Chapter 5

Comparative study of polyaminothiourea- and arginine- based amphiphilic calix[4]arenes and β-cyclodextrins as non-viral vectors

5.1 Introduction

Apart from their inherent properties as nanometric containers,[1] cyclodextrins' ability to improve drug bioavailability has been suggested to benefit from two additional features: (i) their membrane absorption enhancing properties and (ii) their ability to stabilize biomolecules in physiological media by shielding them from non-specific interactions.[2]

Thanks to these two features and after a pioneering work whose authors quantified a 6-fold enhancement in gene expression in rat lung by adding βCD (1%) to the original DNA:lipid formulations[3], many efforts have been devoted to manipulate the properties of pre-existing gene-transfecting polymers[4] (generally namely CD-pendant polymers) or dendrimers [5] by attaching CDs (**Fig 5.1 A**).

Subsequently, polymeric species containing CDs in their backbone (**Fig 5.1 B**), namely CD-embedding polymers, have been applied in gene delivery. The influence of many factors such as the CD cavity size [6] and the distribution and nature of cationic elements (their linkages, distances, and relative dispositions),[7] as well as the polymer size and polydispersity,[6b] has been investigated in order to find the optimal architectures in terms of both high delivery efficiency and low cytotoxicity.

For CD-based polymers or dendrimers, it is generally known that the CD framework imparts biocompatibility, avoids or reduces toxicity[5a] and behaves as a transfection enhancer. Moreover it offers unique possibilities associated with the intrinsic molecular inclusion capabilities of the CD cavity, that have been exploited to decorate DNA polyCDplexes with target ligands[8] or to design dual delivery systems, with a lipophilic core that can allocate hydrophobic drugs, and a cationic shell intended to condense DNA. [9]

Polyrotaxanes consisting of cationic CD derivatives threaded onto poly(ethyleneglycol) (PEG) or poly(ethyleneimine) (PEI) chains have also conceived for DNA complexation and gene delivery purposes. The first example was the threading of linear polycationic polymers (**Fig. 5.1 C**).[10] Subsequently, strategies to build up series of supramolecularly assembled cationic polyrotaxanes grafting oligoethyleneimine branches onto α- or β-CD were developed. α-CDs yielded consistently smaller rotaCDplexes, more suitable for cellular uptake, but, although they were assessed in a variety of cell lines, the transfection efficiency was not always optimal.[11]

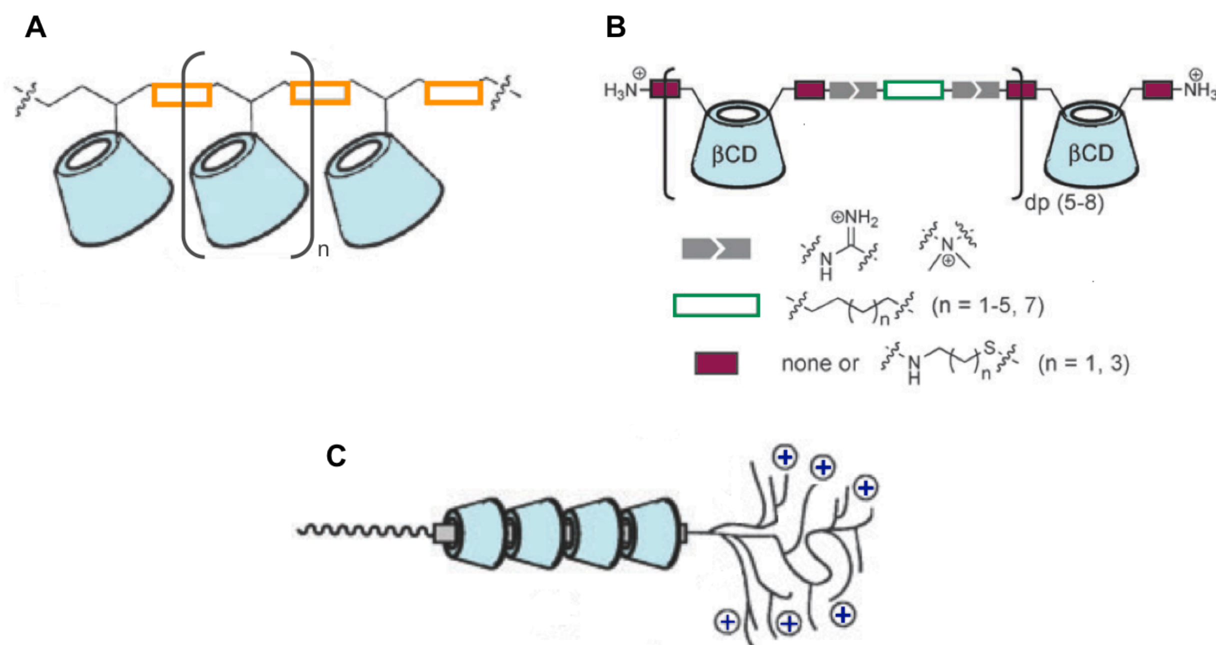


Fig 5.1 Schematic representations of A) CD-pendant polymers; B) CD-embedding polymers and C) CD-based polyrotaxane.

Multifunctional molecular vectors have emerged as a new generation of gene-delivery systems prone to chemical tailoring and systematic structural modification, thereby facilitating the elucidation of structure–activity relationships. A general approach is based on the use of preorganized macrocyclic scaffolds to achieve a precise arrangement of functional elements. Similarly to what we have seen in the case of calixarenes, very recently, several groups have turned their attention to the development of such systems starting in this case from discrete, monodisperse CD derivatives that can self-organize in the presence of DNA to promote its compaction and safe delivery to cells.

The higher accessibility of the primary hydroxyl groups facilitates homogeneous functionalization at the narrower rim, which has been used to create different types of polycationic bundles.[12] Although mono-facially functionalized polycationic CDs present a polarity gradient between the primary and secondary rims, hydrophobicity is limited to the internal walls of the basket-shaped cavity which is relevant for encapsulation of small guests, but, in principle, not to promote self-assembling and intermolecular interactions. But, analogously to calixarenes, the truncated cone of CDs, which exhibit well-differentiated faces, allows accessing compounds with segregated cationic and lipophilic domains for which, according to the ‘‘facial amphiphilicity’’ concept, self-assembly and gene delivery properties could be expected.

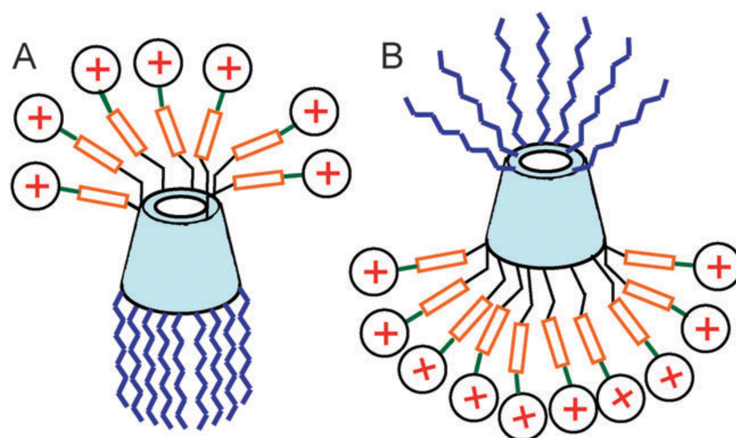


Fig 5.2 Relative orientation of the hydrophilic and hydrophobic domains in skirt-shaped (A) and jellyfish-shaped (B) polycationic amphiphilic CDs (paCDs).

In principle, both possible relative orientations of the cationic groups and the hydrophobic elements, that is, the skirt or the jellyfish arrangements (**Fig 5.2**, left and right, respectively), could be considered. A variety of synthetic strategies for regioselective manipulation of the primary hydroxyl groups (narrower rim) are at hand, on the contrary homogeneous functionalization at the secondary rim has consistently been a sticking point in CD chemistry.

In a pioneering work,[13] Dancy and coworkers presented the first examples of polycationic amphiphilic CDs (paCDs) taking advantage of the differential chemical reactivity between the primary and secondary hydroxyls to install alkyl chains and polar groups at the primary and secondary positions, respectively.

Recently, Garcia Fernandez and coworkers found that esterification of CD hydroxyls with long-chain acyl anhydrides in the presence of DMAP ensures homogeneous products independently of their location at the primary or secondary rim,[14] opening a very convenient route to monodisperse multihead, multitail, facial amphiphiles through bidirectional manipulation strategies.

Synthetic strategies suitable for structure–activity relationship studies to correlate modifications at the atomic level in discrete vectors and gene-delivery efficiencies for the corresponding supramolecular CDplexes were investigated. On the skirt-type architectures, two very robust “Click-type” methodologies have been privileged,[15] namely the thiourea-forming coupling reaction between amine and isothiocyanate counterparts and Cu(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC). In principle, amine and thiourea could act in a cooperative way in the reversible complexation of the phosphate

groups in DNA backbone through electrostatic and hydrogen-bonding interactions. The triazole moiety, on his side, similarly to other nitrogen heterocycles, could also behave as hydrogen bonding acceptor, as a DNA intercalating or as buffering unit (triazole pKa = 6), thereby affecting stability, endosomal release and transfection efficiency of the resulting CDplexes. In both β-CDs libraries, factors such as the density and arrangement of the cationic groups and the hydrogen bond donor centers, the flexibility of the linkers, or the length of the lipophilic chains were evaluated, obtaining some interesting results in terms of DNA-binding and transfection properties.

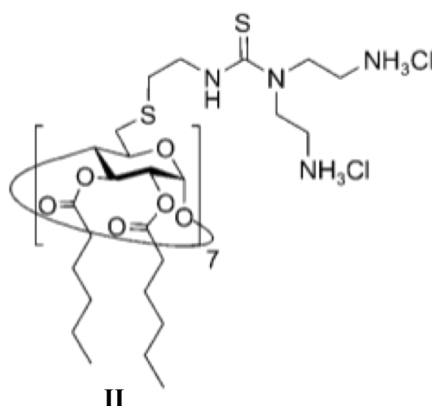


Fig 5.3 Structural formula of cyclodextrin **II**.

In particular, high transfection efficiency, even in presence of serum, and low cytotoxicity profile were achieved to COS-7 and BNL-CL2 cell lines by using the paCD **II** (**Fig 5.3**) possessing a dendritic arrangement of cationic head. The results obtained with this compound suggest that a rigid conformational motif and hydrogen-bonding thiourea centers can participate in cooperative binding to the phosphate groups in the plasmid chain.[16]

On the bases of results obtained in the Professor Garcia Fernandez's research group with compound **II** and in ours with **3** bearing four arginine unit as cationic arms (see Chapter 2), we planned a comparative study between amphiphilc β-cyclodextrins and calix[4]arenes, exchanging the cationic elements on the macrocyclic scaffolds. Polyaminothiourea- and arginine-based amphiphilic calix[4]arenes and β-cyclodextrins, respectively, were thus synthesized and studied as possible new potential non-viral vectors during a period of six months I spent at the Institute for Chemical Research (IIQ-CSIC) and University of Sevilla in the groups of Professor Garcia Fernandez and Professor Ortiz Mellet, respectively.

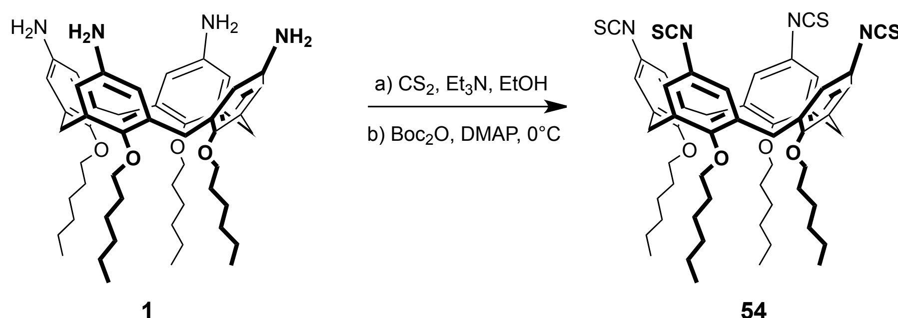
5.2 Results and discussion

5.2.1 Synthesis of polyaminothiourea based calix[4]arenes

Starting from cyclodextrin **II** as reference, we prepared three new amphiphilic polyaminothioureido-calix[4]arenes whose polar domain was finely tuned in terms of number, flexibility of the linkers and distance between phosphate binding motifs (amine and thiourea functionalities) and calixarene cavity, in order to investigate the effects that these factors might have in DNA complexation and delivery.

In principle, all these calix[4]arene based vectors could be synthesized by directly reacting a tetraamino-derivative with appropriately functionalized isothiocyanates. From a practical point of view, the derivative **1** (**Scheme 1**) was thus selected as the macrocyclic scaffold for the reaction with proper isothiocyanate counterparts.

Unfortunately, the low nucleophilicity of aromatic amines severely limited the use of the compound **1** in amine-isothiocyanate coupling reactions. This reactivity problem was easily solved by deciding to reverse the functional groups present on the building blocks involved in the formation of the thiourea connector. The synthesis of corresponding tetraisothiocyanate **54** was carried out using an excess of carbon disulfide and di-*tert*-butyl dicarbonate (Boc_2O)[17] as desulfurylation reagent (**Scheme 1**), proceeding rapidly and in good yields.



Scheme 1: synthesis of calixarene **54**.

The mechanism suggested for this reaction (**Fig 5.5**) involves the formation of dithiocarbamate stabilized by the addition of Et_3N; Boc_2O, subsequently added at 0°C in nearly stoichiometric amounts (0.99 equiv. for each NH_2 group), thus reacts with it to form an unstable “mixed dithiocarbamate/carbonate” adduct that in presence of a catalytic amount of DMAP rapidly decomposes to the desired isothiocyanate, carbonyl sulfide (COS) and *tert*-butanol.

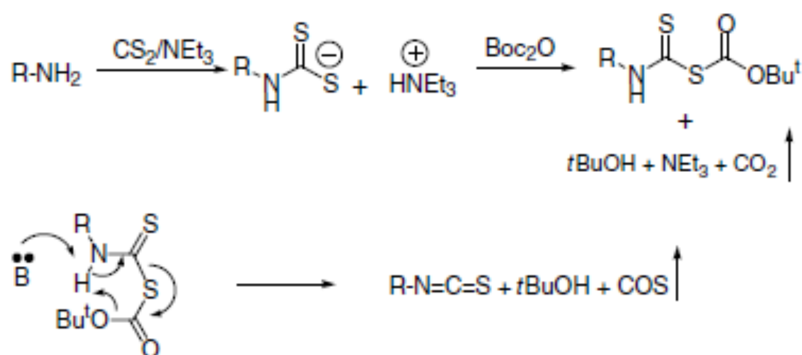
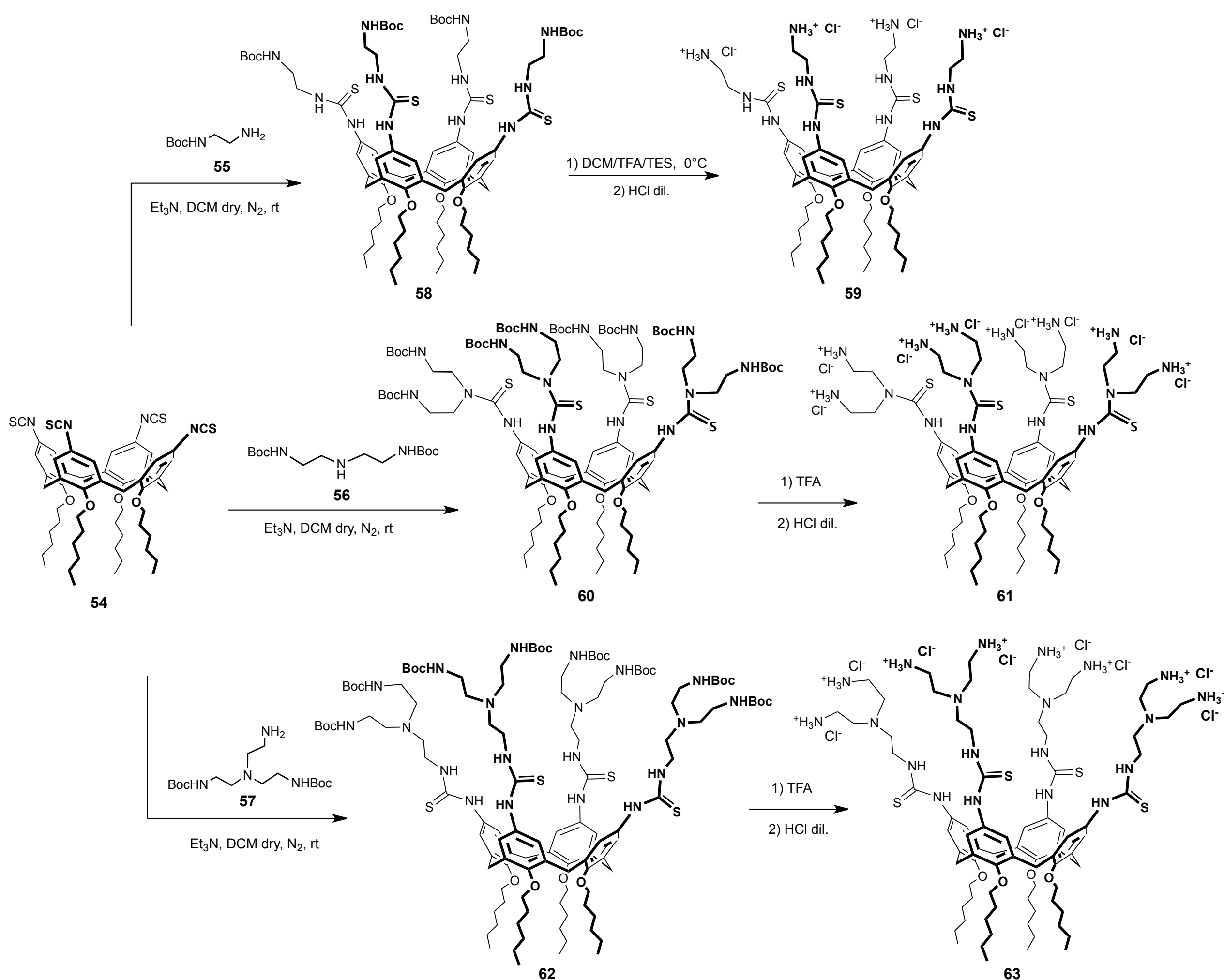


Fig 5.5 Suggested mechanism for the base catalyzed synthesis of isothiocyanates from the corresponding amine using CS_2 and Boc_2O.[17]

Amines **55**, **56** and **57** were chosen as nucleophilic reagents in thiourea-forming coupling reactions.

In all cases the thiourea-forming coupling reaction (**Scheme 2**) proceeded to completion, despite possible problems of steric hindrance, in relatively short times (~16 h) and in high yields from 65% to quantitative. Subsequent trifluoroacetic acid (TFA)-catalyzed cleavage of the carbamate protecting groups and freeze-drying of the crude products from diluted HCl solutions afforded the target amphiphilic polyaminothiourea-calix[4]arenes **59**, **61** and **63**, as hydrochloride salts in quantitative yield.

In detail, the aminothiourea-calixarene library consists of derivatives well suited for a possible structure-gene transfection relationship studies. Calixarene **59** had a linear thiourea belt with two hydrogen-bond donor centers. In calixarene **61** one of these donor centers was sacrificed in order to increase the number of protonable amine groups. A dendritic arrangement was displayed also by derivative **63**, but in this case the distance between the amino and thioureido functions was increased. It contained, additionally, a rim of tertiary amino groups that could give rise to a pH buffer effect.



Scheme 2: synthesis of calixarenes **59**, **61** and **63**.

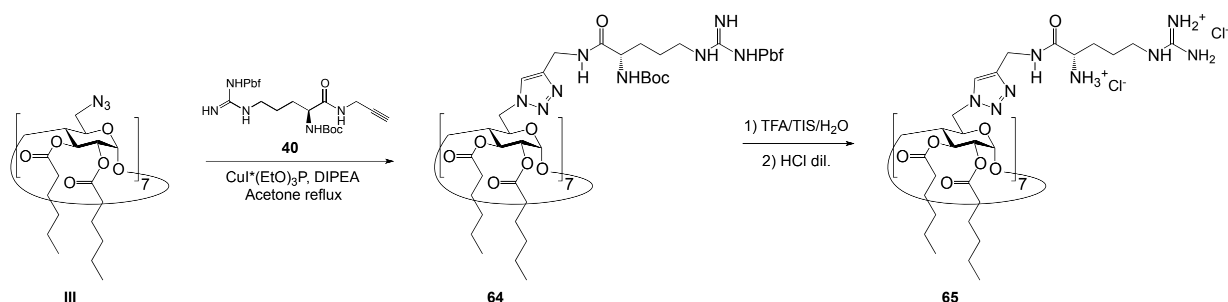
5.2.2 Synthesis of arginine based β-cyclodextrins

In parallel, inspired by the good results on the transfection abilities obtained by clustering arginine units on the calix[4]arene scaffold in cone conformation, we decided to follow the same strategy also for β-CD platform. Focusing on the skirt-type architecture having hexanoyl chains at the wider rim, we thus synthesized two new C-linked arginino-β-CDs to compare their properties as gene delivery systems respect to the arginino-calixarene based vectors.

For the synthesis of the first arginino-β-CD derivative, CuAAC reaction was privileged as key step to couple the cyclodextrin core with arginine arms. This is a well-know synthetic procedure to easily functionalize the β-CD narrower rim and warrants possible comparisons about DNA binding and transfection properties between the product that will

be obtain and the arginino-calixarene **42** (Chapter 3).

We identified as useful reagent the heptakis[6-azido-6-deoxy-2,3-di-*O*-hexyl]cyclomaltoheptaose **III** [15] (**Scheme 3**) and as proper partner for the reaction bearing the carbon-carbon triple bond the N_{α}-Boc-N_{ω}-Pbf-L-arginine-*N*-propargylamide (**40**). The reaction between the two compounds successfully gave derivative **64** with triazole rings as spacers. Deprotection step was carried out in standard conditions (TFA/TIS/H_2O) and furnished the target CD **65** in quantitative yield.



Scheme 3: synthesis of β-cyclodextrins **65**.

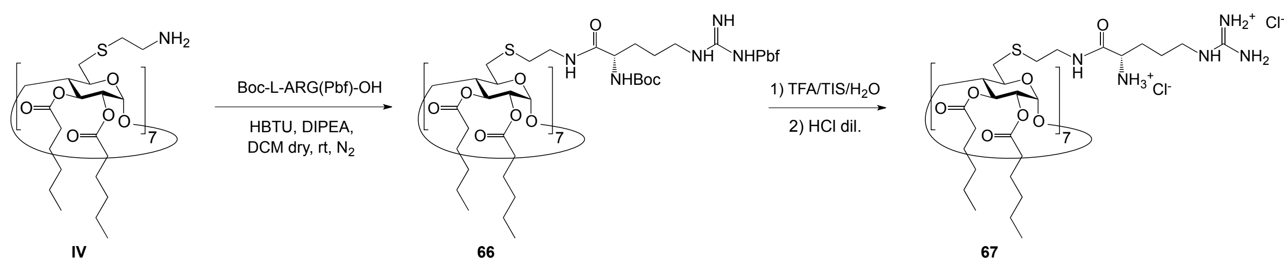
Subsequently, an arginino-β-CD derivative having the α amino acid units anchored to the macrocycle through anamide bond was prepared for a comparison with the calixarene **3** (Chapter 2).

First of all, we tried to reduce the azide groups of β-CD **III** in order to affort an heptamino cyclodextrin derivative. Different reducing conditions, such as PPh_3 in H_2O or H_2 atmosphere in presence of catalytic amount of Pd/C, were tested, but any reaction yielded the desired product.

Not being possible to have the amino groups directly linked to the glucose carbon 6, the introduction of the cysteamine segment in the structure was of importance in improving the accessibility of the amino groups on the CD core. Heptakis[6-(2-aminoethylthio)-6-deoxy-2,3-di-O-hexanoyl]cyclomaltoheptaose **IV** [16] was thus chosen as key starting material for our synthetic purposes.

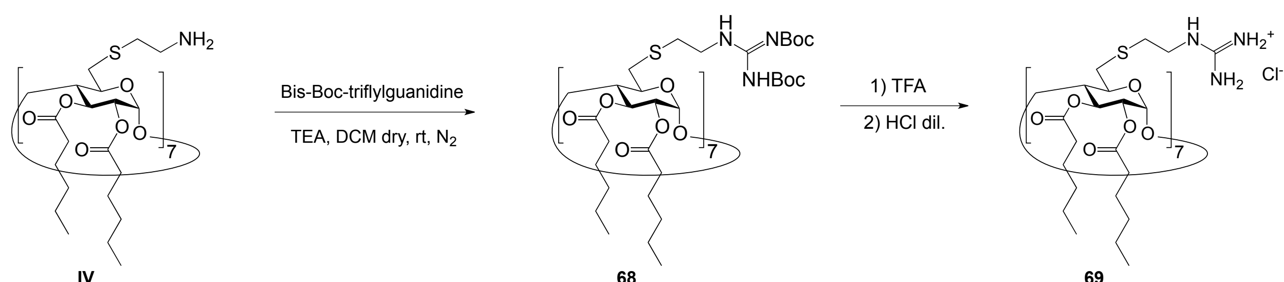
The synthesis (**Scheme 4**) comprised the same steps described in the previous chapters for calixarene derivatives and led to the desired product **67** in good yield. The coupling reaction was performed by using Boc-L-Arg(Pbf)-OH in presence of HBTU [*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate] as coupling reagent and subsequent removal of the protecting groups with a TFA solution in presence

of TIS (triisopropylsilane) as carbocation scavenger.



Scheme 4: synthesis of β-cyclodextrins **67**.

In addition, starting from **IV** and following the scheme **5,** the amphiphilic heptaguanidino-β-CD (**69**) was also prepared, as a control compound to evaluate the relevant contribution of the arginine in cell penetrating and transfection properties.



Scheme 5: synthesis of β-cyclodextrins **69**.

5.2.3 DNA binding properties and ligands-DNA *nanoparticles* characterization

The various calixarene and cyclodextrin based ligands shown in Figure 5.6 were preliminarily studied with different techniques to verify their ability to interact with plasmid DNA (pGL4.13 – 4642 bp).

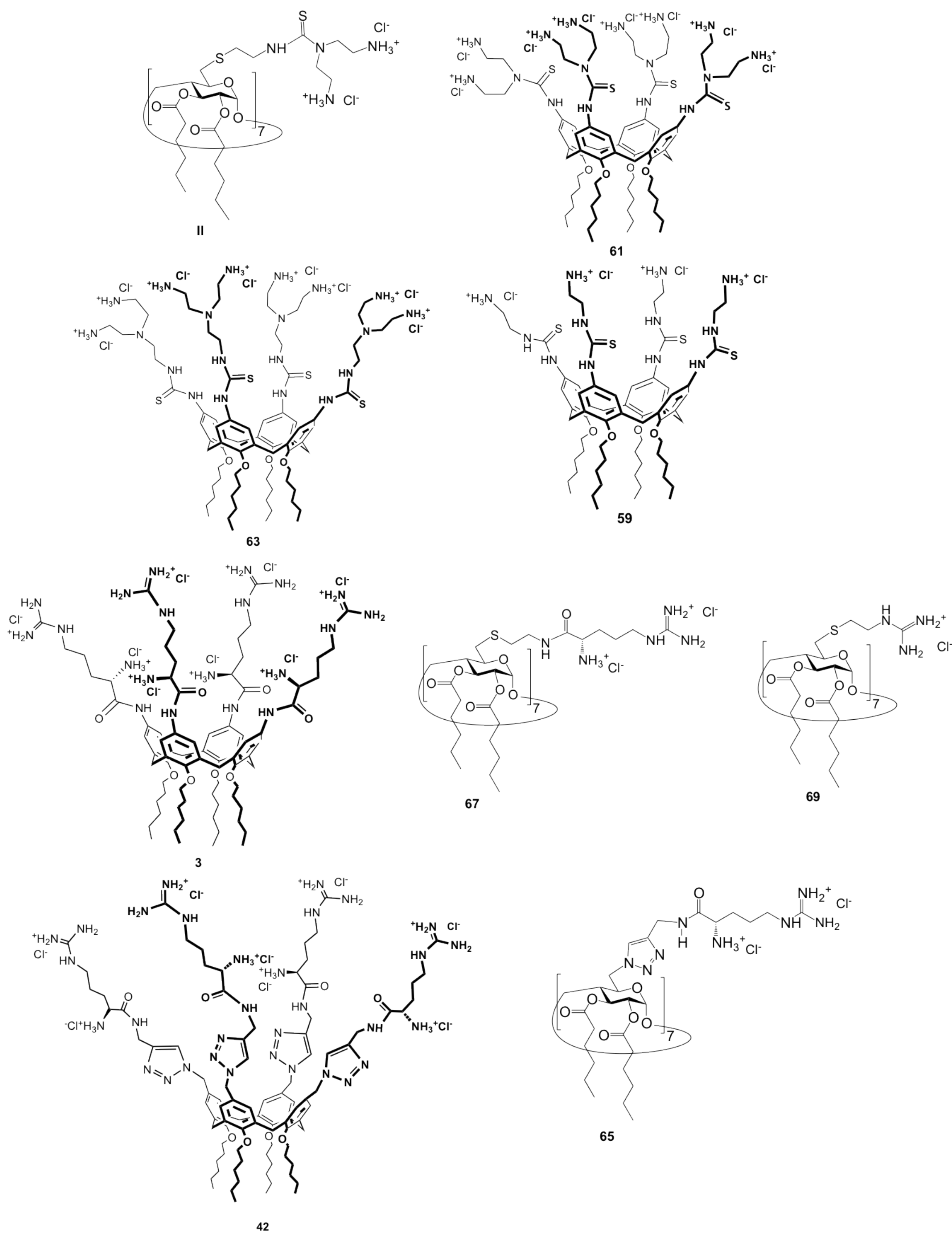


Fig 5.6 Structural formulas of polyaminothioureido- and arginino- calixarene and cyclodextrins.

To avoid self-aggregation phenomena, the calixarene and CD stock solutions were prepared in DMSO and further diluted with the pDNA solution in a HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (the final DMSO content never exceeded 1%v/v) at N/P ratios ranging from 1 to 20. We decided to comment and compare the results obtained using this parameter rather than concentration because calixarene and cyclodextrin derivatives have a different number of protonable nitrogen groups.

First, these samples were analyzed by agarose gel electrophoresis (EMSA), with staining by the ethidium bromide (EB) intercalating agent, for assessing DNA complex formation and protection as well as DNA integrity.

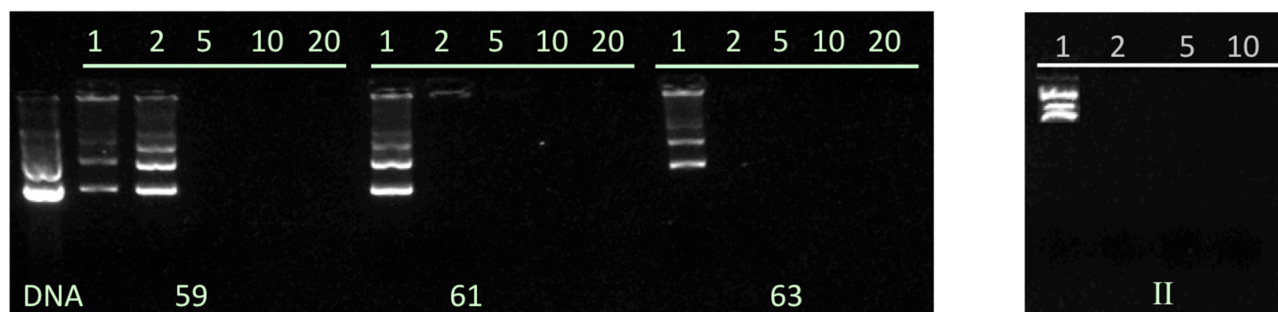


Fig.5.7 EMSA (electrophoretic mobility shift assays) of plasmid DNA (pGL4.13) in presence of compounds **59**, **61**, **63** and **II** at different N/P ratios,

The electrophoretic data revealed that the DNA mobility is strongly affected by the polyaminothiourea based calix[4]arenes (**Fig 5.7**). In particular, for the derivatives **61** and **63** with a dendritic arrangement of the cationic elements, the pDNA complexation resulted more efficient: already at N/P ratio 2 no “free” mobile plasmids were observed. This can be reasonably ascribed to the higher number of ammonium ions that in these cases is double respect to **59**.

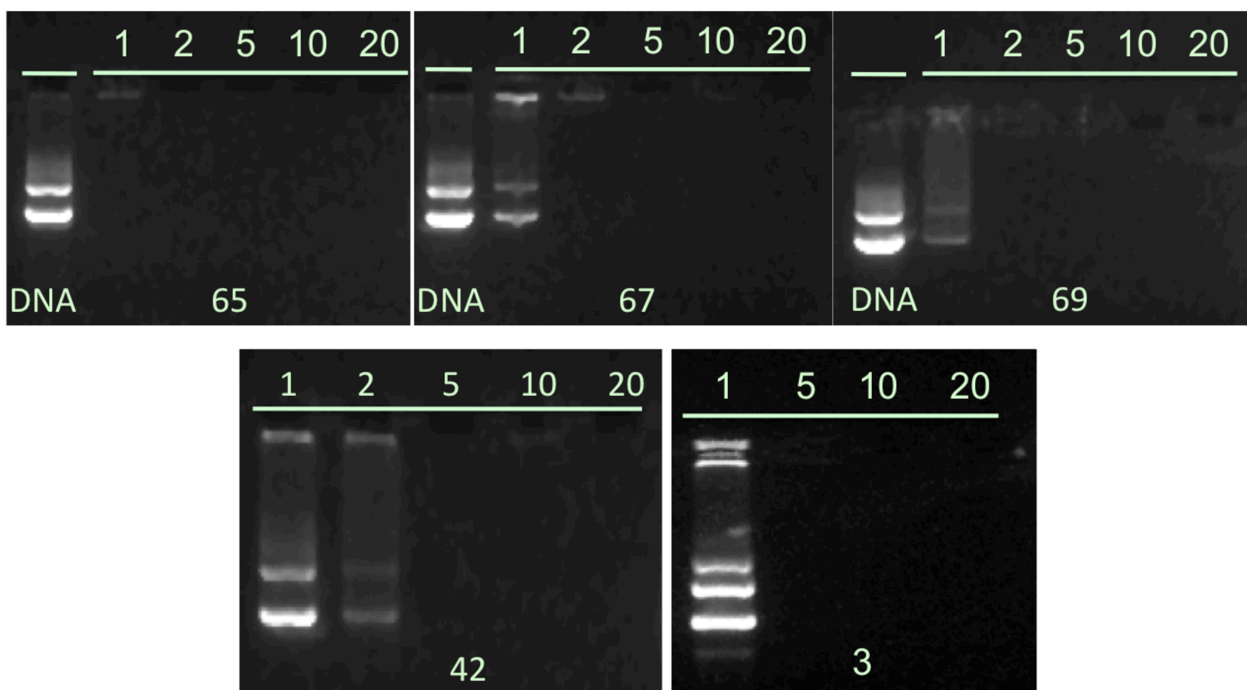


Fig.5.8 EMSA (electrophoretic mobility shift assays) of plasmid DNA (pGL4.13) in presence of compounds **65**, **67**, **69**, **3** and **42** at different N/P ratios,

Also in the cases of the guanidinylated β-CDs **65**, **67** and **69**, pDNA was efficiently compacted and protected (**Fig 5.8**). The plasmid filaments remained in the well and were inaccessible to the intercalating agent used as staining reagent for N/P ratio 2, as demonstrated by the absence of fluorescence in the corresponding lanes.

Concerning the comparison with the arginino-derivatives, for the compounds **3** and **67** with the amide bond connection between macrocyclic scaffold and amino acid, electrophoretic data showed a very similar behavior. On the other hand some differences were evidenced between the two triazole containing clusters: the CD **65** already at N/P ratio 1 was able to fully complex pDNA, while calixarene **42** completely bound the plasmid only at N/P ratio >2.

The calixarene/DNA and CD/DNA complexes were further characterized by dynamic light scattering (DLS) in order to estimate their hydrodynamic diameter (**Fig. 5.9**). Moreover, the surface charge of the complexes was calculated measuring its Z-potential, by applying an electric field to the samples. We determined these two parameters especially at values N/P ratio 5 and 10, to which, from the EMSA data, the pDNA was totally complexed by all the compounds synthesized.

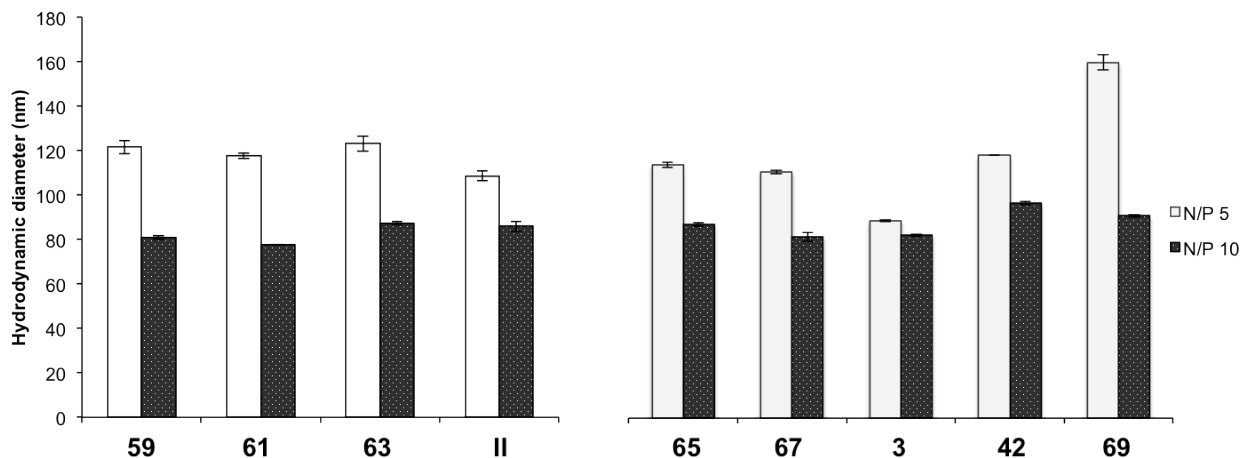


Fig. 5.9 Values of hydrodynamic diameters at two different N/P ratios for polyamino- and guanidinylated calixarenes and cyclodextrins.

Generally, at N/P ratio 5 all the new compounds formed relatively large aggregates with dimensions around or greater than 120 nm, but these values decreased upon increasing the N/P ratio. In fact, remarkably smaller particles were detected at N/P ratio 10, in the range 80–90 nm, indicating that the excess of ligand was able to be again incorporated in the complex nanostructure, generating through further hydrophobic and electrostatic interactions highly condensed nanometric aggregates. Only arginino-calixarene **3** showed substantially unaltered size, around 80 nm, at both N/P ratios. Quasi-monodisperse populations of “nanoparticles” were observed in almost all experiments.

Z-potential measurements (**Fig 5.10**) revealed for all complexes positive surface potentials (40-50 mV), and there were no significant differences between the two N/P ratio values.

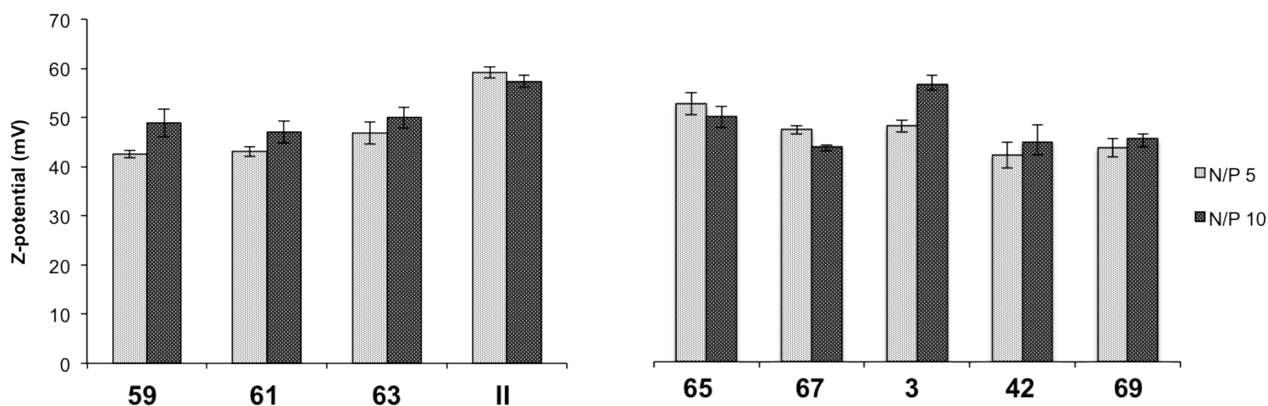


Fig. 5.10 Values of Z-potential at two different N/P ratios for polyamino- and guanidinylated calixarenes and cyclodextrins.

We deepened the investigation on particle size and morphology by using transmission electron microscopy (TEM).

The collected images (**Fig 5.11**) confirmed the proper size for cell penetration and homogeneous distribution of calixarene/DNA or CD/DNA complexes, except in the case of cyclodextrin **67** that already in DLS experiments showed indeed high polydispersity indexes (0.3-0.4). Interactions occurred without doubt but the image (**Fig 5.11 D**) evidenced the scarce propensity of this compound to condense efficiently single filaments of DNA. In fact, some big aggregates constituted by multiple filaments, probably not suitable for transfection, were present; on the contrary, the analogue calixarene **3** originated tight condensates characterized by proper dimensions for the cell membrane crossing.

The polyaminothiourea based calix[4]arenes (**59**, **61**, **63**) and the other two cyclodextrin derivatives **65** and **69** formed highly condensed nanometric aggregates (**Fig 5.11 E** and **F**). In addition, compounds **59** and **61** showed characteristics similar to compound **II** in the mode of DNA binding: at high magnification, an internal structure of the nanoparticles, made of alternating lamellar arrangements of amphiphilic calixarene and pDNA, was observed (**Fig 5.11 A-C**).

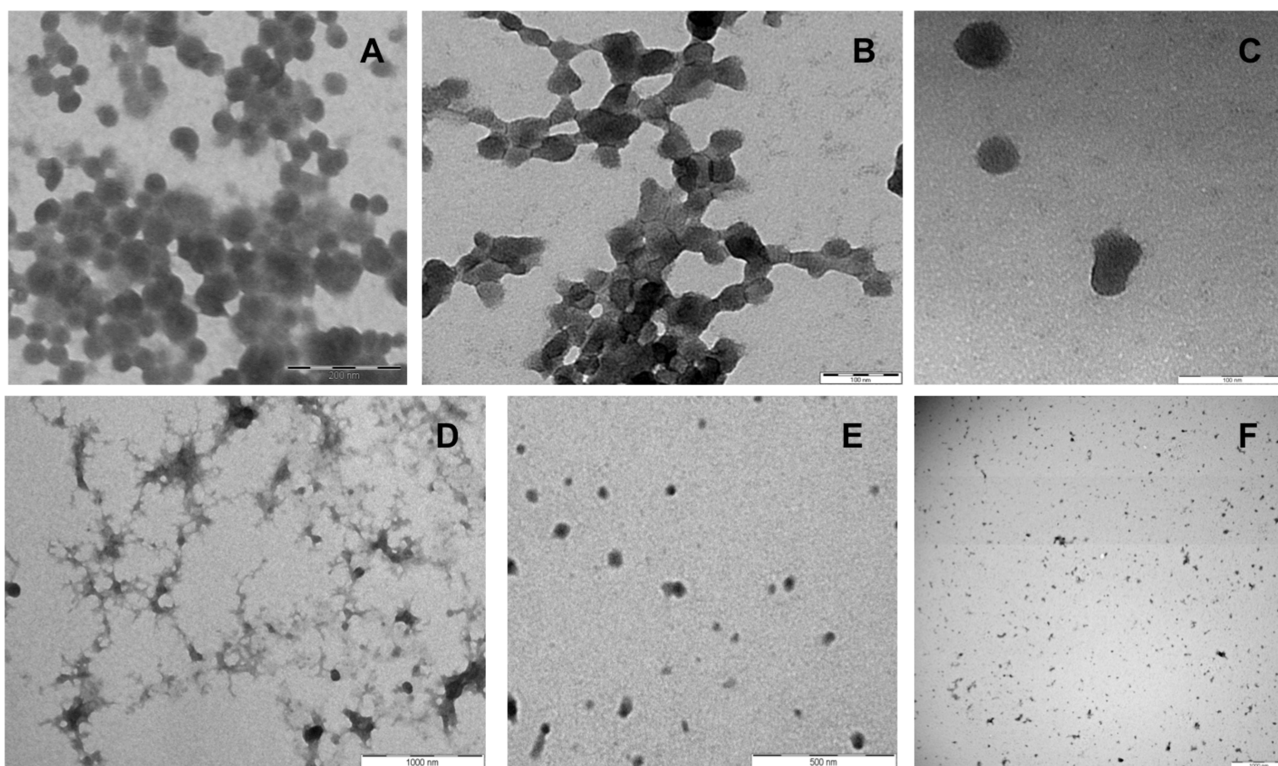


Fig. 5.11 TEM images showing the effects induced on plasmid pGL4.13 by A) ligand **II** B) ligand **59** C) ligand **61** D) ligand **67** E) ligand **69** and F) ligand **65**. All images were registered at N/P ratio 10.

5.2.4 Transfection experiments

First the transfection efficiency and cell viability (**Fig 5.12** and **5.13**) of the new synthesized ligands, at N/P ratio 5 and 10, were evaluated in the Professor Garcia Fernandez's laboratories. The experiments were performed in vitro to COS-7 cells using the same luciferase-encoding plasmid used for pDNA complexation studies (pGL4.13 – 4642 bp), in the absence and presence of 10% serum. Branched PEI (bPEI 25kDa) was used as reference, and for comparative purposes the structurally related thiourea-based CD **II** and arginino-based calixarene **3**, being in the two classes the most promising macrocyclic vectors synthesized so far, were further included in this studies.

Both in the absence and in the presence of serum, all compounds were found to mediate gene transfer in COS-7 cells with much higher efficiency than bPEI. Moreover, the efficiencies were generally better at N/P 10 than at 5, even if toxicity levels were higher.

In particular, focusing on N/P 10, derivative **61** featured a 10-fold increase in luciferase production to the reference bPEI. However, its efficiency was slightly lower than that of the corresponding CD derivative **II**, and, unfortunately, it showed only 40% of cell viability (**Fig**

5.12 right). Calixarene **63,** although possessing the same number of protonable amine groups in a dendritic arrangement, showed a lower transfection activity compared to **61**.Therefore, the gene expression decreased when increasing the distance between the amino and thioureido function, confirming the idea that a little modification in the vector's amphiphilic structure determine notable changes in the lipophilicity-hydrophilicity ratio affecting the biological properties. The presence of 10% of serum in the transfection mixture only partially decreased the efficiency in the cases of compounds **61** and **63**, on the contrary, for compound **59** at N/P 10 an increase was noticed. Remarkably, the linear arrangement present in calixarene **59** thus represented a good compromise between efficiency and toxicity, even in conditions closer to those in vivo.

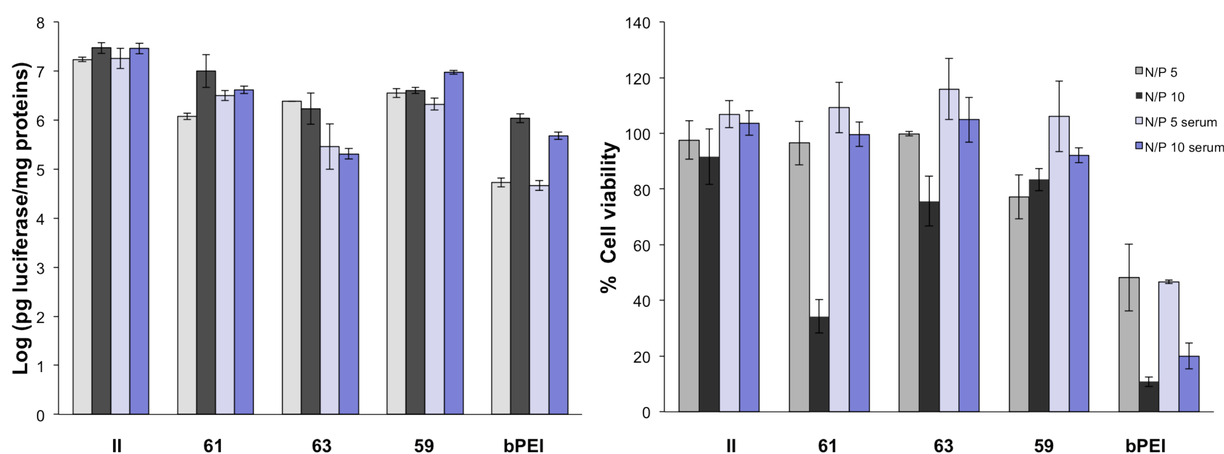


Fig 5.12 In vitro transfection efficiency using plasmid pGL4.13 (left) and cell viability (right) to COS-7 cells of compounds **59**, **61** and **63** and for compound **II** and **bPEI** used as references in comparative studies.

Concerning the influence of the triazole unit in the linker of arginino-derivatives, unlike what was previously observed for the two upper rim arginino-calixarenes **3** and **42** on RD-4 cells (see Chapter 3), different transfection efficiencies (**Fig 5.13** left) were not evidenced in COS-7 cells neither for cyclodextrins **65** and **67** nor for calixarenes**3** and **42**.

At N/P 10, all arginine-based macrocyclic vectors, mediated gene transfer in COS-7 cell line with 10-fold increased efficiency than bPEI. For the two cyclodextrins derivative **65** and **67** the efficiency was high even in presence of 10% serum, at the same N/P ratio. Quite rewarding was also the finding that their cytotoxicity was not relevant (**Fig 5.13** right).

The guanidino-cyclodextrin **69** showed a much lower efficiency in transfection efficiency compared to the arginine based ones, confirming that the presence of this basic α amino acid enhances the cell penetration ability of the lipoplexes and transfection abilities of the vector, strengthening on one hand the DNA binding and on the other one facilitating its release though a possible proton sponge effect.

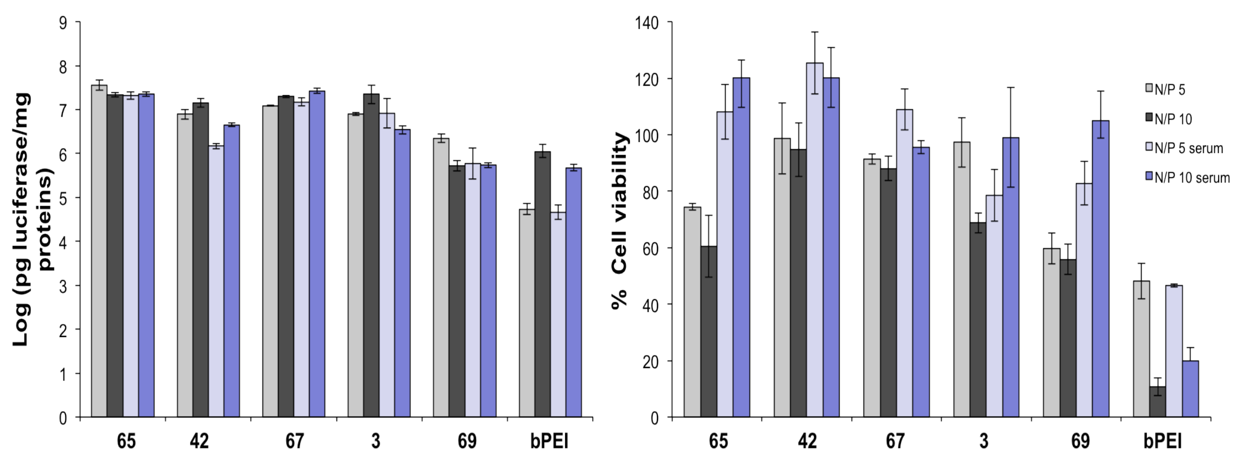


Fig 5.13 In vitro transfection efficiency using plasmid pGL4.13 (left) and cell viability (right) in COS-7 cells of compounds **65**, **67** and **69** and for compounds **3**, **42** and **bPEI** used as references in comparative studies.

Although luciferase-based assays provide valuable information about cell transfection activity of non-viral vectors, this technique, measuring the whole fluorescence intensity after the cell lysis, do not allow the quantification of the real number of transfected cells as parameter for evaluation of the effectiveness of the transfection process.

Therefore, some transfection experiments were also performed in the Professor Donofrio's laboratories with the usual conditions used for all the other compounds in the previous chapters. Then, exploiting again the plasmid pEGFP-C1 encoding for the green fluorescent protein, we performed a series of tests both with COS-7 and RD-4 cells given the common experimental conditions. The collected results could be compared with those in Chapter 2 and 3.

The tests on COS-7 with polyaminothiourea based calix[4]arenes and β-CD **II** (**Fig 5.14**) evidenced that the latter one has a better transfection efficiency. In particular when used without DOPE, it showed a maximum of 55% of transfected cells. In the absence of adjuvant the dendritic calixarene **61** appeared better than the linear analogue **59** and much better than the other dendritic derivative **63**. This, rather surprisingly, matched the

transfection level of **61** thanks to the addition of DOPE.

Among arginine containing compounds, on the contrary, the best vector was the calixarene based derivative **3** with the 52% of transfection. Between the two arginino-cyclodextrins **65** and **67**, the latter, with amide bond as **3**, showed a slightly higher efficiency, reaching the 40% of transfected cells. Significantly, the cyclodextrin **69** functionalized only with guanidinium moieties, was totally unable to give cell transfection, underlining once more the importance of the α amino acid in conferring good gene delivery properties.

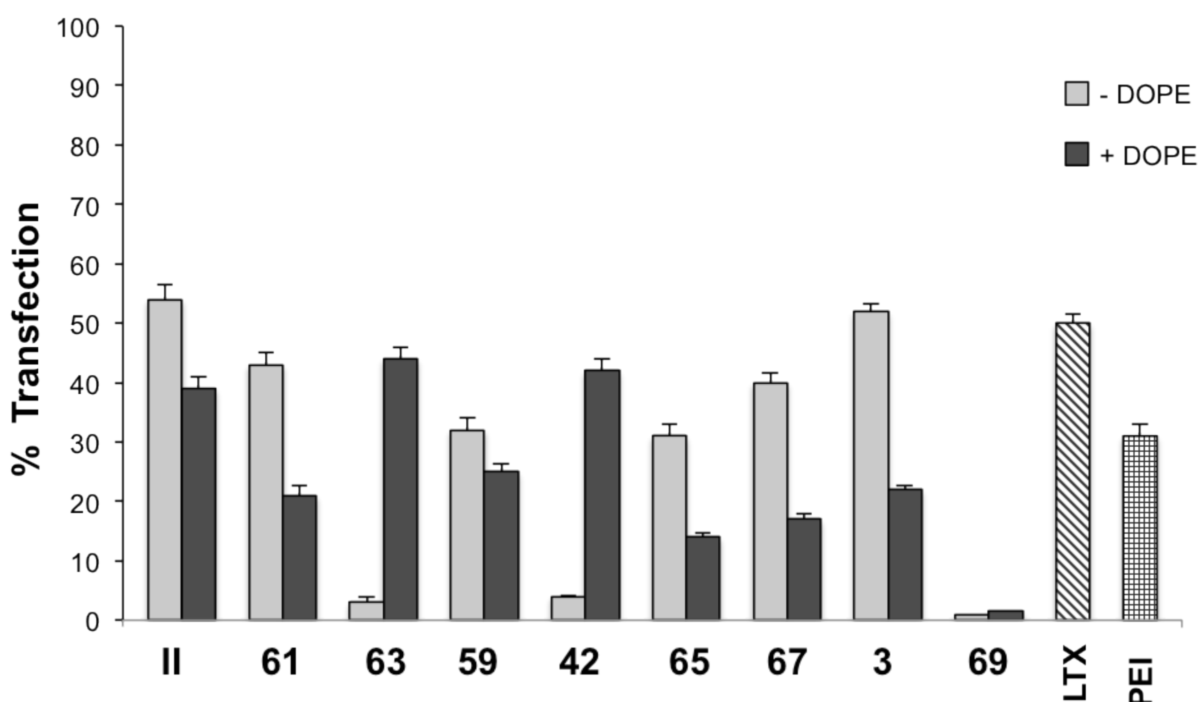


Fig 5.14 In vitro transfection efficiency using plasmid pEGFP-C1 in COS-7 cells of all compounds synthesized in this chapter and for compounds **II**, **3**, **42** and **bPEI** used as references in comparative studies.

Moving to the medically relevant RD-4 cell line, all the new compounds synthesized failed to render efficient transfection (**Fig 5.15**). The reference β-CD **II** resulted the most efficient in the polyaminothiourea based vectors series, but reaching only about 35% of transfected cells, both in absence or in presence of DOPE, an efficiency that, however, was not higher respect to the calixarene analogues **61** and **63** and close to those of LTX and PEI.

The total inability of the guanidino-CD **69** was confirmed also with these cells.

Considering the arginine containing compounds, in the case of RD-4 cells the two cyclodextrins **65** and **67** resulted much less efficient than the already established excellent

transfection of our upper rim arginino-calixarene **3**.

On the whole the low transfection percentages of the polyaminothiourea based calix[4]arenes and the arginine based β-cyclodextrins could also partially be attributed to the high toxicity found for all new synthesized compounds in the treatment of RD-4 cells, at least at concentrations > 2.5 μM.

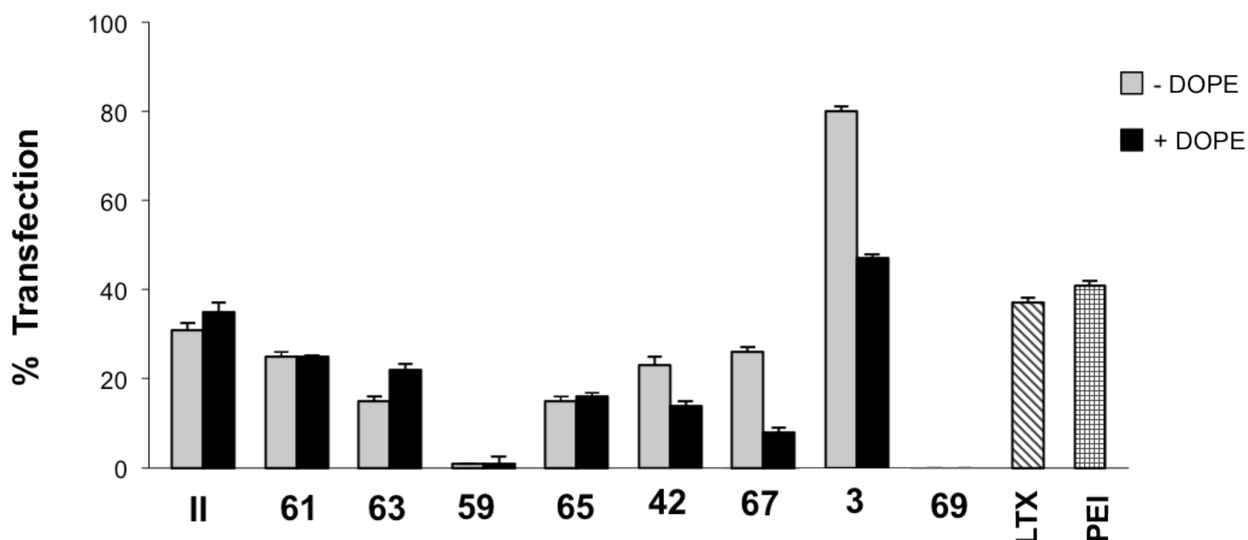


Fig 5.15 In vitro transfection efficiency using plasmid pEGFP-C1 in RD-4 cells of all compounds synthesized in this chapter and for compounds **II**, **3**, **42** and **bPEI** used as references in comparative studies.

The comparison of the results in the two series of tests confirmed the strong dependency of the vectors behavior on the cell line used for the transfection experiments.

5.3 Conclusions

In summary, the first comparison between potential non-viral vectors based on calixarenes and β-CDs was done. Taking as references the previously published the tetradecacationic β-CD **II** and the tetrearginino-calix[4]arene **3**, some new promising calixarene- and cyclodextrin-based systems for the gene delivery were synthesized, attaching aminothiourea arms at the upper rim of the calix[4]arene scaffold and arginine units at the narrower rim of the β-CD one.

As evidenced by electrophoretic, DLS and TEM data, both series confirmed their DNA binding abilities forming nanometric condensates, in principle suitable for cellular uptake. The control of the appropriate hydrophilic/hydrophobic ratio proved to be crucial for the

biological properties of these kind of gene delivery systems, even when relevant differences in DNA binding and condensation are not found by EMSA or TEM experiments. The difference in terms of transfection efficiency between the calixarene and CD derivatives resulted substantially small. Only in the experiments with plasmid DNA pEGFP-C1 and RD-4 cells, the tetraarginino-calix[4]arene **3** showed a largely higher efficiency respect to the other vectors, evidencing at least in that case a clear superiority of arginine compared to ammonium groups as cationic head and of calixarene compared to cyclodextrin as scaffold.

5.4 Experimental section

General information. Reagents and solvents were purchased from commercial sources and used without further purification. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with *Sílica Gel 60 F_{254} Merck* (0.25 mm), with visualization by UV light (λ 254 nm) and by charring with 10% H_2SO_4; ninhydrin 0.1% in ethanol; Mostain (20 g of ammonium (VI) molibdate4·H_2O; 0.4 g of $Ce(SO_4)_2\cdot H_2O$ and 10% H_2SO_4 in 400 mL of H_2O) and heating at 300 ºC. With preparative purposes, column chromatography was carried out on *Sílice 60 A.C.C. Chromagel* (SDS, E. Merck, 230-400 mesh). Optical rotations were measured at 20 ± 2 ºC in 1-cm or 1-dm tubes on a Jasco P-2000 polarimeter using Na D line (λ 589 nm), 0.2-1% (w/v) solutions and 1-cm cells. ^{1}H (and ^{13}C NMR) spectra were recorded at 500 (125.7), 400 (100.6) and 300 (75.5) MHz with, respectively, *Bruker AVANCE DRX 500* (500 MHz), *BrukerAVANCE 400* (400 MHz) and *BrukerAVANCE 300* (300 MHz) spectrometers. COSY, TOCSY and HMQC experiments were used to assist on NMR assignments. Electrospray mass spectra were obtained were recorded with *Bruker Esquire6000* (ESI ionization) for samples dissolved in MeCN, MeOH or H_2O-MeOH mixtures at low µm concentrations.

Synthesis of 5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (54)

To a solution of 5,11,17,23-tetramino-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene **1**[18] (150 mg, 0.18 mmol) in absolute EtOH (4 mL), CS_2 (440 µL, 7.3 mmol) and Et_3N (102 µL, 0.73 mmol) were added. The reaction mixture was stirred for 2 h at rt and then cooled on

an ice bath. A solution of di-*tert*-butyl dicarbonate (51 mg, 0.23 mmol) in absolute EtOH (1 mL) was added followed by the immediate addition of a catalytic amount of DMAP (1.8 mg, 14 μmol). The reaction mixture was kept in the ice bath for 20 min. Then it was allowed to reach rt and stirred for 2 hours. The solvent was removed under reduced pressure and the residue was purified by column chromatography (1:3 DCM-cyclohexane). A white solid (132 mg, 74%) was isolated.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (s, 8H, Ar*H*), 4.37 (d, *J* = 10.2 Hz, 4H, $ArCH_{ax}Ar$), 3.86 (t, *J* = 5.7 Hz, 8H, OCH_2), 3.12 (d, *J* = 10.2 Hz, 4H, $ArCH_{eq}Ar$), 1.93-1.78 (m, 8H, OCH_2CH_2), 1.45-1.12 (m, 24 H, $O(CH_2)_2CH_2CH_2CH_2$), 0.94 (t, *J* = 5.1 Hz, 12H, CH_2CH_3).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.6 and 135.8 (*C* Ar), 134.1 (N*C*S), 125.5 and 125.4 (*C* Ar), 75.7 (O*C*H_2), 31.9 (OCH_2CH_2), 30.7 (Ar*C*H_2Ar), 30.1 ($OCH_2CH_2CH_2$), 25.8 ($CH_2CH_2CH_3$), 22.8 (CH_2CH_3), 14.1 (CH_2CH_3).

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{56}H_{68}N_4O_4S_4$ 1011.4, found 1011.1.

Synthesis of *N-tert*-Butoxycarbonyl-1,2-diaminoethane (55)

It was synthesized according to a literature procedure. [19]

Synthesis of Bis(2-*tert*-butoxycarbonylaminoethyl)amine (56)

It was synthesized according to a literature procedure. [20]

Synthesis of 2-Aminoethyl-Bis(2-*tert*-butoxycarbonylaminoethyl)amine (57)

Triphenylphosphine (116 mg, 0.44 mmol) was added to a solution of 2-azidoethyl-bis(2-*tert*-butoxycarbonylaminoethyl)amine (150 mg, 0.40 mmol) in dioxane (6 mL), at room temperature. The reaction was stirred for 20 min, then concentrated aqueous ammonia (1.8 mL) was added and stirring was continued at room temperature overnight. After removal of solvent under reduced pressure, the crude was purified by flash chromatography ($CH_3CN/H_2O/NH_4OH$ 30:2:1). A colorless oil (138.5 mg, quant. yield) was isolated.

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.27 (bs, 2H, NH_2), 3.17 (q, *J* =5.9 Hz 4H, CH_2NHBoc), 2.73 (t, *J* =5.5 Hz 4H, CH_2NH_2), 2.59-2.43 (m, 6H, CH_2CH_2NHBoc and $CH_2CH_2NH_2$), 1.45 (s, 18H, $C(CH_3)_3$).

^{13}C NMR (75 MHz, $CDCl_3$) δ 156.3 (C=O), 79.1 ($C(CH_3)_3$), 56.7 ($NCH_2CH_2NH_2$), 54.2 (NCH_2CH_2NHBoc), 39.6 (CH_2NH_2), 38.6 (CH_2NHBoc), 28.4 ($C(CH_3)_3$).

Synthesis of 5,11,17,23-Tetrakis{*N'*-[2-(*tert*-butoxycarbonylamino)ethyl]thioureido}-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (58).

A solution of **54** (84.1 mg, 0.085 mmol) in dry DCM (3 mL) was added dropwise to a solution of *N'*-(2-(*tert*-butoxycarbonylamino))ethylamine (**55**, 68 mg, 0.41 mmol) and Et_3N (85 μL, 0.62 mmol) in dry DCM (3 mL). The mixture was stirred overnight at rt. The solvent was removed under vacuum and the residue was purified by flash chromatography (gradient CH_2Cl_2/MeOH from 95:5 to 9:1). The pure product **58** was obtained as white solid in quantitative yield (138.4 mg).

^{1}H NMR (300 MHz, CD_3OD) δ 6.68 (bs, 8H, Ar*H*), 4.46 (d, *J* = 12.7 Hz, 4H, ArCH_{ax}Ar), 3.93 (t, *J*=7.9 Hz, 8H, OCH_2), 3.65 (t, *J*=6.0 Hz, 8H, CSNHCH_2), 3.29-3.16 (m, 12H, ArCH_{eq}Ar and CH_2NHBoc), 2.15-1.88 (m, 8H, OCH$_2$CH_2), 1.55-1.32 (m, 60H, O(CH$_2$)$_2$CH_2CH_2CH_2 and C(CH_3)$_3$), 1.01-0.89 (m, 12H, CH$_2$CH_3).

^{13}C NMR (100 MHz, CD_3OD) δ 180.2 (*C*=S), 157.2 (*C*=O), 154.4, 135.4, 131.6, 124.4 (*C* Ar), 78.8 (*C*(CH$_3$)$_3$), 75.3 (O*C*H$_2$), 44.4 (NHCS*C*H$_2$), 39.2 (*C*H$_2$NHBoc), 31.8 (OCH$_2$CH$_2$*C*H$_2$), 30.4 (Ar*C*H$_2$Ar), 30.1 (OCH$_2$*C*H$_2$), 27.3 (C(*C*H$_3$)$_3$), 25.8 (*C*H$_2$CH$_2$CH$_3$), 22.5 (*C*H$_2$CH$_3$), 13.0 (CH$_2$*C*H$_3$).

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{84}H_{132}N_{12}O_{12}S_4$ 1651.9, found 1652.8.

Synthesis of 5,11,17,23-Tetrakis(2-aminoethylthioureido)-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, tetrahydrochloride (59).

A solution of **58** (40 mg, 25 μmol) in DCM-TFA-TES (87.5 : 10 : 2.5, 0.5 mL) was stirred at 0 °C for 2 h. The solvent was removed under reduced pressure. The residue was precipitated and washed with anhydrous diethyl ether. Then the solid was dissolved in 0.1 M HCl solution followed by evaporation under reduced pressure to obtain the product as hydrochloride salt in quantitative yield (33.8 mg).

^{1}H NMR (500 MHz, CD_3OD) δ 6.72 (bs, 8H, Ar*H*), 4.47 (d, *J* = 13.2 Hz, 4H, ArCH_{ax}Ar), 3.98-3.85 (m, 16H, OCH_2 and CSNHCH_2), 3.22-3.13 (m, 12H, ArCH_{eq}Ar and CH_2NH$_3^+$), 2.02-1.91 (m, 8H, OCH$_2$CH_2), 1.52-1.35 (m, 24H, O(CH$_2$)$_2$CH_2CH_2CH_2), 0.99-0.91 (m, 12H, CH$_2$CH_3).

^{13}C NMR (75 MHz, CD_3OD) δ 182.8 (*C*=S), 156.0, 136.9, 133.1, 125.7 (*C* Ar), 76.6 (O*C*H$_2$), 42.8 (NHCS*C*H$_2$), 40.8 (*C*H$_2$NH$_3^+$), 33.4 (OCH$_2$CH$_2$*C*H$_2$), 31.9 (Ar*C*H$_2$Ar), 31.5 (OCH$_2$*C*H$_2$), 27.3 (*C*H$_2$CH$_2$CH$_3$), 24.0 (*C*H$_2$CH$_3$), 14.5 (CH$_2$*C*H$_3$).

ESI-MS (m/z): $[M + Cu]^+$ calcd for $C_{64}H_{104}Cl_4N_{12}O_4S_4$ 1292.2, found 1292.4.

Synthesis of 5,11,17,23-Tetrakis{*N',N'*-bis[2-(*tert*-butoxycarbonylamino)ethyl] thioureido}25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (60).

A solution of **54** (84.1 mg, 0.085 mmol) in dry DCM (3 mL) was added dropwise to a solution of *N',N'*-bis-[2-(*tert*-butoxycarbonylamino)]ethylamine (**56**, 124 mg, 0.41 mmol) and Et_3N (85 µL, 0.62 mmol) in dry DCM (3 mL). The mixture was stirred overnight at rt. The solvent was removed under vacuum and the residue was purified by flash chromatography (gradient CH_2Cl_2/MeOH from 100:1 to 99:1). The pure product **60** was obtained as white solid in quantitative yield (187 mg).

^{1}H NMR (500 MHz, CD_3OD) δ 6.96 (bs, 8H, Ar*H*), 4.50 (d, *J* = 12.5 Hz, 4H, ArCH_{ax}Ar), 4.05-3.70 (m, 24H, OCH_2 and CSNHCH_2), 3.31 (under the CD_3OD signal, 16H, CH_2NHBoc), 3.16 (d, *J* = 12.5 Hz, 4H, ArCH_{eq}Ar), 2.07 (m, 8H, OCH$_2$CH_2), 1.67-1.28 (m, 96 H, O(CH$_2$)$_2$CH_2CH_2CH_2 and C(CH_3)$_3$), 1.05-0.95 (m, 12H, CH$_2$CH_3).

^{13}C NMR (125 MHz, CD_3OD) δ 183.3 (*C*=S), 158.5 (*C*=O), 155.3, 135.8, 135,7 and 127.3 (*C* Ar), 80.7 (*C*(CH$_3$)$_3$), 76.6 (O*C*H$_2$), 52.4 (NHCS*C*H$_2$), 39.7 (*C*H$_2$NHBoc), 33.3 (OCH$_2$CH$_2$*C*H$_2$), 32.0 (Ar*C*H$_2$Ar), 31.5 (OCH$_2$*C*H$_2$), 29.0 (C(*C*H$_3$)$_3$), 27.2 (*C*H$_2$CH$_2$CH$_3$), 23.9 (*C*H$_2$CH$_3$), 14.4 (CH$_2$*C*H$_3$).

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{112}H_{184}N_{16}O_{20}S_4$ 2224.3, found 2224.7.

Synthesis of 5,11,17,23-Tetrakis[*N',N'*-bis(2-aminoethyl)thioureido]-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, octahydrochloride (61)

A solution of **60 (**25 mg, 14.7 µmol) in TFA (0.2 mL) was stirred at rt for 5 min. Water (10 mL) was added and the solution was freeze-dried. The residue was dissolved in 0.1 M HCl methanolic solution and freeze-dried to obtain the product as hydrochloride salt in quantitative yield (19 mg).

^{1}H NMR (300 MHz, CD_3OD) δ 6.86 (s, 8H, Ar*H*), 4.48 (d, *J* = 12.6 Hz, 4H, ArCH_{ax}Ar), 4.09 (t, *J* = 6.7 Hz, 16H, CSNHCH_2), 3.95 (t, 8H, *J* = 7.8 Hz, OCH_2), 3.26 (t, *J* = 6.7 Hz, 16H, CH_2NH$_3^+$), 3.16 (d, *J* = 12.6 Hz, 4H, ArCH_{eq}Ar), 2.12-1.93 (m, 8H, OCH$_2$CH_2), 1.53-1.31 (m, 24H, O(CH$_2$)$_2$CH_2CH_2CH_2), 1.02-0.90 (m, 12H, CH$_2$CH_3).

^{13}C NMR (125 MHz, CD_3OD) δ 183.0 (*C*=S), 154.2, 134.4, 134.2 and 126.7 (*C* Ar), 75.6 (O*C*H$_2$), 45.2 (NHCS*C*H$_2$), 37.1 (*C*H$_2$NH$_3^+$), 32.1 (OCH$_2$CH$_2$*C*H$_2$), 30.5 (Ar*C*H$_2$Ar), 30.3 (OCH$_2$*C*H$_2$), 25.9 (*C*H$_2$CH$_2$CH$_3$), 22.7 (*C*H$_2$CH$_3$), 13.2 (CH$_2$*C*H$_3$).

ESI-MS (m/z): $[M + Cu]^+$ calcd for $C_{72}H_{128}Cl_8N_{16}O_4S_4$ 1464.8, found 1465.8.

Synthesis of 5,11,17,23-Tetrakis{2-*N'*-[*N,N*-bis-(2-(*tert*-butoxycarbonylamino) ethyl)aminoethyl]thioureido}-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (62)

A solution of **54** (40 mg, 0.040 mmol) in dry DCM (3 mL) was added dropwise to a solution of 2-{[*N',N'*-bis-[2-(*tert*-butoxycarbonylamino)ethylamino]}ethylamine (**57**, 67.4 mg, 0.19 mmol) and Et_3N (85 µL, 0.62 mmol) in dry DCM (3 mL). The mixture was stirred overnight at rt. The solvent was removed under vacuum and the residue was purified by flash chromatography (CH_2Cl_2/MeOH 19:1). The pure product **62** was obtained as white solid (66% yield, 63 mg).

^{1}H NMR (300 MHz, CD_3OD) δ 6.71 (bs, 8H, Ar*H*), 4.46 (d, *J* = 13.3 Hz, 4H, ArCH_{ax}Ar), 3.93 (t, *J*=7.4 Hz, 8H, OCH_2), 3.61 (t, *J*=5.9 Hz, 8H, CSNHCH_2), 3.18 (d, *J* = 13.3 Hz, 4H, ArCH_{eq}Ar), 3.07 (t, *J*=6.4 Hz, 16H, CH_2CH$_2$NHBoc), 2.71 (t, *J*=5.9 Hz, 8H, CSNHCH$_2$CH_2), 2.59 (t, *J*=6.4 Hz, 16H, CH$_2$CH_2NHBoc), 2.05-1.88 (m, 8H, OCH$_2$CH_2), 1.54-1.31 (m, 96H, O(CH$_2$)$_2$CH_2CH_2CH_2 and C(CH_3)$_3$), 1.02-0.89 (m, 12H, CH$_2$CH_3).

^{13}C NMR (75 MHz, CD_3OD) δ 181.7 (*C*=S), 158.4 (*C*=O), 155.9, 136.8, 133.3 and 126.1 (*C* Ar), 80.3 (*C*(CH$_3$)$_3$), 76.6 (O*C*H$_2$), 55.3 (*C*H$_2$NHBoc), 54.4 (CSNHCH$_2$*C*H$_2$) 43.8 (NHCS*C*H$_2$), 40.1 (*C*H$_2$CH$_2$NHBoc), 33.3 (OCH$_2$CH$_2$*C*H$_2$), 32.0 (Ar*C*H$_2$Ar), 31.5 (OCH$_2$*C*H$_2$), 29.0 (C(*C*H$_3$)$_3$), 27.3 (*C*H$_2$CH$_2$CH$_3$), 23.9 (*C*H$_2$CH$_3$), 14.5 (CH$_2$*C*H$_3$).

ESI-MS (m/z): $[M + Na]^+$ calcd for $C_{120}H_{204}N_{20}O_{20}S_4$ 2396.4, found 2397.3.

Synthesis of 5,11,17,23-Tetrakis{2-*N'*-[*N,N*-bis-(2-ethylamino)ethyl)thioureido]}-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene, octahydrochloride (63)

A solution of **62** (52 mg, 22 µmol) in TFA (0.4 mL) was stirred at rt for 5 min. Water (10 mL) was added and the solution was freeze-dried. The residue was dissolved in 0.1 M HCl methanolic solution and freeze-dried to obtain the product as hydrochloride salt in quantitative yield (41 mg).

^{1}H NMR (300 MHz, CD_3OD) δ 7.02 (bs, 8 H, Ar), 4.51 (d, *J* = 12.7 Hz, 4 H, ArCH_{ax}Ar), 4.18 (t, *J* = 7.2 Hz, 8 H, ArOCH_2), 3.96 (t, *J* = 7.7 Hz, 8 H, CSNHCH_2), 3.37 – 3.26 (m, 40 H, CH_2CH$_2$NHCS and CH_2CH_2NH$_3^+$), 3.21 (d, 4 H, ArCH_{eq}Ar), 2.18 – 2.06 (m, 8 H, CH2-2Hex), 1.56-1.34 (m, 24 H, O(CH$_2$)$_2$CH_2CH_2CH_2), 1.05-0.89 (m, 12 H, CH$_2$CH_3).

^{13}C NMR (125.7 MHz, CD_3OD) δ 183.9 (*C*=S), 155.4, 136.9, 133.1 and 127.8 (*C* Ar), 76.5 (O*C*H$_2$), 51.5, 51.8 (*C*H$_2$N(*C*H$_2$CH$_2$)$_2$), 42.2 (NHCS*C*H$_2$), 37.9 (*C*H$_2$NH$_3^+$), 32.5 (OCH$_2$CH$_2$*C*H$_2$), 31.3 (Ar*C*H$_2$Ar), 30.8 (OCH$_2$*C*H$_2$), 26.7 (*C*H$_2$CH$_2$CH$_3$), 23.4 (*C*H$_2$CH$_3$), 14.4 (CH$_2$*C*H$_3$).

ESI-MS (m/z): [M + H]$^+$ calcd for $C_{80}H_{148}Cl_8N_{20}O_4S_4$ 1574.1, found 1574.9, [M + 2H]$^{2+}$ calcd 787.5, found 788.0.

Synthesis of Heptakis{6-[4-(*N*$_α$-Boc-*N*$_ω$-Pbf-L-arginine-*N*-amidomethyl)-1,2,3-triazol-1-yl]-6-deoxy-2,3-di-*O*-hexanoyl}cyclomaltoheptaose (64)

To a solution of heptakis[6-azido-6-deoxy-2,3-di-*O*-hexanoyl]cyclomaltoheptaose[15] (**III**, 88 mg, 33 μmol) in acetone (5 mL), *N*$_α$-Boc-*N*$_ω$-Pbf-L-arginine-*N*-propargylamide (**40**, 142 mg, 0.25 mmol), DIPEA (38μL, 0.24 mmol) and CuI·P(EtO)$_3$ (8.0 mg, 25μmol) were added. The reaction mixture was refluxed for 48 h. The solvent was removed under reduced pressure and the residue was purified by flash chromatography (gradient CH_2Cl_2/MeOH from 20:1 to 9:1). The pure product **64** was obtained as white solid in quantitative yield (218 mg).

$[α]_D$ = +29.7 (*c* 1.0, MeOH)

^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.12 (bs, 7 H, C*H* $_{triazole}$), 7.84 (bs, 7 H, N*H*), 6.69 (bs, 7 H, N*H*), 6.55 (bs, 14 H, N*H*), 5.48-5.35 (m, 14 H, *H*-3 and *H*-1), 4.74-4.59 (m, 21 H, triazol-C*H*$_2$-arginine, *H*-2), 4.57-4.54 (m, 7 H, *H*-5), 4.41-4.36 (m, 7 H, *H*-6a), 4.23-4.11 (m, 7 H, *H*-6b), 3.95 (m, 7 H, COC*H*NHBoc), 3.61 (bt, 7 H, *H*-4), 3.09 (m, 14 H, C*H*$_2$NHCNH), 2.96 (s, 14 H, C*H*$_{2\ Pbf}$), 2.51 (s, 21 H, C*H*$_{3\ Pbf}$), 2.46 (s, 21 H, C*H*$_{3\ Pbf}$), 2.28–2.26 (m, 14 H, OCOC*H*$_2$), 2.27–2.10 (m, 14 H, OCOC*H*$_2$), 2.03 (s, 21 H, C*H*$_{3\ Pbf}$), 1.66-1.18 (217 H, COCHC*H*$_2$C*H*$_2$, OCOCH$_2$C*H*$_2$, C*H*$_2$C*H*$_2$CH$_3$, C(C*H*$_3$)$_2$, C(C*H*$_3$)$_3$), 0.93-0.80 (bs, 42 H, CH$_2$C*H*$_3$).

^{13}C NMR (125.7 MHz, DMSO-*d*$_6$) δ 175.3, 175.1 and 174.3 (C=O), 160.6 (C=N), 159.3 (C=O), 147.6 (*C* $_{triazole}$), 140.3-134.6, (*C* Ar $_{Pbf}$), 127.8 (*C*H $_{triazole}$), 127.3, 119.3 (*C* Ar $_{Pbf}$), 99.0 (*C*-1), 89.2 (*C*(CH$_3$)$_3$), 81.3 (*C*(CH$_3$)$_2$), 79.6 (*C*-4), 72.8 (*C*-3), 72.5 (*C*-5), 72.3 (*C*-2), 57.2 (CO*C*HNHBoc), 52.6 (triazol-*C*H$_2$-arginine), 45.7 (*C*H$_{2\ Pbf}$), 42.4 (N=CNH*C*H$_2$), 37.4 (*C*-6), 36.4 and 36.3 (OCO*C*H$_2$), 33.9 and 33.7 (*C*H$_2$CH$_2$CH$_3$), 32.0 (COCH*C*H$_2$), 31.3, 31.2 (C(*C*H$_3$)$_2$, C(*C*H$_3$)$_3$), 28.7 (COCHCH$_2$*C*H$_2$), 26.9 and 26.8 (OCOCH$_2$*C*H$_2$), 24.8 and 24.9 (*C*H$_2$CH$_3$), 21.8 (*C*H$_{3\ Pbf}$), 20.5 (*C*H$_{3\ Pbf}$), 16.6 (CH$_2$*C*H$_3$), 15.2 (CH$_{3\ Pbf}$).

ESI-MS (m/z): [M + Cu + Na]$^{2+}$ calcd for $C_{315}H_{490}N_{56}O_{84}S_7$ 3355.0, found 3355.6.

Synthesis of Heptakis{6-[4-(L-arginine-*N*-amidomethyl)-1,2,3-triazol-1-yl]-6-deoxy-2,3-di-*O*-hexanoyl}cyclomaltoheptaose, tetradecahydrochloride (65)

A solution of **64** (45.2 mg, 7 μmol), in TFA-TIS-H_2O (95 : 2.5 : 2.5, 4.5 mL) was stirred at rt

for 2 h. a The solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in a 0.1 M HCl solution and freeze-dried to obtain the product as hydrochloride salt in quantitative yield (35.7 mg).

$[\alpha]_D$ = +46.5 (*c* 1 in DMF).

^{1}H NMR (500 MHz, DMSO-*d$_6$*) δ 8.99 (bs, 7 H, N*H*), 7.99 (m, 21 H, C*H* $_{triazole}$ and N*H*), 7.18 (bs, 21 H, N*H*), 5.41-5.37 (m, 14 H, *H*-3 and *H*-1), 4.68-4.63 (m, 21 H, *H*-2, triazol-CH_2-arginine), 4.51 (m, 7 H, *H*-5), 4.36-4.34 (m, 7 H, *H*-6a), 4.23-4.21 (m, 7 H, *H*-6b), 3.92 (m, 7 H, COC*H*NH_3^+), 3.72 (bt, 7 H, *H*-4), 3.19 (m, 14 H, CH_2NHCNH), 2.37–1.93 (m, 28 H, OCOCH_2), 1.86-1.79 (m, 14 H, COCHCH_2CH_2), 1.48-1.38 (m, 42 H, COCHCH_2CH_2, OCOCH_2CH_2), 1.37-2.20 (m, 56 H, CH_2C$H_2$$CH_3$), 0.94-0.80 (bs, 42 H, CH_2CH_3).

^{13}C NMR (125.7 MHz, DMSO-*d$_6$*) δ 172.8, 171.8 and 169.1 (*C*=O), 157.7 (*C*=N), 144.2 (*C* $_{triazole}$), 125.7 (*C*H $_{triazole}$), 102.5 (*C*-1), 77.3 (*C*-4), 70.3 (*C*-3), 70.1 (*C*-5), 69.7 (*C*-2), 52.5 CO*C*HNH_3^+) 49.9 (triazol-*C*H_2-arginine), 40.7 (N=CNH*C*H_2), 34.9 (*C*-6), 33.8 and 33.7 (OCO*C*H_2), 31.2 and 31.1 (OCOCH_2*C*H_2 and COCHCH_2*C*H_2), 28.5 (COCH*C*H_2), 24.3 and 24.2 (*C*$H_2$$CH_2$$CH_3$), 22.2 and 22.1 (*C*$H_2$$CH_3$), 13.9 ($CH_2$*C*$H_3$).

MALDI-MS (m/z): $[M + H - 14HCl]^+$ calcd for $C_{189}H_{336}Cl_{14}N_{56}O_{49}$ 4161.5, found 4162.6.

Synthesis of Heptakis{6-[2-(N_α-Boc-N_ω-Pbf-L-arginine-*N*-amide)ethylthio]-6-deoxy-2,3-di-*O*-hexanoyl}cyclomaltoheptaose (66)

To a solution of heptakis[6-(2-aminoethylthio)-6-deoxy-2,3-di-O-hexanoyl] cyclomaltoheptaose[16] (**IV**, 60 mg, 19 µmol) in dry DCM (4 mL) under N_2 atmosphere, DIPEA (90 µL, 53 µmol, 10.5 eq), Boc-L-Arg(Pbf)-OH (104 mg, 198 µmol, 10.5 eq) and HBTU [*O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate] (75 mg, 198 µmol, 10.5 eq) were added and the mixture was stirred at room temperature for 24 h. The reaction mixture was washed with a solution of $NaHCO_3$ saturated (8 mL), and the organic layer was dried (MgSO4), filtrated and concentrated. The residue was purified by flash chromatography (gradient CH_2Cl_2/MeOH from 25:1 to 18:1). The pure product **64** was obtained as white solid (yield 68%, 83 mg).

$[\alpha]_D$ = +31.9 (*c* 1 in MeOH)

^{1}H NMR (500 MHz, CD_3OD): δ 5.54 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.5 Hz, *H*-3), 5.36 (d, $J_{1,2}$ = 3.7 Hz, 7 H, *H*-1), 5.02 (dd, 7 H, *H*-2), 4.41 (m, 7 H, *H*-5), 4.33 (m, 7 H, COC*H*NHBoc), 4.12 (m, 7 H, *H*-4), 3.76-3.67 (m, 7 H, SCH_2C*H*NHCO), 3.64-3.55 (m, 7 H, SCH_2C*H*NHCO), 3.48-3.28 (m, 28 H, CH_2NHCNH, *H*-6a and *H*-6b), 3.17 (s, 14 H, CH_2 $_{Pbf}$), 3.07-2-96 (m, 14H,

SCH_2), 2.77 (s, 21 H, CH_3 Pbf), 2.71 (s, 21 H, CH_3 Pbf), 2.57–2.31 (m, 28 H, OCOCH_2), 2.26 (s, 21 H, CH_3 Pbf), 2.03-1.93 (m, 7 H, COCH*H*), 1.91–1.71 (m, 35 H, COCH*H*, COCHCH_2CH_2, OCOCH_2CH_2), 1.63 (bs, 42 H, C(CH_3)$_2$), 1.61 (bs, 63 H, C(CH_3)$_3$), 1.59–1.44 (m, 28 H, $CH_2CH_2CH_3$), 1.20–1.05 (m, 42 H, CH_2CH_3).

^{13}C NMR (75.5 MHz, $CDCl_3$) δ 174.9, 174.7 and 173.5 (*C*=O), 159.9 (*C*=N), 158.1 (*C*=O), 157.7-118.4, (*C* Ar Pbf), 98.2 (*C*-1), 87.7 (*C*(CH_3)$_3$), 80.7 (*C*(CH_3)$_2$) 71.7 (*C*-4), 73.5 (*C*-5), 71.7 and 71.6 (*C*-2 and *C*-3), 55.8 (CO*C*HNHBoc), 44.1 (CH_2 Pbf), 41.5 (N=CNHCH_2), 40.5 (SCH_2CH_2NHCO), 35.1 and 35.0 (OCOCH_2 and *C*-6), 34.1 (CH_2S), 32.6 and 32.5 ($CH_2CH_2CH_3$), 30.9 (COCHCH_2), 28.9 and 28.8 (C(CH_3)$_2$ and C(CH_3)$_3$), 27.1 (COCHCH_2CH_2), 25.6 (OCOCH_2CH_2), 23.6 (CH_2CH_3), 19.8 (CH_3 Pbf), 18.6 (CH_3 Pbf), 14.6 (CH_2CH_3), 12.7 (CH_3 Pbf).

ESI-MS (m/z): $[M + 2Na]^{2+}$ calcd for $C_{308}H_{497}N_{35}O_{84}S_{14}$ 3262.0, found 3263.3.

Synthesis of Heptakis{6-[2-(L-arginine-*N*-amide)ethylthio]-6-deoxy-2,3-di-*O*-hexanoyl}cyclomaltoheptaose, tetradecahydrochloride (67)

A solution of **66** (37.5 mg, 9 µmol) in TFA-TIS-H_2O (95 : 2.5 : 2.5, 4 mL) was stirred at rt for 1 h. a The solvent was removed under reduced pressure and coevaporated several times with water. The residue was dissolved in a 0.1 M HCl solution and freeze-dried to obtain the product as hydrochloridesalt in quantitative yield (40.7 mg).

$[\alpha]_D$ = +9.8 (*c* 1 in MeOH).

^{1}H NMR (500 MHz, DMSO-d_6): δ 8.96 (bs, 7 H, N*H*) 8.51-8.29 (m, 14 H, N*H*), 7.44-7.09 (m, 21 H, N*H*), 5.25 (t, $J_{2,3} = J_{3,4}$ = 9.04 Hz, 7 H, *H*-3), 5.09 (bs, 7 H, *H*-1), 4.69 (bd, 7 H, *H*-2), 4.17 – 4.07 (m, 14 H, *H*-5), 4.07 – 3.99 (m, 14 H, COC*H*NH_3^+ and *H*-4), 3.43-3.32 (bs, 14 H, SCH_2CH_2NHCO), 3.29-3.19 (bs, 14 H, CH_2NHCNH), 3.15-2.99 (m, 14 H, *H*-6a and *H*-6b), 2.87-2.66 (m, 14 H, SCH_2), 2.44-2.29 (m, 28 H, OCOCH_2), 1.92–1.80 (m, 14 H, COCHCH_2), 1.70–1.58 (m, 14 H, COCHCH_2CH_2), 1.58-1.45 (m, 28 H, OCOCH_2CH_2), 1.35-1.19 (bs, 56 H, $CH_2CH_2CH_3$), 0.93–0.78 (m, 42 H, CH_2CH_3).

^{13}C NMR (125.7 MHz, DMSO-d_6) δ 175.7, 174.5 and 177.8 (*C*=O), 161.2 (C=N), 99.4 (*C*-1), 81.3 (*C*-4), 74.3 (*C*-5), 73.1 (*C*-2), 72.9 (*C*-3), 55.1 (CO*C*HNH_3^+), 43.7 (N=CNHCH_2), 42.4 (SCH_2CH_2NHCO), 36.5 and 36.3 (OCOCH_2 and *C*-6), 35.3 (CH_2S), 33.9 and 33.8 ($CH_2CH_2CH_3$), 31.4 (COCHCH_2), 27.2 (COCHCH_2CH_2), 26.9 and 26.8 (OCOCH_2CH_2), 24.89 and 24.85 (CH_2CH_3), 16.65 and 16.63 (CH_2CH_3).

MALDI-MS (m/z): $[M + H-14HCl]^+$ calcd for $C_{182}H_{343}Cl_{14}N_{35}O_{49}S_7$ 4016.5,found 4017.6

Synthesis of Heptakis{6-[2-(di-*tert*-butoxycarbonyl-guanidine)ethylthio]-6-deoxy-2,3-di-*O*-hexanoyl}cyclomaltoheptaose (68)

To a solution of **IV** (50 mg, 15 μmol) in dry DCM (5mL) under N_2 atmosphere, triethylamine (61 μL, 440 μmol, 28 eq) and *N-N'*-di-*tert*-butoxycarbonyl-*N''*-triflylguanidine (86 mg, 220 μmol, 14 eq) were added. The reaction mixture was stirred overnight. The reaction mixture was washed with a solution of 2M $KHSO_4$ and the organic layer was dried ($MgSO_4$), filtrated and concentrated. The residue was purified by flash chromatography (gradient from AcOEt/cyclohexane 1:4 to CH_2Cl_2). The pure product **68** was obtained as white solid in quantitative yield (67 mg).

$[\alpha]_D$ = +30.7 (*c* 1 in MeOH).

^{1}H NMR (300 MHz, CD_3OD): δ 5.33 (t, 7 H, $J_{2,3} = J_{3,4}$ = 8.6 Hz, *H*-3), 5.14 (d, $J_{1,2}$ = 3.7 Hz, 7 H, *H*-1), 4.88 (dd, 7 H, *H*-2), 4.36–4.27 (m, 7 H, *H*-5), 3.93 (t, 7 H, *H*-4), 3.78–3.53 (m, 14 H, *H*-6a and *H*-6b), 3.16 (bs, 14 H, CH_2NH), 2.98–2.85 (m, 14 H, SCH_2), 2.51–2.14 (m, 28 H, OCOCH_2), 1.74–1.56 (m, 28 H, $OCOCH_2CH_2$), 1.57-1.43 (bs, 126 H, $C(CH_3)_3$), 1.45–1.24 (m, 56 H, $CH_2CH_2CH_3$), 1.02-0.85 (m, 42 H, CH_2CH_3).

^{13}C NMR (75.5 MHz, CD_3OD) δ 174.5 and 173.4 (*C*=O), 164.6 (*C*=N), 157.3 and 154.2 (*C*=O), 98.6 (*C*-1), 84.5 (*C*-4), 80.4 ($C(CH_3)_3$), 73.0 (*C*-5), 71.9 (*C*-3), 71.5 (*C*-2), 41.4 (*C*-6), 35.2 and 35.0 ($OCOCH_2$ and N=CNHCH_2), 34.1 (CH_2S), 32.6 and 32.5 ($CH_2CH_2CH_3$), 28.9 and 28.6 ($C(CH_3)_3$), 25.6 ($OCOCH_2CH_2$), 23.6 and 23.5 (CH_2CH_3), 14.5 (CH_2CH_3).

ESI-MS (m/z): $[M + 2Na]^{2+}$ calcd for $C_{217}H_{371}N_{21}O_{70}S_7$ 2330.7, found 2332.2, $[M + 3Na]^{3+}$ calcd 1561.5, found 1562.7.

Synthesis of Heptakis{6-[(2-guanidine-ethyl)thio]-6-deoxy-2,3-di-*O*-hexanoyl}-cyclomaltoheptaose, heptahydrochloride (69)

A solution of **68** (60 mg, 13 μmol) in DCM-TFA (1:1, 2 mL) was stirred at rt for 3 h the residue was eliminated under reduced pressure and coevaporated several times with water. The residue was disolved in a 0.1 M HCl solution and freeze-dried to yield the product as hydrochloride salt in quantitative yield (45 mg).

$[\alpha]_D$ = + 68.2 (*c* 1 in DMF).

^{1}H NMR (300 MHz, CD_3OD): δ 5.39 (t, 7 H, $J_{2,3} = J_{3,4}$ = 9.4 Hz, *H*-3), 5.18 (d, $J_{1,2}$ = 3.5 Hz, 7 H, *H*-1), 4.86 (m, 7 H, *H*-2), 4.16–4.07 (m, 7 H, *H*-5), 3.96 (t, 7 H, *H*-4), 6.53 (t, 14 H, CH_2NH), 3.20-3.11 (m, 14 H. *H*-6a and *H*-6b), 2.97–2.85 (m, 14 H, SCH_2), 2.55–2.21 (m,

28 H, OCOCH_2), 1.72–1.55 (m, 28 H, OCOCH_2CH_2), 1.41-1.26 (m, CH_2CH_2CH_3), 0.99-0.87 (m, 42 H, CH_2CH_3).
^{13}C NMR (125.7 MHz, DMSO-d$_6$) δ 172.2 and 171.2 (C=O), 156.8 (C=N), 96.1 (*C*-1), 78.0 (*C*-4), 71.2 (*C*-5), 69.7 (*C*-3), 69.8 (*C*-2), 45.5 (*C*-6), 40.6 (N=CNHCH_2), 33.1 and 32.9 (OCO*C*H_2) 32.1 (*C*H_2S), 30.6 and 30.4 (*C*H_2CH_2CH_3), 23.6 and 23.5 (OCOCH_2*C*H_2), 21.5 and 21.5 (*C*H_2CH_3), 13.3 and 13.2 (CH_2*C*H_3).
ESI-MS (m/z): [M + 2H-7HCl]$^{2+}$ calcd for $C_{147}H_{266}Cl_7N_{21}O_{42}S_7$ 1610.6, found 1611.8.

Preparation of complexes composed of calixarene or CD derivatives and plasmid. The plasmid pGL4.13 used for the preparation of the DNA complexes and for transfection assay, is a plasmid of 4642 bp (base pairs). The quantities of compound used were calculated according to the desired DNA concentration of 0.1 mg mL^{-1} (303 μM phosphate), the N/P ratio, the molar weight, and the number of protonable nitrogen atoms in the selected calixarene or CD derivative or cationic polymer (bPEI, 25 kDa). Experiments were performed for N/P 1,2, 5, 10 and 20. For the preparation of the DNA complexes from CD derivatives and PEI, DNA was diluted in HEPES (20 mM, pH 7.4) to a final concentration of 303 μM, then the desired amount of CD derivative was added from 10 or 20 mM stock solution (DMSO) and bPEI (25 kDa) was added from a 0.1 M stock solution (H_2O). The preparation was vortexed for 2 h and used for characterization or transfection experiments.

Electrophoresis mobility shift assay (EMSA). Each calixarene derivative/DNA sample or CD derivative/DNA sample (20 μL, 0.4 μg of plasmid) was submitted to electrophoresis for about 30 min under 150 V through a 0.8 % agarose gel in 1 × tris(hydroxymethyl)aminomethane (Tris)/acetate/ethylenediaminetetraacetic acid (EDTA) (TAE)buffer and stained by spreading an ethidium bromide (Sigma) solution in TAE buffer (20 μL ethidium bromide of a 10 mg mL^{-1} solution in 200 mL TAE). The DNA was then visualized after photographing using an UV transilluminator.

Measurement of the Size of the Complexes by Dynamic Light Scattering (DLS) and of the ζ-Potential. The average sizes of the calix-pDNA complexes were measured using a Zetasizer nano with the following specification: sampling time, automatic; number of

measurements, 3 per sample; medium viscosity, 1.054 cP; refractive index, 1.33; scattering angle, 173°; λ = 633 nm; temperature, 25°C. Data were analyzed using the multimodal number distribution software included in the instrument. Results are given as volume distribution of the major population by the mean diameter with its standard deviation. Zeta-potential measurements were made using the same apparatus with "mixed-mode measurement" phase analysis light scattering (M3-PALS). M3-PALS consists of both slow field reversal and fast field reversal measurements, hence the name "mixed-mode measurement"; it improves accuracy and resolution. The following specifications were applied: sampling time, automatic; number of measurements, 3 per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25°C. Before each series of experiments, the performance of the instruments was checked with either a 90 nm monodisperse latex beads (Coulter) for DLS or with DTS 50 standard solution (Malvern) for ζ potentials.

Transmission Electron Microscopy (TEM). Formvar-carboncoated grids were placed on top of small drops of the calix-pDNA complex (HEPES 20 mM, pH 7.4, DNA 60 μM phosphate). After 1-3 min of contact, grids were negatively stained with a few drops of 1% aqueous solution of uranyl acetate. The grids were then dried and observed using an electron microscope working under standard conditions.

Cell culture and transient transfection assay. Twenty-four hours before transfection, COS-7 cells were grown at a density of 2×10^4 cells per well in 96-well plates in Dulbelcco modified Eagle culture medium (DMEM; Gibco-BRL) containing 10% fetal calf serum (FCS; Sigma), 100 mg mL^{-1} streptomycin for COS-7cells, in a wet (37°C) and 5% CO_2/95% air atmosphere. The above described complexes and PEI:pDNA polyplexes were diluted to 100mL in DMEM or in DMEM supplemented with 10 % FCS so as to have 0.5 mg of pDNA in the well (15 mm phosphate). The culture medium was removed and replaced by these 100 μL of the complexes. After 4 and 24 h, DMEM (50 and 100 μL) supplemented with 30 % and 10 % FCS, respectively, were added. After 48 h, the transfection was stopped, the culture medium was discarded, and the cells were washed twice with PBS (100 μL) and lysed with lysis buffer (50 mL; Promega, Charbonniéres, France). The lysates were frozen at -80°C before the analysis of luciferase activity. This measurement was performed using a luminometer (GENIOS PRO, Tecan France S.A.) in

dynamic mode, for 10 s on the lysis mixture (20 μL) and using the "luciferase" determination system (Promega) in 96-well plates. The total protein concentration per well was determined by the BCA test (Pierce, Montlucon, France). Luciferase activity was calculated as femtograms (fg) of luciferase per mg of protein. The percentage of cell viability was calculated as the ratio of the total protein amount per well of the transfected cells relative to that measured for untreated cells $\times 100$. The data were calculated from three or four repetitions.

RD-4 [human Rhabdomyosarcoma cell line (obtained from David Derse, National Cancer Institute, Frederick, Maryland)] were grown in EMEM medium containing NEAA, 10% FBS, 2 mM l-glutamine, 100 IU/mL penicillin and 100 μg/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 μg 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 μM. 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.

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