino]-25,26,27-trihexyloxy-28-phthalimidohexyloxy-calix[4]arene (48)

To a solution in dry DMF (2.5 mL) of Boc-L-Arg(Pbf)-OH (0.38 g, 0.72 mmol) and HOBt (0.11 g, 0.82 mmol), DCC (0.15 g, 0.72 mmol) was added. After 15 min amino calix[4]arene **47** (0.11 g, 0.12 mmol) in 3 mL of DMF was added. The mixture was stirred at room temperature for 24 h. Ethyl acetate was added (10 mL), DCU was filtered off by gravity on a PTFE filter, and the solvent was removed under reduced pressure. The crude was dissolved in ethyl acetate (20 mL) and washed with a saturated $NaHCO_3$ aqueous solution (20 mL), brine (20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure giving a crude material that was purified by flash column chromatography (gradient from DCM to DCM/MeOH 95:5) to obtain the pure product as a white solid in 11% yield (39.5 mg).

^{1}H NMR (300 MHz, CD_3OD/CD_3Cl= 19:1) δ 7.85-7.72 (m, 4H, Ar*H* $_{Pht}$), 7.10 (bs, 2H, Ar*H*), 7.02 (bs, 2H, Ar*H* $_{calix}$), 6.65 (bs, 2H, Ar*H* $_{calix}$), 6.55 (bs, 2H, Ar*H* $_{calix}$), 4.43 (d, *J* = 12.6 Hz, 2H, ArC*H*$_{ax}$Ar), 4.41 (d, *J* = 13.2 Hz, 2H, ArC*H*$_{ax}$Ar), 4.09 (bs, 4H, COC*H*NH), 3.86 (bs, 8H, OC*H*$_2$), 3.69 (t, *J* = 6.9 Hz, 2H, C*H*$_2$N(CO)$_2$), 3.28-3.00 (m, 10H, C*H*$_2$NH and ArC*H*$_{eq}$Ar), 2.96 (s, 8H, C*H*$_{2\ Pbf}$), 2.55 (s, 12H, C*H*$_{3\ Pbf}$), 2.49 (s, 12H, C*H*$_{3\ Pbf}$), 2.04 (s, 12H, C*H*$_{3\ Pbf}$), 1.91 (bs, 8H, OCH_2CH_2), 1.80-1.20 (m, 100H, $O(CH_2)_2CH_2CH_2CH_2CH_2$, $COCHCH_2CH_2$, $C(CH_3)_{3\ Boc}$ and $C(CH_3)_{2\ Pbf}$), 0.99-0.80 (m, 12H, CH_2CH_3).

^{13}C NMR (75 MHz, CD_3OD/CD_3Cl= 19:1) δ 171.4, 168.4, 158.5, 156.7, 156.5, 153.3, 138.1, 134.9, 134.0, 132.8, 132.2, 131.9, 131.6, 124.6, 122.8, 121.4, 120.4, 117.1, 86.3, 79.4, 75.1, 54.7, 42.7, 40.2, 37.5, 32.0, 31.7, 30.9, 30.0, 29.9, 29.8, 29.4, 29.1, 28.5, 27.7, 27.6, 26.8, 25.9, 22.6, 22.4, 18.5, 17.3, 13.3, 11.4.

ESI-MS (m/z): $[M + 2Na]^{2+}$ calcd for $C_{156}H_{223}N_{21}O_{30}S_4$; 1522.3, found 1522.7.

Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Arg(Pbf))amino]-25-26,27-trihexyloxy-28-aminohexiloxycalix[4]arene (49)

To a solution of **48** (0.18 g, 0.06 mmol) in ethanol (4 mL), hydrazine monohydrate (5.8 mL, 0.12 mmol) was added and the reaction mixture was stirred and refluxed for 7 hours. The solvent was then removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL) and washed with water (3x10 mL). The organic layers were dried over anhydrous

Na_2SO_4 and pure product **49** was obtained after evaporation of the solvent under reduced pressure as light yellow solid in 76% yield (130 mg).

^{1}H-NMR (300 MHz, CD_3OD): δ 7.08 (bs, 4H, Ar*H*), 6.54 (bs, 4H, Ar*H*), 4.48 (d, *J* = 13.2 Hz, 4H, ArCH_{ax}Ar), 4.08 (bs, 4H, COC*H*NH), 3.85 (bs, 6H, OCH_2), 3.67 (bs, 2H, OCH_2), 3.31-3.08 (m, 12H, CH_2NHC(NH)NH and ArCH_{eq}Ar), 2.94 (s, 8H, C$H_{2\ Pbf}$), 2.55 (s, 12H, C$H_{3\ Pbf}$), 2.50 (s, 12H, C$H_{3\ Pbf}$), 2.04 (s, 12H, C$H_{3\ Pbf}$), 1.92 (bs, 8H, OCH_2CH_2), 1.58-1.24 (m, 102H, $C(CH_3)_{3\ Boc}$, $C(CH_3)_{2\ Pbf}$, $COCHCH_2CH_2$, $O(CH_2)_2CH_2CH_2CH_2CH_2$ and $O(CH_2)_2CH_2CH_2CH_2CH_2CH_2$), 0.94 (m, 9H, CH_2CH_3).

^{13}C-NMR (75 MHz, $CDCl_3/CD_3OD$: 4/1): δ 171.6, 158.7, 156.5, 155.9, 153.4, 138.3, 135.1, 132.6, 132.2, 124.7, 121.1, 86.4, 75.3, 54.4, 43.2, 40.3, 31.9, 31.1, 30.0, 25.8, 22.8, 19.3, 17.9, 14.1, 12.4.

ESI-MS (m/z): $[M + 2Na]^{2+}$ calcd for $C_{148}H_{221}N_{21}O_{28}S_4$; 1457.3, found 1458.5.

Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Arg(Pbf))amino]-25-26,27-trihexyloxy-28-(7-hydroxycoumarin-3-carboxy)aminohexiloxycalix[4]arene (50)

To a solution in dry DMF (2 mL) of EDC [(1-Ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride] (6.54 mg, 0.03 mmol) and DMAP [4-Dimethylaminopyridine] (8.32 mg, 0.07 mmol), 7-hydroxy-coumarin-3-carbaxylic acid (7.02 mg, 0.03 mmol) was added. After 15 min calix[4]arene **49** (65.2 mg, 0.02 mmol) was added. The mixture was stirred at room temperature for 48 h. The solvent was then removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL) and washed with a saturated $NaHCO_3$ aqueous solution (3x10 mL), brine and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure giving a crude material that was purified by flash column chromatography (eluent CH_2Cl_2/MeOH 97:3). The pure product was isolated as yellow solid in 15% yield.

^{1}H-NMR (400 MHz, $CDCl_3/CD_3OD$: 1/4): δ 8.76 (s, 1H, $H^4_{coumarin}$), 7.62 (d, *J* = 8.8 Hz, 1H, $H^8_{coumarin}$), 7.07 (bs, 4H, Ar*H*), 6.88 (bs, 1H, $H^9_{coumarin}$), 6.77 (s, 1H, $H^6_{coumarin}$), 6.64 (bs, 4H, Ar*H*), 4.45 (d, *J* = 12.8 Hz, 4H, ArCH_{ax}Ar), 4.11 (bs, 4H, COC*H*NH), 3.90 (bs, 6H, OCH_2), 3.46 (bs, 2H, OCH_2), 3.22-3.15 (m, 12H, CH_2NHC(NH)NH and ArCH_{eq}Ar), 2.97 (s, 8H, C$H_{2\ Pbf}$), 2.57 (s, 12H, C$H_{3\ Pbf}$), 2.51 (s, 12H, C$H_{3\ Pbf}$), 2.06 (s, 12H, C$H_{3\ Pbf}$), 1.94 (bs, 8H, OCH_2CH_2), 1.69-1.29 (m, 102H, $C(CH_3)_{3\ Boc}$, $C(CH_3)_{2\ Pbf}$, $COCHCH_2CH_2$, $O(CH_2)_2CH_2CH_2CH_2$ and $O(CH_2)_2CH_2CH_2CH_2CH_2$), 0.92 (m, 9H, CH_2CH_3).

^{13}C-NMR (75 MHz, $CDCl_3/CD_3OD$: 1/4): δ 172.8, 158.2, 154.5, 150.1, 139.7, 136.5, 135.1, 134.4, 133.7, 133.1, 130.2, 129.8, 129.6, 126.5, 126.2, 122.9, 122.1, 120.5, 116.2, 113.0, 103.6, 87.9, 76.8, 56.1, 44.4, 41.9, 41.0, 33.5, 33.3, 32.6, 32.6, 31.6, 31.0, 29.4, 28.6, 27.4, 24.8, 24.2, 24.0, 20.2, 19.0, 15.1, 14.9, 13.2.
ESI-MS (m/z): $[M + 2Na]^{2+}$ calcd for $C_{158}H_{225}N_{21}O_{32}S_4$; 1551.3, found 1552.1.

Synthesis of 5,11,17,23-Tetrakis[(L-Arg)amino]-25-26,27-trihexyloxy-28-(7-hydroxycoumarin-3-carboxy)aminohexiloxycalix[4]arene, octahydrochloride (51)

A solution of calix[4]arene **50** (10.2 mg, 0.003 mmol) in TFA/TIS/H_2O (95/2.5/2.5, 10 mL) was stirred at room temperature. The progression of the reaction was followed using mass spectroscopy. After completion (10 h), the volatiles were removed under reduced pressure and the residue washed with ethyl acetate (3×5 mL) to remove the exceeding TFA. The crude material was precipitated, washed and centrifuged with anhydrous diethyl ether (3×7 mL). The trifluoroacetate anion of the resulting TFA salts was exchanged by adding 10 mM HCl solution (3×5 mL) followed by evaporation under reduced pressure. The pure product was isolated as white solid in 70% yield (4 mg).
^{1}H-NMR (400 MHz, CD_3OD): δ 8.78 (s, 1H, $H^4_{coumarin}$), 7.69 (d, J = 8.8 Hz, 1H, $H^8_{coumarin}$), 7.20 (bs, 2H, Ar*H*), 7.16 (bs, 2H, Ar*H*), 6.97-6.91 (m, 5H, Ar*H* e $H^9_{coumarin}$), 6.803 (s, 1H, $H^6_{coumarin}$), 4.48 (d, J = 12.4 Hz, 4H, ArCH_{ax}Ar), 4.09 (bs, 4H, COC*H*NH), 3.93 (bs, 6H, OCH_2), 3.48 (bs, 2H, OCH_2), 3.20-3.17 (m, 12H, CH_2NHC(NH)NH and ArCH_{eq}Ar), 2.007 (bs, 16H, OCH_2CH_2 and $COCHCH_2$), 1.77 (bs, 8H, $COCHCH_2CH_2$), 1.55-1.31 (m, 32H, $O(CH_2)_2CH_2CH_2CH_2$ and $O(CH_2)_2CH_2CH_2CH_2CH_2$), 0.92 (m, 9H, CH_2CH_3).
^{13}C-NMR (75 MHz, CD_3OD): δ 166.3, 164.3, 157.2, 156.9, 153.5, 148.3, 134.8, 131.6, 121.0, 120.5, 114.4, 113.1, 111.4, 101.7, 75.3, 53.2, 40.6, 39.2, 30.7, 30.0, 28.5, 24.2, 13.1.
ESI-MS (m/z): $[M + 2H]^{2+}$ calcd for $C_{86}H_{137}N_{21}O_{12}Cl_8$ 825.0; found 825.8.

Synthesis of 5,11,17,23-Tetrakis[(Boc-L-Arg(Pbf))amino]-25-26,27-trihexyloxy-28-(NBD)aminohexiloxycalix[4]arene (52)

To a solution in dry DMF (2 mL) of calix[4]arene **49** (51.4 mg, 0.018 mmol) and DIPEA (2.54 mg, 0.02 mmol), 7-Cloro-4-nitrobenzofurazano (3.6 mg, 0.018 mmol). was added. The mixture was stirred at room temperature for 24 h. The solvent was then removed under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL) and washed with

water (3x10 mL) and dried over anhydrous $MgSO_4$. The solvent was removed under reduced pressure giving a crude material that was purified by flash column chromatography (eluent CH_2Cl_2/MeOH 93:7). The pure product was isolated as orange solid in 46% yield.

^{1}H-NMR (400 MHz, $CDCl_3$/CD_3OD: 1/1): δ 8.48 (d, *J* = 8.8 Hz, 1H, Ar*H* $_{NBD}$), 7.02 (bs, 4H, Ar*H*), 6.57 (bs, 4H, Ar*H*), 6.22 (d, *J* = 7.6 Hz, 1H, Ar*H* $_{NBD}$), 4.41 (d, *J* = 12.8 Hz, 4H, ArC*H*$_{ax}$Ar), 4.12 (bs, 4H, COC*H*NH), 3.87 (bs, 6H, OC*H*$_2$), 3.51 (bs, 2H, OC*H*$_2$), 3.14-3.11 (m, 12H, C*H*$_2$NHC(NH)NH and ArC*H*$_{eq}$Ar), 2.96 (s, 8H, C*H*$_{2\ Pbf}$), 2.56 (s, 12H, C*H*$_{3\ Pbf}$), 2.50 (s, 12H, C*H*$_{3\ Pbf}$), 2.06 (s, 12H, C*H*$_{3\ Pbf}$), 1.90 (bs, 8H, OCH_2CH_2), 1.74-1.26 (m, 102H, C(C*H*$_3$)$_{3\ Boc}$, C(C*H*$_3$)$_{2\ Pbf}$, $COCHCH_2CH_2$, $O(CH_2)_2CH_2CH_2CH_2$, $O(CH_2)_2CH_2CH_2CH_2CH_2$), 0.90 (m, 9H, CH_2CH_3).

^{13}C-NMR (75 MHz, $CDCl_3$/CD_3OD: 1/1): δ 171.1, 158.7, 156.5, 156.2, 153.8, 138.2, 137.2, 134.9, 132.7, 132.2, 131.4, 124.7, 121.3, 120.7, 98.3, 75.3, 54.5, 43.0, 40.3, 32.0, 31.1, 30.0, 29.5, 27.0, 25.8, 25.4, 22.7, 18.9, 17.7, 13.7, 12.0.

ESI-MS (m/z): $[M + 3Na]^{3+}$ calcd for $C_{154}H_{222}N_{24}O_{31}S_4$; 1033.9, found 1034.8.

Synthesis of 5,11,17,23-Tetrakis[(L-Arg)amino]-25-26,27-trihexyloxy-28-(NBD)aminohexiloxycalix[4]arene, octahydrochloride (53)

A solution of calix[4]arene **52** (22.4 mg, 0.007 mmol) in TFA/TIS/H_2O (95/2.5/2.5, 16 mL) was stirred at room temperature. The progression of the reaction was followed using mass spectroscopy. After completion (10 h), the volatiles were removed under reduced pressure and the residue washed with ethyl acetate (3×5 mL) to remove the exceeding TFA. The crude material was precipitated, washed and centrifuged with anhydrous diethyl ether (3×7 mL). The trifluoroacetate anion of the resulting TFA salts was exchanged by adding 10 mM HCl solution (3×5 mL) followed by evaporation under reduced pressure. The pure product was isolated as white solid in 72% yield (9.6 mg).

^{1}H-NMR (300 MHz, CD_3OD): δ 8.49 (d, *J* = 8.7 Hz, 1H, Ar*H* $_{NBD}$), 7.22 (bs, 2H, Ar*H*), 7.10 (bs, 2H, Ar*H*), 7.01 (bs, 2H, Ar*H*), 6.82 (bs, 2H, Ar*H*), 6.37 (d, *J* = 8.7 Hz, 1H, Ar*H* $_{NBD}$), 4.49-4.42 (m, 4H, ArC*H*$_{ax}$Ar), 4.09 (bs, 4H, COC*H*NH), 3.88 (bs, 6H, OC*H*$_2$), 3.61 (bs, 2H, OC*H*$_2$), 3.19-3.10 (m, 12H, C*H*$_2$NHC(NH)NH and ArC*H*$_{eq}$Ar), 1.98 (bs, 8H, OCH_2CH_2), 1.77 (bs, 8H, $COCHCH_2$), 1.62 (bs, 8H, $COCHCH_2CH_2$), 1.38-1.31 (m, 32H, $O(CH_2)_2CH_2CH_2CH_2$, $O(CH_2)_2CH_2CH_2CH_2CH_2$), 0.94 (m, 9H, CH_2CH_3).

^{13}C-NMR (75 MHz, CD_3OD): δ 164.8, 155.7, 151.6, 143.8, 135.6, 133.1, 129.9, 119.2, 96.1, 73.7, 67.1, 51.7, 38.9, 30.4, 29.1, 28.4, 27.8, 26.9, 24.2, 22.6, 21.0, 11.5.
ESI-MS (m/z): $[M + 3H]^{3+}$ calcd for $C_{82}H_{134}N_{24}O_{11}Cl_8$; 542.0, found 542.1, $[M + 2H]^{2+}$ calcd 812.5, found 812.9.

DNA preparation and storage. Plasmid DNA (pEGFP-C1) was purified through cesium chloride gradient centrifugation (Maniatis, T., Fritsch, E. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, New York). A stock solution of the plasmid 0.35 mM in milliQ water (Millipore Corp., Burlington, MA) was stored at -20 °C.

Cell culture and transient transfection assay. RD-4 [human Rhabdomyosarcoma cell line (obtained from David Derse, National Cancer Institute, Frederick, Maryland)], was grown in EMEM medium containing NEAA, 10% FBS, 2 mM l-glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. All cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were subcultured to a fresh culture vessel when growth reached 70-90% confluence (i.e. every 3-5 days) and incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2. Transfections were performed in 24 well plates, when cells were 80% confluent (approximately 5×10^4 cells) on the day of transfection. 2.5 mg of plasmid and different concentrations of ligands were added to 1 mL of serum-free medium (DMEM, 2 mM L-glutamine and 50 μg/ml), mixed rapidly and incubated at room temperature for 20 min. When used, serum was added at this point to the transfection solution. Following the removal of the culture medium from the cells, 0.5 mL of transfection mixture were carefully added to every well. Lipoplex formulations with helper lipid were prepared adding a 2 mM ethanol solution of DOPE to plasmid-ligand mixture at 1:2 ligand:DOPE molar ratio, where ligand concentration was kept to 10 mM. These solutions administered to the cells were completely clear and homogeneus. LTX™ transfection reagent was used according to manufacturer's protocol as positive transfection control. The mixture and cells were incubated at 37 °C in a humidified atmosphere of 95% air-5% CO_2 for 5 h. Finally, transfection mixture was removed and 1 mL of growth medium added to each transfected well and left to incubate for 72 h. Five fields were randomly selected from each well without viewing the cells (one in the centre

and one for each quadrant of the well) and examined. The transfected cells were observed under fluorescence microscope for EGFP expression. Each experiment was done three times. Statistical differences between treatments were calculated with Student's test and multifactorial ANOVA.

4.5 References

[1] Wender, P. A.; Wesley C. G.; Goun, E. A.; Jones, L. R.; Pillow, T. H. *Adv. Drug Del. Rev.* **2008**, *60*, 452-472.

[2] a) Suzuki, T.; Futaki, S.; Niwa, M.; Tanaka, S.; Ueda, K.; Sugiura, Y. *J. Biol. Chem*. **2002**, *277*, 2437–2443; b) Nakase, I.; Tadokoro, A.; Kawabata, N.; Takeuchi, T.; Katoh, H.; Hiramoto, K.; Negishi, M.; Nomizu, M.; Sugiura, Y.; Futaki, S. *Biochemistry* **2007**, *46*, 492–501.

[3] Zuhorn, I. S.; Engbrts, J.; Hoekstra, D. Eur. Biophys. J. 2007, 36, 349-362.

[4] Richard, J.P.; Melikov, K.; Brook, H.; Prevot, P.; Lebleu, B.; Chernomordik, L.V. *J. Biol. Chem.* **2005**, *280*, 15300–15306.

[5] a) Kaplan, I.M., Wadia, J.S. and Dowdy, S.F. *J. Control. Release* **2005**, *102*, 247–253; b) Jones, A. T. *Journal of Cellular and Molecular Medicine,* **2007**, *11*, 670–684. J. Cell. Mol. Med.

[6] Khalil, I.A.; Kogure, K.; Futaki, S.; Harashima, H *J. Biol. Chem*. **2006**, *281*, 3544–3551.

[7] Vercauteren, D.; Rejman, J.; Martens, T. F.; Demeester, J.; De Smedt, S. C.; Braeckmans, K. *J. Control Release* **2012,** *161*, 566−581.

[8] a) Lalor, R.; Baillie-Johnson, H.; Redshaw, C.; Matthews, S. E.; Mueller, A. *J. Am. Chem. Soc.* **2008**, *130*, 2892-2893; b) Mueller, A.; Lalor, R.; Cardaba, C. M.; Matthews, S. E. *Cytometry Part A*, **2011**, *79A*, 126-136.

[9] Diaz-Moscoso, A., Vercauteren, D., Rejman, J., Benito, J. M., Mellet, C. De Smedt, S. C., Garcia Fernandez, J. M. *J. Control Release* **2010,** *143*, 318−325.

[10] Zyryanov, G. V.; Kang, Y.; Rudkevich, D. M. *J. Am. Chem. Soc*. **2003,** *125*, 2997-3007.