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Cyclodextrin- and calixarene-based polycationic amphiphiles as gene delivery systems: a structure—activity relationship study†

Laura Gallego-Yerga, a Michela Lomazzi, b Valentina Franceschi, c Francesco Sansone,*b Carmen Ortiz Mellet,*a Gaetano Donofrio,*c Alessandro Casnatib and José M. García Fernández*d

Multi-head/multi-tail facial amphiphiles built on cyclodextrin (CD) and calixarene (CA) scaffolds are paradigmatic examples of monodisperse gene delivery systems. The possibility to precisely control the architectural features at the molecular level offers unprecedented opportunities for conducting structureactivity relationship studies. A major requirement for those channels is the design of a sufficiently diverse ensemble of compounds for parallel evaluation of their capabilities to condense DNA into transfection nanoparticles where the gene material is protected from the environment. Here we have undertaken the preparation of an oriented library of β -cyclodextrin (β CD) and calix[4]arene (CA₄) vectors with facial amphiphilic character designed to ascertain the effect of the cationic head nature (aminothiourea-, arginine- or quanidine-type groups) and the macrocyclic platform on the abilities to complex plasmid DNA (pDNA) and in the efficiency of the resulting nanocomplexes to transfect cells in vitro. The hydrophobic domain, formed by hexanoyl or hexyl chains, remains constant in each series, matching the overall structure found to be optimal in previous studies. DLS, TEM and AFM data support that all the compounds selfassemble in the presence of pDNA through a process that involves initially electrostatic interactions followed by formation of βCD or CA₄ bilayers between the oligonucleotide filaments. Spherical transfectious nanoparticles that are monomolecular in DNA are thus obtained. Evaluation in epithelial COS-7 and human rhabdomyosarcoma RD-4 cells evidenced the importance of having primary amino groups in the vector to warrant high levels of transfection, probably because of their buffering capacity. The results indicate that the optimal cationic head depends on the macrocyclic core, aminothiourea groups being preferred in the βCD series and arginine groups in the CA₄ series. Whereas the transfection efficiency relationships remain essentially unchanged within each series, irrespective of the cell type, the optimal platform (βD or CA₄) strongly depends on the cell type. The results illustrate the potential of monodisperse vector prototypes and diversity-oriented strategies on identifying the optimal candidates for gene therapy applications.

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Introduction

Gene therapy represents a potent tool for the therapeutic treatment of a broad range of genetic and acquired diseases. After some controversial failures, the last few years have witnessed the first really successful applications of this technique, opening the possibility to fight pathologies like severe immunodeficiencies, the Wiskott-Aldrich syndrome, beta-thalassaemia, haemophilia, adrenoleukodystrophy and several types of cancer, with an ever increasing number of clinical trials under way.2 In most cases, the delivery of genetic material aimed at the correction of defects in the patients' cell genome is performed by using viruses as vectors in the context of an "ex vivo" strategy. Adenoviruses and retroviruses are properly modified to

^aDept. Química Orgánica, Facultad de Química, Universidad de Sevilla, c/Profesor García González 1, 41012 Sevilla, Spain. E-mail: mellet@us.es; Fax: (+34) 954624960

^bDipartimento di Chimica, Università degli Studi di Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy. E-mail: francesco.sansone@unipr.it;

^cDipartimento di Scienze Medico-Veterinarie, Università degli Studi di Parma, Via del Taglio 10, 43126 Parma, Italy. E-mail: gaetano.donofrio@unipr.it; Fax: (+39) 0521902672

^dInstituto de Investigaciones Químicas (IIQ), CSIC - Universidad de Sevilla, Avda. Américo Vespucio 49, 41092 Sevilla, Spain. E-mail: jogarcia@iiq.csic.es;

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eliminate their infectiousness and to incorporate the therapeutic sequences. Their efficiency in transfecting cells, due to their own nature, does the rest. Yet, the use of viral vectors is still accompanied by some not negligible risks, such as violent adverse immune responses and genotoxicity,3 and limits like complicate and expensive preparation processes, production in rather scarce quantities, restricted dimensions of the nucleic acid filaments that can be transported or possible compromised bioavailability because of their large molecular size. These not vet resolved problems fuelled the research for alternatives that materialized, in the years, in the development of non-viral gene vectors based initially on cationic lipids and then on cationic polymers, dendrimers and nanoparticles.⁴ Among them, some have shown relevant efficiency and relatively low toxicity and are widely used in transfection protocols. Nevertheless, although some outstanding examples are on record,5 the systems proposed so far cannot really replace viruses. For these reasons, the efforts in the development of new molecules and formulations able to deliver nucleic acids into cells with increasing efficiency and safety as of today are still ongoing.

A main difficulty in non-viral gene carrier optimization strategies is the multicomponent or polydisperse nature and random conformational properties of most of the systems that are currently available, which handicaps establishing reliable relationships between chemical structure and transfection efficiency. The design of monodisperse, molecularly well-defined gene vector prototypes, while more challenging, offers unprecedented opportunities in this respect. In recent years, cyclodextrins (CDs) and calixarenes (CAs) have been proposed

as central frameworks allowing the controlled incorporation of nucleic acid complexation elements. While some positive results using polycationic hydrophilic derivatives have been reported, we and others have shown that endowing the vector architecture with facial amphiphilicity, by installing segregated clusters of cationizable and hydrophobic groups at opposite rims in the macrocyclic cores, significantly improves the nucleic acid condensation abilities and the transfection efficiency of the resulting supramolecular nanocomplexes (Fig. 1).

Structure-activity relationship studies independently conducted in both series of compounds allowed identification of some favourable structural features. Thus, β-cyclodextrin (βCD)-based architectures were generally superior to αCD or γCD derivatives¹³ and dispositions having the cationic heads at the primary face and the lipophilic tails at the secondary hydroxyls (skirt-type arrangement)¹⁴ performed better than analogues exhibiting the reversal orientation. 15 In the polycationic amphiphilic CA family, the reports point to the supremacy of the calix[4] arene (CA₄) core in the cone conformation with the protonable groups at the upper rim. 16 The combination of thiourea and amine groups at the cationic domain was found optimal for βCD derivatives, 17 whereas arginine clustering imparted the highest DNA delivery efficiency in the CA₄ series. 16 Copper(1)-catalyzed azide-alkyne cycloaddition (CuAAC) amine-isothiocyanate coupling and implemented for "click" multiconjugation, both ligation chemistries warranting full homogeneity. Interestingly, in both types of macrocycles incorporation of linear six-carbon chains

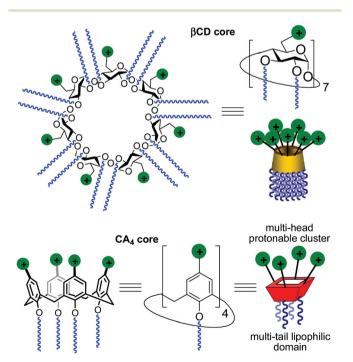


Fig. 1 Schematic representation of the amphiphilic polycationic β -cyclodextrin (β CD) and calix[4]arene (CA₄) gene vector prototypes in the optimal skirt-type and upper-rim protonable cone arrangements, respectively.

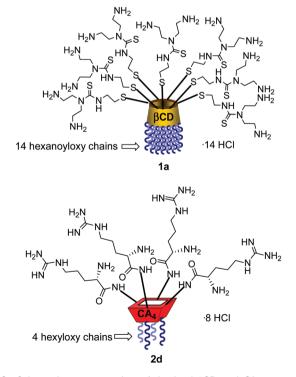


Fig. 2 Schematic representation of the lead βCD and CA_4 vectors 1a and 2d.

into the hydrophobic domain provided the best results. The tetradecacationic βCD derivative 1a, displaying a dendritic presentation of primary amino groups and fourteen hexanoyl chains at the secondary hydroxyls, and the tetraarginine-CA₄ conjugate 2d, bearing four hexyl ether substituents, were identified as lead compounds within each category (Fig. 2).

In our ongoing efforts to develop CD and CA-based artificial viruses for drug and gene delivery, 16 evaluating the properties of structurally related series of CD and CA vectors in the same cell systems was highly sought. By combining our expertise on the chemistry and supramolecular properties of both archetypes we are in the position to underpin whether or not the

BCD scaffold

CA₄ scaffold

Fig. 3 Structures of the polycationic amphiphilic βCD and CA₄ derivatives synthesized and evaluated in this work.

2f. connector C

 $R = s^{\xi} \underbrace{N \xrightarrow{NH} \cdot HCI}_{NH_2}$

macrocyclic platform determines the optimal architectural requirements for gene therapy applications.

Herein we report the synthesis of a collection of polycationic amphiphilic βCD (1a-f) and CA₄ (2a-f) homogeneously functionalized with the same polar heads at the narrower and upper rim, respectively, and distinguished by the cationic species introduced: ammonium, arginine and guanidinium groups (sub-libraries I-III, Fig. 3). The lipophilic domains, in contrast, were maintained unvaried within a series, namely hexanoyl ester groups for the BCD derivatives and hexyl ether chains for the CA₄ counterparts. The relative orientation of the multi-head and multi-tail domains thus remains constant within each family. A parallel evaluation of the ability of these regioselectively functionalized macrocycles to condense pDNA into discrete cationic nanoparticles and of their in vitro transfection capabilities toward the COS-7 African green monkey kidney epithelial and RD-4 human rhabdomyosarcoma cell lines have been conducted.

Results and discussion

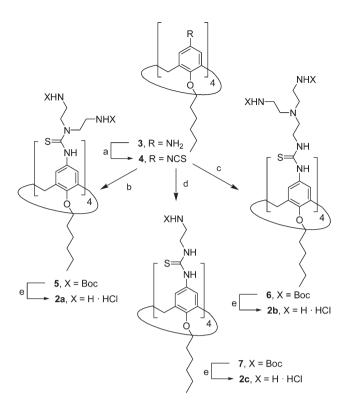
Synthesis

The known polycationic amphiphilic β CD derivatives **1a-c**, bearing aminothiourea head groups, have been previously shown to exhibit promising transfection capabilities both in vitro^{9b} and, in the case of **1a**, also in vivo. ^{14d,f} For this reason, they were selected as the βCD representatives in sublibrary I for the purpose of this study. The synthesis of the new analogues 2a-c (Scheme 1), incorporating the same cationic heads in the CA₄ series, started from the known 5,11,17,23tetraamino-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene 3, 10b which was transformed into the pivotal tetraisothiocyanate 4 using an excess of the isothiocyanation reagent carbon disulfide/bis-(tert-butyl) carbonate. 18 Reaction of 4 with bis(2tert-butyloxycarbonylaminoethyl) amine $(\rightarrow 5)$, bis(2-tertbutyloxycarbonylaminoethyl) 2-aminoethyl amine $(\rightarrow 6)$ and 2*tert*-butyloxycarbonylaminoethylamine (\rightarrow 7), in the presence of triethylamine, and the removal of the carbamate protecting group in the thiourea adducts by trifluoroacetic acid (TFA)-promoted hydrolysis in the presence of triethylsilane (TES) afforded the target CA₄ facial amphiphiles 2a-c in high yield. The final compounds were isolated as the corresponding octa-(2a and 2b) or tetra-hydrochloride salts (2c) after repeated dissolution/evaporation cycles from methanolic HCl and final lyophilisation (Scheme 1).

The lead calixarene derivative 2d was the reference compound that inspired sub-library II. The homologous-βCD representative 1d was obtained by hepta-amidation of the per-(C-6)cysteaminyl-per-(O-2,O-3)-hexanoyl βCD precursor 8, accessible in only three steps from βCD , βb , 19 with the commercial protected arginine derivative 9 $(\rightarrow 10)$ and final deprotection (Scheme 2).

The presence of the cysteaminyl connector releases the steric constrain at the primary BCD rim and warrants homogeneous multiconjugation even for hyperbranched architec-

1f, connector A



Scheme 1 Synthesis of polyaminothioureido CA₄ derivatives **2a–c**. Reagents and conditions: (a) (i) CS₂, Et₃N, EtOH, rt, 2 h; (ii) Boc₂O, DMAP, EtOH, 0 °C \rightarrow rt, 2 h, 88%; (b) NH(CH₂CH₂NHBoc)₂, Et₃N, DCM, rt, 12 h, quantitative; (c) H₂NCH₂CH₂N(CH₂CH₂NHBoc)₂, Et₃N, DCM, rt, 12 h, 99%; (d) NH₂CH₂CH₂NHBoc, Et₃N, DCM, rt, 1.2 h, 99%; (e) (i) TFA–TES–DCM, 5 min, rt; (ii) HCl, 99%.

Scheme 2 Synthesis of amide-linked polyarginine β CD derivative 1d. Reagents and conditions: (a) HBTU, DIPEA, DCM, rt, 12 h, 68%; (b) (i) 95:2.5:2.5 TFA-TIS-DCM, rt, 1 h; (ii) 0.1 M HCl, quantitative. Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl. HBTU: o-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate. DIPEA: Diisopropyl ethyl amine. TIS: triisopropylsilane.

tures.20 Sub-library II has been further enriched by synthesizing the corresponding 1,2,3-triazol-linked isosters 1e and 2e (Scheme 3). Whereas the resulting triazole segment is considered to be isosteric of the amide functionality, it imparts a higher rigidity thereby influencing the conformational properties. Moreover, the triazole moiety might actively participate in DNA complexation and release by behaving as the hydrogen bonding acceptor, and/or intercalating DNA, thereby affecting transfection efficiency.²¹ The key "click" multiconjugation step involved the copper(1)-catalyzed azide-alkyne cycloaddition reaction (CuAAC)²² of the known hexanoylated heptaazide β CD 12^{9c} or the hexyloxy tetraazide CA_4 derivative 18 with the propargyl-armed arginine derivative 11 (→13 and 19, respectively). Alkyne 11 was prepared by standard amide coupling of the commercial protected amino acid 9 with propargylamine, whereas installation of the azidomethyl substituents onto the upper rim of the CA₄ scaffold, to access tetraazide 18, was accomplished in four-steps from the tetrahexyl ether precursor 14²³ through a reaction sequence involving formulation (\rightarrow 15), reduction $(\rightarrow 16)$, replacement of the resulting primary hydroxyl groups into chloro groups $(\rightarrow 17)$ and nucleophilic displacement of the latter by an azide anion. Acid-promoted removal of the carbamate and sulfonyl protecting groups in the triazole adducts 13 and 19 afforded the target polycationic amphiphilic clicked clusters 1e and 2e (Scheme 3).

In order to evaluate possible synergies between the amino and the guanidino groups of polyarginine facial amphiphiles in DNA complexation and delivery, the inclusion in our study of the polyguanidine analogues 1f and 2f, grouped in sublibrary III, as controls lacking any amine functionality was considered appropriate. Their synthesis was achieved by guanidinylation of the β CD heptaamine 8 and the CA_4 tetraamine derivative 20, obtained by the reduction of tetraazide 18 (see Experimental), respectively, with bis-Boc-triflyl guanidine and the final hydrolysis of the carbamate protecting groups (Scheme 4).

pDNA complexation abilities and nanocomplex characterization

The ability of the βCD- and CA₄-based polycationic amphiphiles 1a-f and 2a-f to complex and protect DNA was first examined by electrophoresis mobility shift assays (EMSA) and by determining their capability to prevent intercalation of GelRedTM (Biotium) used to stain the DNA filaments. The luciferase-encoding plasmid DNA (pDNA) pTG11236 (pCMV-SV40luciferase-SV40pA, 5739 base pairs) was employed in this experimental setting at protonable nitrogen/phosphorous (N/P) ratios 1, 2, 5, 10 and 20. The data (Fig. 4) evidenced that all the tested derivatives were able to fully complex and protect pDNA at N/P > 2 as indicated by the absence of free mobile plasmid in the corresponding lanes. As a general trend, sublibrary I compounds 1a,b or 2a,b, displaying a dendritic presentation of primary amino groups, were more efficient at condensing pDNA than the linear aminothioureido derivatives 1c or 2c. In sub-library II, amide connectors (1d or 2d) performed better than the triazol linkers (1e or 2e). Overall, βCD-

Paper

Scheme 3 Synthesis of triazol-linked polyarginine βCD and CA₄ "clicked" clusters 1e and 2e. Reagents and conditions: (a) Cul-P(OEt)3, DIPEA, acetone, reflux, 48 h, 99% (for 13) or 24 h, 71% (for 19); (b) (i) 95: 2.5: 2.5 TFA-TIS-H₂O, rt, 1 h; (ii) 0.1. M HCl, quantitative; (c) (i) hexamethylenetetramine, TFA, reflux, 2 d; (ii) HCl, 3 h, 96%; (d) NaBH₄, EtOH, 0 °C \rightarrow rt, 18 h, 86%; (e) SOCl₂, DCM, 4 h, rt, 95%; (f) NaN₃, DMF, 16 h, 40 °C, 95%.

scaffolded polycations achieved full neutralization of pDNA and fully blocked GelRedTM intercalation at lower N/P ratios as compared with the respective CA₄ counterparts.

Nanocondensates formulated with 1a-f or 2a-f and the pTG11236 plasmid at N/P 5 and 10, for which EMSA indicated full pDNA complexation and protection, were characterized by dynamic light scattering (DLS) to determine the average hydrodynamic size and by mixed-mode measurement-phase analysis light scattering (M3-PALS) to measure the ζ -potential (Fig. 5). Apart from the two guanidino macrocycles 1f and 2f at N/P = 5that formed aggregates of 160 and 175 nm, respectively, all the other compounds gave rise to supramolecular species of

Scheme 4 Synthesis of polyguanidino βCD and CA₄ derivatives 1f and 2f. Reagents and conditions: (a) Et₃N, DCM, rt, 12 h, 99% (for 21) or 48 h, 91% (for 22); (b) (i) 1:1 TFA-DCM, rt, 3 h; (ii) 0.1 M HCl, 99%; (c) TES, dioxane, 0.1 M HCl, rt, 24 h, quantitative.

similar size in the range of 80-120 nm hydrodynamic diameter with a rather low polydispersity. The observed size decrease on going from N/P = 5 to N/P = 10 for most of the nanoparticles, in spite of the equivalent results obtained in EMSA, is indicative of different compaction states. Accordingly, the ζ-potential, which is positive for both N/P values, was higher at N/P = 10 (Fig. 5 and Tables S1 and S2 in the ESI†). No significant changes in size or ζ-potential were observed at higher N/P ratios (data not shown).

Transmission electron microscopy (TEM) of the nanocomplexes formulated at N/P 10 confirmed their relatively small size and low polydispersity (Fig. 6). As previously observed for amphiphilic βCD aminothiourea polycations, ^{13,14b} a snake-like ultra-thin structure revealing an alternating arrangement of high (dark) and low (light) electron density regions was apparent, independently of the sub-library or the macrocyclic scaffold. The dark regions account for the DNA chain, whereas the lighter regions probably correspond to bilayers of the facial amphiphile. This scenario strongly suggests a compaction mechanism involving the polynucleotide chain acting as a template for the alignment of the cationic clusters, a process driven initially by electrostatic interactions. Zipping of the bilayers with simultaneous expulsion of hydration water must then take place through hydrophobic contacts implying the hydrophobic domains, leading to the final nanocondensates (Fig. 6).

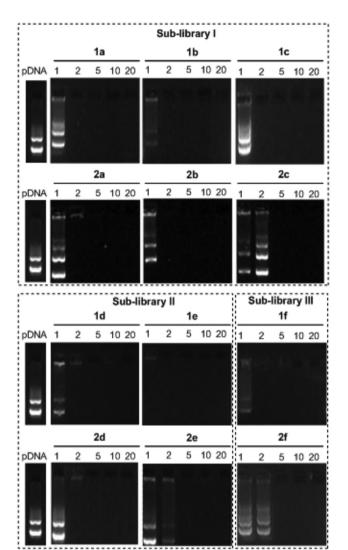


Fig. 4 Electrophoresis mobility shift assays (EMSA) for pDNA complexes formulated with 1a-f and 2a-f at different N/P ratios (1, 2, 5, 10 and 20). GelRedTM was used as the staining reagent. Naked pDNA was used as the negative control.

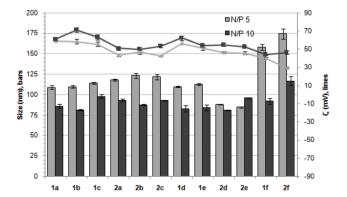


Fig. 5 Hydrodynamic diameter (bars, nm) and ζ potential (lines, mV) of complexes pDNA-polycationic amphiphilic derivatives determined by DLS and M3-PALs analysis.

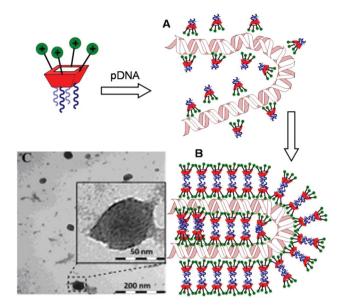


Fig. 6 Schematic representation of the proposed mechanism for pDNA complexation by polycationic amphiphilic macrocycles (the general cartoon for CA_4 derivatives in Fig. 1 has been used) involving electrostatically-driven templating (A) and bilayer zipping/nanocondensation (B). The TEM micrograph corresponding to the nanocomplexes formulated with compound 2d at N/P 10, is also presented (C). The typical snake-like ultra-thin structure can be appreciated in the insert.

Atomic force microscopy (AFM) further confirmed the size, spherical shape and monomolecular DNA character of the nanocomplexes formulated with **1a–f** or **2a–f**, irrespective of the nature of the protonable groups or the scaffold. Most interestingly, this technique allowed the monitoring of DNA compaction by increasing concentrations of the vector. The green fluorescence protein (GFP)-encoding plasmid pEGFP-C1 (4731 bps) was used for this purpose. As an example, Fig. 7 shows the images obtained for sub-library III polyguanidine derivatives **1f** and **2f**. At N/P 0.5 complexation is incomplete and free pDNA filaments of about 0.5 μ m in length can be observed together with partially shrunk plasmids. At N/P 5 all DNA molecules appear as individual nanocondensates.

In vitro transfection of COS-7 cells

The β CD and CA₄:pDNA nanocomplexes (CDplexes and calixplexes) obtained by formulation of **1a-f** or **2a-f** and the luciferase-encoding plasmid pTG11236 at N/P 5 and 10, were first tested in gene delivery assays towards the COS-7 cell line. For comparative purposes, naked pDNA and polyplexes formulated with commercial branched polyethyleneimine (bPEI) were used as negative and positive controls, respectively. The suitability of the vectors for potential systemic applications was assessed by additionally performing experiments in the presence of 10% serum. The total amount of luciferase expressed was normalized to the total amount of proteins produced by untreated cells. Transfection efficiencies are thus presented as logarithm of picograms of luciferase with respect to the number of milligrams of total proteins (Fig. 8).

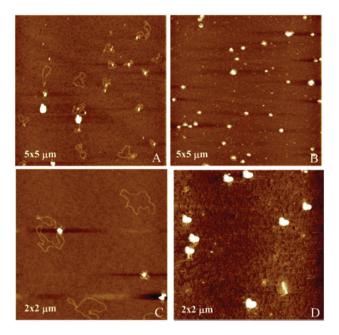


Fig. 7 AFM images recorded in tapping mode on air showing the effects induced on EGFP-C1 plasmid DNA folding by incubation with polyguanidino βCD and CA₄ derivatives 1f and 2f at N/P 0.5 (A and C) or N/P 5 (B and D).

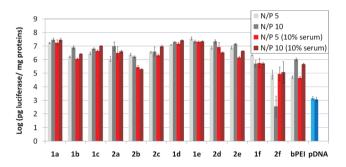


Fig. 8 In vitro transfection efficiency in COS-7 cells for CDplexes and calixplexes formulated with 1a-f and 2a-f and pTG11236 plasmid at N/P 5 and 10, in the absence or in the presence of serum (10%). Polyplexes formulated with branched PEI (bPEI) and naked pDNA in the absence (light blue) and presence (dark blue) of 10% serum were included as positive and negative controls, respectively.

Barring the guanidine CA₄ cluster 2f, all the βCD:pDNA and CA4:pDNA formulations showed transfection efficiencies that compare favourably with the results obtained for bPEI polyplexes and all, without exception, exhibited more favorable cell viabilities (Fig. 9). Within sub-library I and III derivatives, βCDscaffolded vectors 1a-c and 1f proved superior to the homologous CA₄ partners 2a-c and 2f, but excepting for the latter pair, differences remained within one order of magnitude. In the group of vectors displaying arginine groups (sub-library II) the influence of the scaffold is less evident. On the other hand, in the CA₄ series the presence of amide connectors (compound 2d) was somehow more favourable as compared to triazol linkers (compound 2e). Indeed, compounds 1d, 1e and 2d were as efficient as the lead aminothiourea representative

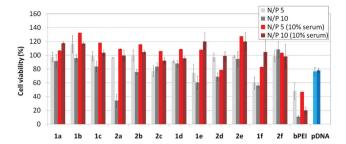


Fig. 9 Cell viability of COS-7 cells in the presence of CDplexes and calixplexes formulated with 1a-f and 2a-f and pTG11236 plasmid at N/P 5 and 10, in the absence or in the presence of serum (10%). Data for polyplexes formulated with branched PEI (bPEI) and naked pDNA in the absence (light blue) and presence (dark blue) of 10% serum are also included.

1a and similarly preserved the transfection capabilities in serum-containing medium. The most striking observation is the dramatic drop in transfection efficiency on comparing sublibraries II and III. The compounds bearing the simple guanidinium units showed by far the lowest transfection efficacy among all the synthesized compounds. This result suggests that the presence in the vectors of nitrogen atoms with the ability to reversibly shift from protonated to neutral state in a physiological pH window is advantageous, probably by imparting buffering capabilities to the corresponding nanoparticles, thereby facilitating endosome escape through the so-called proton sponge mechanism.24

Transfection efficiency towards COS-7 cells was also determined using the GFP-encoding plasmid pEGFP-C1 and directly monitoring by fluorescence microscopy the expression of the GFP protein into the cells as a consequence of successful transfection. Rather than the amount of protein produced, this experiment aims at evaluating the proportion of cells that is effectively transfected by each polycationic cluster:pDNA formulation. Transfection efficiency is then reported as percentage of transfected cells (Fig. 11). Given that cytotoxicity has a strong impact in this assay, for each vector the concentration producing the best compromise between cell viability and transfection efficiency in the range of 1.25-10 µM, was used. At concentrations lower than those selected the cell viability was comparable or higher but the percentage of transfected cells was lower while at higher concentrations cytotoxicity was too high. Cell viabilities, determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (see the ESI† for Experimental details) at the concentrations studied in this experimental setting, are collected in Fig. 10. In addition to PEI-formulated polyplexes, lipoplexes formulated with commercial Lipofectamine® (LTX) were included as positive controls. Parallel experiments were also conducted in the presence of dioleoylphosphatidylethanolamine (DOPE), a commonly used transfection adjuvant²⁵ that has been previously found to improve the transfection efficiency of CA4-scaffolded polycationic clusters in some cases. 10a-c,16 Cells treated only with DOPE were then used as negative controls.

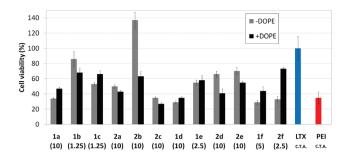


Fig. 10 Cell viability of COS-7 cells (MTT determination) in the presence of nanocomplexes formulated with the pEGFP-C1 and β CD or CA4 polycations 1a–f or 2a–f at their optimal concentration (μ M) with and without DOPE (1:2 vector–DOPE molar ratio). Data for lipoplexes formulated with Lipofectamine® (LTX), and polyplexes formulated with polyethyleneimine (PEI) are also shown. C.T.A.: commercial transfection agent.

Overall, the results obtained using this evaluation protocol (Fig. 11) qualitatively paralleled those previously obtained with the luciferase-encoding plasmid. Thus, the two guanidino derivatives 1f and 2f included in sub-library III exhibited the poorer transfection abilities among all vectors assayed, with a percentage of transfected cells close to zero. In the polyaminothiourea series (sub-library I) βCD-scaffolded derivatives 1ac proved superior to the corresponding CA₄ analogues 2a-c bearing identical cationic heads, whereas in sub-library II amide-linked derivatives 1d and 2d provided higher transfection efficiencies as compared with triazol-linked analogues 1e and 2e. In any case, only the nanocomplexes formulated with the two lead compounds in sublibraries I and II, namely the dendritic βCD aminothiourea 1a and the amide-linked CA₄ tetraarginine derivative 2d, rivalled lipoplexes formulated with LTX. Co-formulation with DOPE was detrimental in most cases with the notable exceptions of the calixplexes obtained from compounds 2b and 2e, for which a quite significant enhancement in the percentage of transfected COS-7 cells was observed.

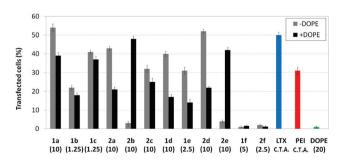


Fig. 11 In vitro transfection efficiency in COS-7 cells in the presence of nanocomplexes formulated with the pEGFP-C1 and β CD or CA₄ polycations 1a–f or 2a–f at their optimal concentration (μ M) with and without DOPE (1:2 vector–DOPE molar ratio). Data for lipoplexes formulated with Lipofectamine® (LTX), and polyplexes formulated with polyethylene-imine (PEI) are also shown. C.T.A.: commercial transfection agent.

In vitro transfection of the human rhabdomyosarcoma RD-4 cell line

Human rhabdomyosarcoma RD-4 cells are a kind of cancer cells of connective tissues. The difficulties of the treatment and the impossibility, in many cases, of removing the tumor make this cell line of high medical relevance for gene therapy. Moreover, it is a cell line which is particularly very difficult to transfect. The protocol based on the use of the pEGFP-C1 and fluorescence microscopy monitoring of the percentage of cells expressing GFP after treatment with the nanocomplexes formulated with the optimal concentration of each molecular vector, eventually co-formulated with DOPE, was applied. This choice is consistent with previous studies on the ability of calixplexes to mediate transfection in this particular cell line.¹⁶ The corresponding cell viability and transfection data are collected in Fig. 12 and 13, respectively. Formulations prepared with LTX and PEI were used as positive controls whereas parallel experiments with DOPE alone were conducted as a negative control.

Most of the nanocomplexes formulated with the β CD or CA₄ polycationic amphiphiles **1a–f** or **2a–f** led to GFP expression in the RD-4 cell line, but only the lead compound

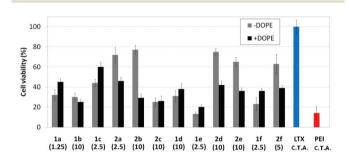


Fig. 12 Cell viability of RD-4 cells (MTT determination) in the presence of nanocomplexes formulated with the pEGFP-C1 and β CD or CA $_4$ polycations 1a-f or 2a-f at their optimal concentration (μ M) with and without DOPE (1:2 vector–DOPE molar ratio). Data for lipoplexes formulated with Lipofectamine® (LTX), and polyplexes formulated with polyethyleneimine (PEI) are also shown. C.T.A.: commercial transfection agent.

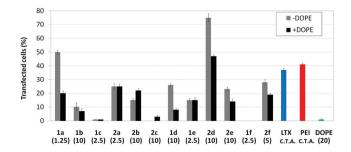


Fig. 13 In vitro transfection efficiency in RD-4 cells in the presence of nanocomplexes formulated with the pEGFP-C1 and β CD or CA₄ polycations 1a–f or 2a–f at their optimal concentration (μ M) with and without DOPE (1:2 vector–DOPE molar ratio). Data for lipoplexes formulated with Lipofectamine® (LTX), and polyplexes formulated with polyethyleneimine (PEI) are also shown. C.T.A.: commercial transfection agent.

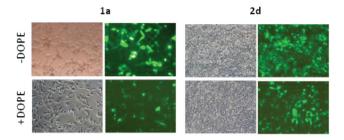


Fig. 14 Optical (left columns) and fluorescent (right columns) microscopy images of human RD-4 cells transfected (in green) upon treatment with EGFP-C1 pDNA (1 nM) formulated with polyaminothiourea βCD derivative 1a and polyarginine CA₄ derivative 2d.

in each series, namely the dendritic aminothiourea βCD derivative 1a and the arginine CA4 derivative 2d, performed better than the references LTX and PEI. Both 1a and 2d showed their best efficiency when formulated without the adjuvant (Fig. 14). Actually, as already observed in COS-7 cells, the incorporation of DOPE was generally detrimental for the activity of the tested ligands. The data confirms the high transfection efficiency of the argininocalixarene 2d in RD-4 cells. While in the experiments with COS-7 cells differences in transfection efficiencies between vectors in the tested library remained modest, in the case of RD-4 cells the transfection efficiency of the argininocalizarene 2d stands out of the rest, with an exceptional 75% of cells successfully transfected. The dendritic aminothiourea βCD derivative 1a, which was the best performing system in the COS-7 cell line, achieved 50% of RD-4 cells transfected, meaning that the optimal vector depends on the target cell. In any case, with few exceptions, such as the significant transfection level observed for the tetraguanidine CA4 derivative 2f in this assay, the data within a series follow the same trends already observed in COS-7 cells, but differences are quantitatively more pronounced. Thus, the proportion of transfected cells drops from 50% or 25% to almost zero on going from the dendritic tetradecacationic derivatives 1a or octacationic 2a to the linear heptacationic 1c or tetracationic 2c analogues. A high cationic density thus seems to be critical for the successful transfection of RD-4

When comparing homologous βCD and CA₄ derivatives in each sub-library, barring the 1a/2a pair the calixarene derivatives were generally superior to the cyclodextrins vectors, which is the opposite situation to that encountered in COS-7 cells. It has been previously advanced that BCD facial amphiphiles can interact with cholesterol in the cell membrane through inclusion complex formation and that this interaction facilitates caveolin-mediated endocytosis (CME) of the corresponding CDplexes. 13,14a Indeed, CME has been shown to be by far the most productive internalization route for CDplexes. 14a Since caveolin is down-regulated in rhabdomyosarcoma, ²⁶ it can be expected that the efficiency of BCD-based vector will be decreased in RD-4 as compared with COS-7 cells. It is interesting to speculate that calixplexes can enter the cell through alternative caveolin-independent routes that remain productive

regarding transfection, which may be at the origin of the outstanding result obtained with compound 2d. Exploring this hypothesis is currently underway in our laboratories.

Conclusions

In summary, we have demonstrated that the approach based in the installation of counter-directional multi-head/multi-tail cationizable/O-hexanoyl or O-hexyl domains onto a β-cyclodextrin or a calix[4] arene platform provides facial amphiphiles with gene delivery capability. Total control of the homogeneity at the molecular level is warranted in homologous series of compounds, allowing reliable structure-activity relationship studies. The components of the three sub-libraries considered in this study, namely compounds featuring aminothiourea, arginine and guanidine clusters were all able to condense pDNA into self-assembled nanocomplexes through a process that involves electrostatic vector-DNA interactions and hydrophobic vector-vector interactions, resulting in a well-ordered arrangement of alternated DNA chains and vector bilayers. As a general trend, increasing the density of protonable groups had a beneficial impact in transfection capabilities provided that amino groups, with buffering capabilities, were present in the structure. The effect of the macrocyclic core on this was more evident when comparing different cell lines. In epithelial COS-7 cells from apes, BCD formulated CDplexes exhibited a higher transfection efficiency than the homologous CA₄-formulated calixplexes, while the reverse situation was encountered in human RD-4 cells. This switch can be tentatively ascribed to the operation of different cell uptake mechanisms that affect in a dissimilar manner the fate of CDplexes and calixplexes. In any case, this work provides clues for the rational design of new molecular gene delivery systems and validates the strategy based on systematic structural modifications in CD and CA-based facial amphiphiles and structureactivity relationship studies for the identification of optimal candidates for gene therapy applications.

Experimental

General methods

Bis(2-tert-butyloxycarbonylaminoethyl) amine and bis(2-tertbutyloxycarbonylaminoethyl) 2-aminoethyl amine obtained according to literature procedures. 9b Optical rotations were measured at 20 ± 2 °C in 1 dm tubes on a Jasco P-2000 polarimeter. ¹H (and ¹³C NMR) spectra were recorded at 500 (125.7) MHz with a Bruker 500 DRX magnet. 1D TOCSY, 2D COSY, HMQC and HSQC experiments were used to assist on NMR assignments. Thin-layer chromatography (TLC) was carried out on aluminum sheets coated with silica gel 60 F254 Merck with visualization by UV light and by charring with ethanolic 10% H₂SO₄ or 0.1% ninhydrin. Column chromatography was carried out on Silica Gel 60. ESI mass spectra were recorded on a Bruker Daltonics Esquire6000TM ion-trap mass

spectrometer; in some cases, CuI was added as the cationizing agent. MALDI-TOF mass spectra were registered in a Bruker Daltonics Autoflex instrument in the linear positive mode with pulse ion extraction; 2,5-dihydroxybenzoic acid (DHB) was used as a desorption matrix. Elemental analyses were carried out at the Instituto de Investigaciones Químicas (Sevilla, Spain).

5,11,17,23-Tetraisothiocyanate-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene (4). To a solution of 5,11,17,23-tetraamino-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (3, 150 0.183 mmol) in absolute EtOH (4 mL), CS_2 (440 μ L, 7.3 mmol) and Et₃N (102 µL, 0.73 mmol) were added. The reaction mixture was stirred for 2 h at rt and then cooled in an ice bath. A solution of Boc₂O (158 mg, 0.72 mmol) in absolute EtOH (1 mL) was added followed by the addition of a catalytic amount of DMAP (1.8 mg, 15 µmol). The reaction mixture was kept in the ice bath for 20 min. Then it was allowed to reach rt and further stirred for 2 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (1:3 DCM-cyclohexane). Yield: 181 mg (88%); $R_f = 0.67$ (1:3 DCM-cyclohexane); ¹H NMR (400 MHz, CDCl₃): δ 6.56 (s, 8 H, Ar), 4.35 (d, J = 10.2 Hz, 4 H, ArC H_{ax} Ar), 3.83 (t, J = 5.7 Hz, 8 H, CH_2-1_{Hex}), 3.09 (d, J = 10.2 Hz, 4 H, $ArCH_{eq}Ar$), 1.89–1.77 (m, 8 H, CH_2 - 2_{Hex}), 1.41–1.28 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 0.97-0.84 (m, 12 H, CH₃-6_{Hex}); 13 C NMR (100.6 MHz, CDCl₃): δ 155.7, 135.9 (Ar), 134.3 (NCS), 125.7, 125.6 (Ar), 75.9 (C-1_{Hex}), 32.1 (C-3_{Hex}), 30.9 (ArCH₂Ar), 30.2 (C-2_{Hex}), 25.9 (C-4_{Hex}), 22.9 (C-5_{Hex}), 14.2 (C-6_{Hex}); ESI-MS: m/z 1011.1 [M + Na]⁺; Anal. calcd for C₅₆H₆₈N₄O₄S₄: C 67.98, H 6.93, N 5.66, S 12.96. Found: C 68.07, H 6.88, N 5.61, S 12.79.

5,11,17,23-Tetra-[N',N'-bis-(2-(tert-butoxycarbonylamino)ethyl)thioureido]-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene solution of 4 (84.1 mg, 0.085 mmol) in dry DCM (3 mL) was added dropwise to a solution of bis(2-tert-butyloxycarbonylaminoethyl) amine (124 mg, 0.41 mmol) and Et₃N (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 column chromatography (100:1 \rightarrow 99:1 DCM-MeOH). Yield: 187 mg (99%); R_f = 0.58 (9:1 DCM-MeOH); ¹H NMR (500 MHz, CD₃OD): δ 6.96 (bs, 8 H, Ar), 4.48 $(d, J = 12.5 \text{ Hz}, 4 \text{ H}, \text{ArC}H_{ax}\text{Ar}), 4.05-3.70 \text{ (m, 24 H, CH}_2-1_{Hex})$ CH_2NCS), 3.31 (bs, 16 H, CH_2NHBoc), 3.14 (d, J = 12.5 Hz, 4 H, ArCH_{eq}Ar), 2.07 (m, 8 H, CH₂-2_{Hex}), 1.67–1.28 (m, 96 H, CMe₃, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.05-0.95 (m, 12 H, CH₃-6_{Hex}); ¹³C NMR (125.7 MHz, CD₃OD, 333 K): δ 183.2 (CS), 158.5 (CO), 155.3-127.3 (Ar), 80.7 (CMe₃), 76.6 (C-1_{Hex}), 52.4 (CH₂NHCS), 39.7 (CH₂NHBoc), 33.3 (C-3_{Hex}), 32.0 (ArCH₂Ar), 31.5 (C-2_{Hex}), 29.0 (CMe₃), 27.2 (C-4_{Hex}), 23.9 (C-5_{Hex}), 14.4 (C-6_{Hex}); ESI-MS: m/z 2265.9 [M + Cu]⁺, 1144.9 [M + Cu + Na]²⁺; Anal. calcd for $C_{112}H_{184}N_{16}O_{20}S_4$: C 61.06, H 8.42, N 10.17, S 5.82. Found: C 61.30, H 8.30, N 9.88, S 5.88.

5,11,17,23-Tetra-[bis-2-(aminoethyl)thioureido]-25,26,27,28-tetrakis-(n-hexyloxy)calix[4]arene octahydrochloride (2a). A solution of 5 (25 mg, 11 μ mol) in DCM-TFA-TES (87.5:10:2.5, 3 mL) was stirred at 0 °C for 2 h. The solvent was removed under reduced pressure. The residue was precipi-

tated and washed with Et₂O (5 mL). Then the solid was dissolved in an aqueous 0.1 M HCl solution and concentrated to give the product as hydrochloride. Yield: 19 mg (99%); 1 H NMR (300 MHz, CD₃OD): δ 6.86 (s, 8 H, Ar), 4.50 (d, J = 12.6 Hz, 4 H, ArC H_{ax} Ar), 4.09 (t, J = 6.7 Hz, 16 H, C H_{2} NHCS), 3.96 (t, J = 7.8 Hz, 8 H, CH₂-1_{Hex}), 3.26 (t, J = 6.7 Hz, 16 H, C H_{2} NH₂), 3.16 (d, J = 12.6 Hz, 4 H, ArC H_{eq} Ar), 2.11–1.95 (m, 8 H, CH₂-2_{Hex}), 1.56–1.32 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.02–0.90 (m, 12 H, CH₃-6_{Hex}); 13 C NMR (125.7 MHz, CD₃OD, 313 K): δ 184.3 (CS), 155.5–127.9 (Ar), 76.9 (C-1_{Hex}), 48.65 (CH₂NHCS), 38.4 (CH₂NH₂), 33.4 (C3_{Hex}), 31.8 (ArC H_{2} Ar), 31.6 (C-2_{Hex}), 27.1 (C-4_{Hex}), 23.9 (C-5_{Hex}), 14.5 (C-6_{Hex}); ESI-MS: m/z 1463.9 [M + Cu] $^{+}$; Anal. calcd for C₇₂H₁₂₈Cl₈N₁₆O₄S₄: C 51.06, H 7.62, N 13.23, S 7.57. Found: C 50.77, H 7.59, N 12.91, S 7.19.

5,11,17,23-Tetra-[N'-(2-(N,N-bis-(2-tert-butoxycarbonylaminoethyl)amino)ethyl)thioureido]-25,26,27,28-tetrakis-(n-hexyloxy)-calix[4]arene (6). A solution of 4 (40 mg, 0.040 mmol) in dry DCM (3 mL) was added dropwise to a solution of bis(2-tertbutyloxycarbonylaminoethyl) 2-aminoethyl amine (67.4 mg, 0.19 mmol) and Et₃N (40 μ L, 0.28 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 column chromatography (19:1 DCM-MeOH). Yield: 95 mg (99%); $R_f = 0.56$ (9:1 DCM-MeOH); ¹H NMR (300 MHz, CD₃OD): δ 6.721 (bs, 8 H, Ar), 4.47 (d, J = 12.9 Hz, 4 H, ArCH-_{ax}Ar), 3.93 (m, 8 H, CH₂-1_{Hex}), 3.69–3.53 (m, 8 H, CH₂NHCS), 3.20 (d, J = 13.3 Hz, 4 H, ArC H_{eq} Ar), 3.07 (t, J = 6.4 Hz, 16 H, CH₂CH₂NHBoc), 2.77-2.65 (m, 8 H, CH₂CH₂NHCS), 2.64-2.53 (m, 16 H, CH_2CH_2NHBoc), 2.06–1.89 (m, 8 H, CH_2-2_{Hex}), 1.62-1.25 (m, 96 H, CMe₃, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.02-0.87 (m, 12 H, CH₃-6_{Hex}); ¹³C NMR (75 MHz, CD₃OD, 313 K): δ 181.7 (CS), 158.4 (CO), 155.9–126.1 (Ar), 80.3 (CMe₃), 76.6 (C-1_{Hex}), 55.3 (CH₂NHBoc), 54.4 (CH₂CH₂NHCS), 43.8 (CH_2NHCS) , 40.1 (CH_2CH_2NHBoc) , 33.3 $(C-3_{Hex})$, 32.0 $(ArCH_2Ar)$, 31.5 $(C-2_{Hex})$, 29.0 (CMe_3) , 27.3 $(C-4_{Hex})$, 23.9 $(C-5_{Hex})$, 14.4 $(C-6_{Hex})$; ESI-MS: m/z 2397.3 $[M + Na]^+$, 1209.6 $[M + 2Na]^{2+}$; Anal. calcd for $C_{120}H_{204}N_{20}O_{20}S_4$: C 60.68, H 8.66, N 11.79, S 5.40. Found: C 60.52, H 8.58, N 11.62, S 5.24.

5,11,17,23-Tetra-[N'-(2-(N,N-bis-(2-aminoethyl)amino)ethyl)thioureido]-25,26,27,28-tetrakis-(n-hexyloxy)calix[4]arene (2b). A solution of 6 (52 mg, 22 µmol) in TFA (0.4 mL) was stirred at rt for 5 min. H₂O (10 mL) was added to it 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. Yield: 41 mg (99%); 1 H NMR (300 MHz, CD₃OD): δ 7.02 (bs, 8 H, Ar), 4.51 (d, J = 12.7 Hz, 4 H, ArC H_{ax} Ar), 4.18 (t, J = 7.2 Hz, 8 H, CH₂-1_{Hex}), 3.96 (t, J = 7.7 Hz, 8 H, CH_2 NHCS), 3.37–3.26 (m, 40 H, CH_2CH_2NHBoc , CH_2CH_2NHCS , CH₂CH₂NHBoc), 3.21 (d, 4 H, ArCH_{eq}Ar), 2.18-2.06 (m, 8 H, CH₂-2_{Hex}), 1.56-1.34 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.05–0.89 (m, 12 H, CH_3 - 6_{Hex}); ¹³C NMR (125.7 MHz, CD_3OD): δ 183.9 (CS), 155.4–127.8 (CAr), 76.5 (C-1_{Hex}), 51.5, 51.8 (CH₂N $(CH_2CH_2)_2$, 42.2 (CH_2NHCS) , 37.9 (CH_2NH_2) , 32.5 $(C-3_{Hex})$, 31.3 (ArCH₂Ar), 30.8 (C-2_{Hex}), 26.7 (C-4_{Hex}), 23.4 (C-5_{Hex}), 14.4 $(C-6_{Hex})$; ESI-MS: m/z 1574.9 $[M + H]^+$, 788.0 $[M + 2H]^{2+}$; Anal.

calcd for $C_{80}H_{152}Cl_{12}N_{20}O_4S_4$: C 47.76, H 7.62, N 13.92, S 6.38. Found: C 47.67, H 7.72, N 13.90, S 6.29.

5,11,17,23-Tetra-[N'-(2-(tert-butoxycarbonylamino)ethyl)thioureido]-25,26,27,28-tetrakis-(n-hexyloxy)calix[4]arene (7). A solution of 4 (84.1 mg, 0.085 mmol) in dry DCM (3 mL) was added dropwise to a solution of commercial 2-tert-butyloxycarbonylaminoethylamine (67 mg, 0.41 mmol) and Et₃N (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 column chromatography (95:5 \rightarrow 9:1 DCM-MeOH). Yield: 138 mg (99%); $R_f = 0.61$ (9:1 DCM-MeOH); ¹H NMR (400 MHz, CD₃OD): δ 6.68 (bs, 8 H, Ar), 4.46 $(d, J = 12.7 \text{ Hz}, 4 \text{ H}, \text{ArC}H_{ax}\text{Ar}), 3.93 (t, J = 7.9 \text{ Hz}, 8 \text{ H}, \text{CH}_2$ 1_{Hex}), 3.65 (t, J = 6.0 Hz, 8 H, CH_2 NHCS), 3.34–3.16 (m, 12 H, ArCH_{eq}Ar, CH₂NHBoc), 2.06-1.91 (m, 8 H, CH₂-2_{Hex}), 1.55-1.32 (m, 60 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}, CMe₃), 1.01-0.92 (m, 12 H, CH₃-6_{Hex}); 13 C NMR (100.6 MHz, CD₃OD): δ 181.6 (CS), 158.8 (CO), 155.8-125.9 (Ar), 80.2 (CMe₃), 76.7 (C-1_{Hex}), 45.9 (CH₂NHCS), 40.8 (CH₂NHBoc), 33.4 (C-3_{Hex}), 31.9 (ArCH₂Ar), 31.6 (C-2_{Hex}), 28.9 (CMe₃), 27.3 (C-4_{Hex}), 24.1 (C-5_{Hex}), 14.5 $(C-6_{Hex})$; ESI-MS: m/z 1651.8 $[M + Na]^+$, 1667.8 $[M + K]^+$; Anal. calcd for $C_{84}H_{132}N_{12}O_{12}S_4$: C 61.88, H 8.16, N 10.31, S 7.87. Found: C 61.85, H 8.29, N 10.24, S 7.79.

5,11,17,23-Tetraaminoethylthioureido-25,26,27,28-tetrakis(nhexyloxy)calix[4]arene (2c). A solution of 7 (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 and the residue was precipitated and washed with Et2O. Then the solid was dissolved in an aqueous 0.1 M HCl solution and concentrated to yield the product as hydrochloride. Yield: 33.8 mg (99%); ¹H NMR (500 MHz, CD₃OD): δ 6.72 (bs, 8 H, Ar), 4.47 (d, J = 13.2 Hz, 4 H, ArC H_{ax} Ar), 3.98–3.85 (m, 16 H, CH₂-1_{Hex}, C H_2 NHCS), 3.26-3.13 (m, 12 H, ArCH_{eq}Ar, CH₂NH₂), 2.02-1.88 (m, 8 H, CH₂-2_{Hex}), 1.56-1.35 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 0.99-0.91 (m, 12 H, CH₃-6_{Hex}); ¹³C NMR (75 MHz, CD₃OD): δ 182.8 (CS), 156.0–125.7 (Ar), 76.7 (C-1_{Hex}), 42.8 (CH₂NHCS), 40.8 (CH₂NH₂), 33.4 (C-3_{Hex}), 31.9 (ArCH₂Ar), 31.5 (C-2_{Hex}), 27.3 (C-4_{Hex}), 24.0 (C-5_{Hex}), 14.5 (C-6_{Hex}); ESI-MS: m/z 1291.4 $[M + Cu]^+$; Anal. calcd for $C_{64}H_{100}N_{12}O_4S_4\cdot 4HCl$: C 55.88, H 7.62, N 12.22, S 9.32. Found: C 55.59, H 7.56, N 11.92, S 9.03.

Heptakis $[6-(2-(N_{\alpha}-tert-but oxy carbonyl-N_{\omega}-(2,2,4,6,7-penta$ methyldihydrobenzofurane-5-sulfonyl)-L-arginine-N-amide)ethylthio)-2,3-di-O-hexanoyl]cyclomaltoheptaose (10). To a solution of heptakis[2,3-di-O-hexanoyl-6-(2-aminoethylthio)]cyclomaltoheptaose heptahydrochloride (8, 154, 60 mg, 19 µmol) in dry DCM (4 mL), under an Ar atmosphere, DIPEA (90 µL, μ mol), N_{α} -tert-butoxycarbonylamino- N_{ω} -(2,2,4,6,7-pentamethyldihydro-benzofurane-5-sulfonyl)-L-arginine (9, N_{α} -Boc- N_{ω} -Pbf-L-arginine) (104 mg, 198 µmol) and O-(benzotriazol-1yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (75 mg, 198 μmol) were added and the mixture was stirred overnight. The reaction mixture was washed with an aqueous solution of NaHCO3 (8 mL), the organic layer was dried (MgSO₄), filtered and concentrated. The residue was purified by column chromatography (25:1 \rightarrow 18:1 DCM-MeOH). Yield: 83 mg (68%); $R_f = 0.43$ (17:1 DCM-MeOH). $[\alpha]_D = +31.9$

(c 1 in MeOH); 1 H NMR (500 MHz, CD₃OD): δ 5.54 (t, 7 H, $J_{2,3} = J_{3,4} = 9.5 \text{ Hz}, \text{ H-3}, 5.36 (d, J_{1,2} = 3.7 \text{ Hz}, 7 \text{ H}, \text{ H-1}), 5.02$ (dd, 7 H, H-2), 4.41 (m, 7 H, H-5), 4.33-4.23 (m, 7 H, CH-2_{Arg}), 4.12 (m, 7 H, H-4), 3.76-3.67 (m, 7 H, CH₂NHCO), 3.64-3.55 (m, 7 H, CH₂NCO), 3.48-3.28 (s, 28 H, CH₂-5_{Arg}, H6a, H6b), 3.17 (s, 14 H, Me_{Pbf}), 3.07-2-96 (m, 14 H, CH₂S), 2.77 (s, 21 H, Me_{Pbf}), 2.71 (s, 21 H, Me_{Pbf}), 2.57-2.38 (m, 28 H, CH₂-2_{Hex}), 2.26 (s, 21 H, Me_{Pbf}), 2.03-1.93 (m, 7 H, CH_2 -3_{Arg}), 1.91-1.71 (m, 49 H, CH_2 - 3_{Arg} , CH_2 - 4_{Arg} , CH_2 - 3_{Hex}), 1.63 (bs, 42 H, CMe_{2Pbf}), 1.61 (bs, 63 H, CMe₃), 1.59–1.44 (m, 56 H, CH₂-4_{Hex}, CH_2 -5_{Hex}), 1.20–1.05 (m, 42 H, CH_3 -6_{Hex}); ¹³C NMR (75 MHz, CD₃OD): δ 174.9, 174.7 (CO ester), 173.5 (CO amide), 159.9 (CN), 158.1 (CO carbamate), 157.7-118.4, (Cq_{Pbf}), 98.2 (C-1), 87.7 (CMe₃), 80.7 (CMe_{2Pbf}), 71.7 (C-4), 73.5 (C-5), 71.7, 71.6 (C-2, C-3), 55.8 (CH₂-2_{Arg}), 44.1 (CH_{2Pbf}), 41.5 (CH-5_{Arg}), 40.5 (CH₂NHCO), 35.1, 35.0 (C-2_{Hex}, C-6), 34.1 (CH₂S), 32.6, 32.5 (C- $^{4}_{Hex}$), 30.9 (CH₂- $^{3}_{Arg}$), 28.9, 28.8 (CMe_{2Pbf}, CMe₃), 27.1 (CH₂-4_{Arg}), 25.6 (C-3_{Hex}), 23.6 (C-5_{Hex}), 19.8, 18.6 (Me_{Pbf}) , 14.6 $(C-6_{Hex})$, 12.7 (Me_{Pbf}) ; ESI-MS: m/z 3263.3 $[M + 2Na]^{2+}$, 2182.6 $[M + 3Na]^{3+}$; Anal. calcd for C₃₀₈H₄₉₇N₃₅O₈₄S₁₄: C 57.06, H 7.73, N 7.56, S 6.92. Found: C 56.88, H 7.64, N 7.34, S 6.63.

Heptakis[6-(2-(L-arginine-N-amide)ethylthio)-2,3-di-O-hexanoyl]cyclomaltoheptaose tetradecahydrochloride (1d). A solution of 10 (37.5 mg, 6 μmol) in TFA-TIS-H₂O (95:2.5:2.5, 4 mL) was stirred at rt for 1 h. The solvent was removed under reduced pressure and coevaporated several times with H2O. The residue was dissolved in an aqueous 0.1 M HCl solution and freeze-dried to obtain the product as hydrochloride. Yield: 41 mg (99%); $[\alpha]_D = +9.7$ (c 1.0 in DMSO); ¹H NMR (500 MHz, DMSO-d₆, 323 K): δ 8.96 (bs, 7 H, NHCO), 8.51-8.29 (m, 14 H, NH_{2Arg}), 7.93-7.77 (bs, 7 H, NH guanidine), 7.44-7.09 (m, 21 H, NH, NH₂ guanidine), 5.25 (t, $J_{2,3} = J_{3,4} = 9.0$ Hz, 7 H, H-3), 5.09 (bs, 7 H, H-1), 4.69 (bd, 7 H, H-2), 4.17-4.07 (m, 7 H, H-5), 3.99-3.83 (m, 14 H, CH₂-5_{Arg}, H-4), 3.43-3.32 (bs, 14 H, CH₂N), 3.29-3.19 (bs, 7 H, CH₂-5_{Arg}), 3.15-2.99 (m, 14 H, H-6a, H-6b), 2.87-2.66 (m, 14 H, CH₂S), 2.44-2.29 (m, 7 H, CH₂-2_{Hex}), 2.26-2.11 (m, 7 H, CH_2-2_{Hex}), 1.92-1.80 (m, 14 H, CH_2-3_{Arg}), 1.70-1.58 (m, 14 H, CH₂-4_{Arg}), 1.58-1.45 (m, 28 H, CH₂-3_{Hex}), 1.35-1.19 (bs, 56 H, CH₂-4_{Hex}, CH₂-5_{Hex}), 0.93-0.78 (m, 42 H, CH₃-6_{Hex}); 13 C NMR (125.7 MHz, DMSO-d₆, 323 K): δ 175.7, 174.5 (CO ester), 177.8 (CO amide), 161.2 (CN), 99.4 (C-1), 81.3 (C-4), 74.3 (C-5), 73.1 (C-2), 72.9 (C-3), 55.1 (CH₂-2_{Arg}), 43.7 (CH₂-5_{Arg}), 42.4 (CH₂NHCO), 36.5, 36.3 (C-2_{Hex}, C-6), 35.3 (CH_2S) , 33.9, 33.8 $(C-4_{Hex})$, 31.4 (CH_2-3_{Arg}) , 27.2 (CH_2-4_{Arg}) , 26.9, 26.8 (C-3_{Hex}), 24.9, 24.8 (C-5_{Hex}), 16.65, 16.63 (C-6_{Hex}); MALDI-MS: m/z 4017.86 [M + H]⁺; Anal. calcd for $C_{182}H_{343}Cl_{14}N_{35}O_{49}S_{7}\cdot 7H_{2}O$: C 46.98, H 7.73, N 10.54, S 4.82. Found: C 47.01, H 7.50, N 10.48, S 4.75.

 N_{α} -tert-Butoxycarbonyl- N_{ω} -(2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl)- ι -arginine-N-propargylamide (11). A mixture of N_{α} -Boc- N_{ω} -Pbf- ι -arginine (1 g, 1.9 mmol), DIPEA (0.78 mL, 4.5 mmol) and HBTU (865 mg, 2.28 mmol) were dissolved in dry DMF (15 mL), under an Ar atmosphere. After stirring for 30 min at rt, propargylamine (0.146 mL, 2.28 mmol) was added and the mixture was stirred for 15 h.

The solvent was removed under reduced pressure and the residue was dissolved in EtOAc (15 mL), the organic layer was washed with aqueous saturated NaHCO3 (15 mL), H2O (15 mL), dried (MgSO₄), filtered and concentrated. The residue was purified by column chromatography $(1:2 \rightarrow 3:1)$ EtOAccyclohexane). Yield: 935 mg (87%); $R_f = 0.41$ (19:1 DCM-MeOH); $[\alpha]_D$ = +0.9 (c 1.0 in MeOH). ¹H NMR (500 MHz, CDCl₃): δ 7.34 (bs, 1 H, NHCO), 6.27 (m, 3 H, NH), 5.59 (bs, 1 H, NHBoc), 4.20 (bs, 1 H, CH-2_{Arg}), 3.99 (m, 2 H, CH₂-alkyne), 3.27 (bs, 2 H, CH₂-5_{Arg}), 2.96 (s, 2 H, CH_{2Pbf}), 2.51 (s, 3 H, Me_{Pbf}), 2.50 (s, 3 H, Me_{Pbf}), 2.18 (t, J = 2.5 Hz, 1 H, CH-alkyne), 2.09 (s, 3 H, Me_{Pbf}), 1.81 (m, 2 H, CH₂-3_{Arg}), 1.60 (m, 2 H, CH₂- 4_{Arg}), 1.46 (s, 6 H, CMe_{2Pbf}), 1.41 (s, 9 H, CMe₃); ¹³C NMR (100.6 MHz, CDCl₃): δ 165.9 (CO amide), 159.0 (CAr), 156.8 (CN), 156.1 (CO carbamate), 138.3-117.6 (CAr), 86.4 (CMe₂), 80.2 (CMe₃), 79.6 (Cq alkyne), 71.4 (CH alkyne), 60.4 (CHNHBoc), 43.2 (CH_{2Pbf}, CH₂- 5_{Arg}), 29.9 (CH₂- 4_{Arg}), 29.1 (CH₂-6_{Arg}), 28.6 (CMe₂), 28.3 (CMe₂), 19.3, 17.9, 12.5 (Me_{Pbf}); ESI-MS: m/z 586.3 [M + Na]⁺, 602.1 [M + K]⁺; Anal. calcd for C₂₇H₄₁N₅O₆S: C 57.53, H 7.33, N 12.42, S 5.69. Found: C 57.58 H 7.24, N 12.31, S 5.36.

Heptakis [6-deoxy-2,3-di-O-hexanoyl-6-(4- $(N_{\alpha}$ -tert-butoxycarbonyl- N_{ω} -(2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl)-L-arginine-N-amidomethyl)-1H-1,2,3-triazol-1-yl)]cyclomaltoheptaose (13). To a solution of heptakis[6-azido-6-deoxy-2,3di-O-hexanoyl]cyclomaltoheptaose (12, 88 mg, 33 µmol) in acetone (5 mL), N_{α} -tert-12- N_{ω} -(2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl)-L-arginine-N-propargylamide (11, 142 mg, 0.25 mmol), N,N-diisopropylethylamine (38 μL, 0.24 mmol) and CuI·P(OEt)₃ (8.0 mg, 25 µmol) were added. The reaction mixture was refluxed for 48 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (20:1 \rightarrow 9:1 DCM-MeOH). Yield: quantitative; $R_f = 0.60 \ (9:1 \ DCM-MeOH); \ [\alpha]_D = +29.7 \ (c \ 1.0)$ in MeOH); ¹H NMR (500 MHz, DMSO-d₆) δ 8.12 (bs, 7 H, CH_{triazole}), 7.84 (bs, 7 H, Ar), 6.69 (bs, 7 H, NH_{2 guanidine}), 6.55 (bs, 14 H, NH_{2 guanidine}), 5.48-5.35 (m, 14 H, H-3, H-1), 4.74-4.59 (m, 21 H, CH₂NHCO amide, 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, CH_{Arg}), 3.61 (bt, 7 H, H-4), 3.09 (m, 14 H, CH₂-5_{Arg}), 2.96 (s, 14 H, CH_{2Pbf}), 2.51 (s, 21 H, CH_{3 Pbf}), 2.46 (s, 21 H, CH_{3 Pbf}), 2.28-2.26 (m, 7 H, CH₂-2_{Hex}), 2.27-2.10 (m, 7 H, CH₂-2_{Hex}), 2.03 (s, 21 H, CH_{3 Pbf}), 1.66-1.18 (217 H, CH₂-2_{Arg}, CH₂-4_{Arg}, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}, CH₃ _{Pbf}, C(CH₃)₃), 0.93-0.80 (bs, 21 H, CH₃-6_{Hex}); ¹³C NMR (125.7 MHz, DMSO d_6) δ 175.3, 175.1 (CO _{ester}), 174.3 (CO amide), 160.6 (CN guanidine), 159.3 (CO carbamate), 147.6 (C-4_{triazole}), 140.3-134.6 (Cq Pbf), 127.8 (C-5 triazole), 127.3, 119.3 (CqPbf), 99.0 (C-1), 89.2 (CMe₃), 81.3 (CMe_{2 Pbf}), 79.6 (C-4), 72.8 (C-3), 72.5 (C-5), 72.3 (C-2), 57.2 (CH₂-2_{Arg}), 52.6 (CH₂NHCO), 45.7 (CH_{2Pbf}), 42.4 (CH-5-Arg), 37.4 (C-6), 36.4, 36.3 (C-2_{Hex}), 33.9, 33.7 (C-4_{Hex}), 32.0 (CH_2 -3_{Arg}), 31.3, 31.2 (CMe_{2Pbf} , CMe_3), 28.7 (CH_2 -4_{Arg}), 26.9, 26.8 (C-3_{Hex}), 24.8, 24.9 (C-5_{Hex}), 21.8, 20.5 (Me _{Pbf}), 16.6 $(C-6_{Hex})$, 15.2 (Me_{Pbf}) ; ESI-MS: m/z 3336.4 $[M + 2Na]^{2+}$; Anal. calcd for C₃₁₅H₄₉₀N₅₆O₈₄S₇: C 57.06, H 7.45, N 11.83, S 3.39. Found: C 56.78, H 7.20, N 11.47, S 2.92.

Heptakis[6-deoxy-2,3-di-O-hexanovl-6-(4-L-arginine-N-amidomethyl)-1H-1,2,3-triazol-1-yl)]cyclomaltoheptaose tetradecahydrochloride (1e). A solution of 13 (45.2 mg, 7 µmol), in TFA-TIS-H₂O (95:2.5:2.5, 4.5 mL) was stirred at rt for 2 h. 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. Yield: 35.7 mg (quantitative); $[\alpha]_D = +46.5$ (c 1.0) in DMF); 1 H NMR (500 MHz, DMSO-d₆) δ 8.99 (bs, 7 H, NH_{amide}), 7.99 (m, 21 H, CH_{triazole}, NH_{2 arginine}), 7.18 (bs, 21 H, NH₂ guanidine, NH guanidine), 5.41-5.37 (m, 14 H, H-3, H-1), 4.68-4.63 (m, 21 H, H-2, CH₂NHCO amide), 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, CH_{Arg}), 3.72 (bt, 7 H, H-4), 3.19 (m, 14 H, CH₂-5_{Arg}), 2.37-1.93 (m, 28 H, CH₂-2_{Hex}), 2.27-2.10 (m, 14 H, CH₂-2_{Hex}), 1.86-1.79 (m, 14 H, CH₂-2_{Arg}), 1.48-1.38 (m, 42 H, CH₂-4_{Arg}, CH₂-3_{Hex}), 1.37-2.20 (m, 56 H, CH₂-4_{Hex}, CH₂-5_{Hex}), 0.94-0.80 (bs, 21 H, CH₃-6_{Hex}); 13 C NMR (125.7 MHz, DMSO-d₆): δ 172.8, 171.8 (CO ester), 169.1 (CO amide), 157.7 (CN guanidine), 144.2 (C-4_{triazole}), 125.7 (C-5_{triazole}), 102.5 (C-1), 77.3 (C-4), 70.3 (C-3), 70.1 (C-5), 69.7 (C-2), 52.5 (CH-2_{Arg}), 49.9 (CH₂NHCO), 40.7 (CH-5-Arg), 34.9 (C-6), 33.8, 33.7 (C-4_{Hex}), 31.2, 31.1 (C-4_{Hex}), 28.5 (CH₂-3_{Arg}), 24.3, 24.2 (C-3_{Hex}, CH₂-4_{Arg}), 22.2, 22.1 (C-5_{Hex}), 13.9 (C-6_{Hex}); MALDI-MS: m/z 4163.60 [M + H]⁺; Anal. calcd for C₁₈₉H₃₂₉Cl₁₄N₅₆O₄₉: C 48.57, H 7.25, Cl 10.62, N 16.78, O 16.78. Found: C 48.33, H 6.82, N 16.49.

5,11,17,23-Tetraformyl-25,26,27,28-tetrakis(n-hexyloxy)calix-[4] arene (15). A solution of hexamethylenetetramine (16.57 g, 118.3 mmol) in TFA (150 mL) was stirred at 100 °C for 10 min. Then, 25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene (14, 2.5 g, 3.29 mmol) was added and the mixture was stirred at reflux for 2 days. The reaction was quenched by the addition of HCl 1 M (400 mL) and stirred for 3 h. The aqueous layer was extracted twice with DCM (250 mL); the combined organic phases were washed with a saturated aqueous solution of NaHCO3 (200 mL) and brine (200 mL), dried (Na₂SO₄) and filtered. The solvent was removed under reduced pressure. The product was purified by crystallization from hexane (50 mL). Yield: 2.75 g (96%); $R_f = 0.20 (2:3 \text{ EtOAc-cyclohexane}); {}^{1}\text{H NMR} (300 \text{ MHz},$ CDCl₃) δ 9.58 (s, 4 H, CHO), 7.15 (s, 8 H, Ar), 4.49 (d, J = 13.8Hz, 4 H, ArC H_{ax} Ar), 3.96 (t, J = 7.5 Hz, 8 H, CH₂-1_{Hex}), 3.34 (d, $J = 13.8 \text{ Hz}, 4 \text{ H}, \text{ArC}H_{eq}\text{Ar}), 2.00-1.80 \text{ (m, 8 H, CH}_2-2_{Hex}),$ 1.50-1.20 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.00-0.80 (m, 12 H, CH₃-6_{Hex}); 13 C NMR (100.6 MHz, CDCl₃) δ 191.3 (CHO), 162.0-130.2 (Ar), 75.8 (C-1_{Hex}), 31.9 (C-3_{Hex}), 30.9 $(ArCH_2Ar)$, 30.3 $(C-2_{Hex})$, 25.8 $(C-4_{Hex})$, 22.8 $(C-5_{Hex})$, 14.0 $(C-6_{Hex})$; ESI-MS: m/z 895.9 $[M + Na]^+$; Anal. calcd for $C_{56}H_{72}O_8$: C 77.03, H 8.31. Found: C 76.78, H 8.04.

5,11,17,23-Tetrahydroxymethyl-25,26,27,28-tetrakis(n-hexyloxy)-calix[4]arene (16). To a suspension of 15 (910 g, 1.04 mmol) in absolute EtOH (50 mL) at 0 °C, NaBH₄ (0.24 g, 6.25 mmol) was added. The mixture was stirred at rt for 18 h. Then, 1 M HCl (20 mL) was added and the solvents were evaporated under reduced pressure. EtOAc (50 mL) was added to this and the organic layer was washed with a saturated aqueous solution of NaHCO₃ (50 mL) and H₂O (50 mL), dried over Na₂SO₄, and

concentrated. Yield: 788 mg (86%); $R_f = 0.16$ (19:1 DCM-MeOH); ¹H NMR (300 MHz, CD₃OD) δ 6.66 (s, 8 H, Ar), 4.46 (d, J = 13.2 Hz, 4 H, ArC H_{ax} Ar), 4.24 (s, 8 H, C H_2 OH), 3.90 (t, J =7.2 Hz, 8 H, CH_2-1_{Hex}), 3.15 (d, J = 13.2 Hz, 4 H, $ArCH_{eq}Ar$), 2.00-1.87 (m, 8 H, CH₂-2_{Hex}), 1.52-1.34 (m, 24 H, CH₂-3_{Hex}, CH_2-4_{Hex} , CH_2-5_{Hex}), 0.95 (t, J = 6.6 Hz, 12 H, CH_3-6_{Hex}); ¹³C NMR (100 MHz, CD₃OD) δ 157.1–128.3 (Ar), 76.0 (C-1_{Hex}), 65.1 (CH₂OH), 33.1 (C-3_{Hex}), 32.1 (ArCH₂Ar), 31.5 (C-2_{Hex}), 27.3 $(C-4_{Hex})$, 24.0 $(C-5_{Hex})$, 14.5 $(C-6_{Hex})$; ESI-MS: m/z 903.8 [M + Na^{+} ; Anal. calcd for $\text{C}_{56}\text{H}_{80}\text{O}_{8}$: C 76.33, H 9.15. Found: C 76.41, H 9.20.

5,11,17,23-Tetrachloromethyl-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene (17). To a solution of 16 (600 mg, 0.68 mmol) in dry DCM (10 mL), SOCl₂ (0.99 mL, 13.60 mmol) was added dropwise and the mixture was stirred at rt for 4 h. Then, the solvent was evaporated under reduced pressure. Yield: 622 mg (95%); ¹H NMR (400 MHz, CDCl₃) δ 6.64 (s, 8 H, Ar), 4.40 (d, J = 13.2 Hz, 4 H, ArC H_{ax} Ar), 4.29 (s, 8 H, CH₂Cl), 3.88 (t, J =7.6 Hz, 8 H, CH_2 - 1_{Hex}), 3.12 (d, J = 13.2 Hz, 4 H, $ArCH_{eq}Ar$), 1.95-1.80 (m, 8 H, CH₂-2_{Hex}), 1.49-1.19 (m, 24 H, CH₂-3_{Hex}, CH_2 -4_{Hex}, CH_2 -5_{Hex}), 1.01–0.77 (m, 12 H, CH_3 -6_{Hex}); ¹³C NMR (100 MHz, CDCl₃) δ 157.0–128.6 (Ar), 75.4 (C-1_{Hex}), 46.6 (CH_2Cl) , 32.0 $(C-3_{Hex})$, 30.9 $(ArCH_2Ar)$, 30.2 $(C-2_{Hex})$, 25.9 $(C-4_{Hex})$, 22.8 $(C-5_{Hex})$, 14.1 $(C-6_{Hex})$; ESI-MS: m/z 977.7 [M +Na] $^{+}$; Anal. calcd for C₅₆H₇₆Cl₄O₄: C 70.43, H 8.02. Found: C 70.54, H 8.16.

5,11,17,23-Tetraazidomethyl-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene (18). To a solution of 17 (192 mg, 0.20 mmol) in dry DMF (7 mL), NaN₃ (78.5 mg) was added. The mixture was stirred, under an Ar atmosphere, at rt, overnight. Then, the solvent was evaporated and the crude was dissolved in EtOAc (15 mL). The organic layer was washed with H₂O (15 mL), dried over MgSO₄, filtered and concentrated. The residue was purified by column chromatography (cyclohexane → 19:1 cyclohexane-EtOAc). Yield: 186 mg (95%); $R_f = 0.29$ (19:1 cyclohexane-EtOAc); ¹H NMR (300 MHz, CDCl₃) δ 6.61 (bs, 8 H, Ar), 4.45 (d, J = 13.2 Hz, 4 H, ArC H_{2ax} Ar), 3.95 (s, 8 H, CH_2N_3), 3.89 (t, J = 7.5 Hz, 8 H, CH_2-1_{Hex}), 3.16 (d, J = 13.2 Hz, 4 H, ArCH_{2eq}Ar), 1.98-1.85 (m, 8 H, CH₂-2_{Hex}), 1.48-1.30 (m, 24 H, CH_2 -3_{Hex}, CH_2 -4_{Hex}), 0.93 (t, J = 6.9 Hz, 12 H, CH_3 -6_{Hex}); ¹³C NMR (75 MHz, CDCl₃) δ 162.1–128.2 (Ar), 75.0 (C-1_{Hex}), 53.8 (CH_2N_3) , 31.7 $(C-3_{Hex})$, 30.4 $(ArCH_2Ar)$, 29.9 $(C-2_{Hex})$, 25.5 $(C-4_{Hex})$, 22.4 $(C-5_{Hex})$, 13.7 $(C-6_{Hex})$; ESI-MS: m/z 1004.1 [M + Na^+ , 1020.0 $[\text{M} + \text{K}]^+$; Anal. calcd for $\text{C}_{56}\text{H}_{76}\text{N}_{12}\text{O}_4$: C 68.54, H 7.81, N 17.13, O 6.52. Found: C 68.60, H 7.67, N 17.09.

5,11,17,23-Tetra- $[4-(N_{\alpha}-tert-butoxycarbonyl-N_{\omega}-(2,2,4,6,7-4)]$ pentamethyldihydro-benzofurane-5-sulfonyl)-L-arginine-Namidomethyl)-1H-1,2,3-triazol-1-methyl]-25,26,27,28-tetrakis (n-hexyloxy)calix[4]arene (19). To a solution of 11 (35 mg, 35.7 µmol) in acetone (5 mL), 18 (104.5 mg, 0.185 mmol), DIPEA (25 μL, 0.143 mmol) and CuI·P(OEt)₃ (5.1 mg, 14.3 µmol) were added. The reaction mixture was refluxed for 24 h. The solvent was removed under reduced pressure and the residue was purified by column chromatography (99:1 \rightarrow 9:1 DCM-MeOH). Yield: 82 mg (71%); $R_f = 0.65$ (9:1 DCM-MeOH); $[\alpha]_D = +2.69$ (c 1.0 in MeOH), ¹H NMR (500 MHz,

CD₃OD, 323 K): δ 7.79 (bs, 4 H, CH_{triazole}), 6.60 (bs, 8 H, Ar), 5.25 (bs, 8 H, CH₂NHCO amide), 4.50-4.36 (m, 12 H, CH₂Ar, $ArCH_{ax}Ar$), 4.02 (bs, 4 H, CH-2-Arg), 3.86 (t, J = 7.4 Hz, 8 H, CH₂-1_{Hex}), 3.19-3.02 (m, 12 H, ArCH_{eq}Ar, CH₂-5-Arg), 2.96 (s, 8 H, CH_{2Pbf}), 2.55 (s, 12 H, Me_{Pbf}), 2.50 (s, 12 H, Me_{Pbf}), 2.05 (s, 12 H, Me_{Pbf}), 1.94-1.85 (m, 8 H, CH₂-2_{Hex}), 1.79-1.29 (m, 100 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}, CMe₃, CMe_{2Pbf}, CH₂-3-Arg, CH₂-4-Arg), 0.98-0.88 (m, 12 H, CH₃-6_{Hex}), ¹³C NMR (100.6 MHz, CD₃OD): δ 173.5 (CO amide), 158.4 (CN guanidine), 156.4 (Ar), 156.2 (CO carbamate), 145.0 (C-4_{triazole}), 138.0-128.8 (Cq_{Pbf}, Ar), 127.9, 124.5 (Ar), 122.6 (C-5_{triazole}), 116.9 (Ar), 86.2 (CMe_{2 Pbf}), 79.1 (CMe₃), 75.1 (C-1_{Hex}), 54.2 (CH-2-Arg), 53.3 (CH₂NHCO), 42.4 (Me_{Pbf}), 39.8 (CH₂-5-Arg), 34.3 (CH₂Ar), 31.8 (C-3_{Hex}), 30.1 (ArCH₂Ar), 30.0 (C-2_{Hex}), 29.0 (CH₂-3-Arg), 27.2 (CMe_{2Pbf}, CMe₃), 25.8 (C-4_{Hex}), 25.5 (CH₂-4-Arg), 22.5 (C-5_{Hex}), 18.2, 17.0 (Me_{Pbf}), 13.0 (C-6_{Hex}), 11.1 (Me_{Pbf}) ; ESI-MS: m/z 1640.4 $[M + 2Na]^{2+}$; Anal. calcd for C₁₆₄H₂₄₀N₃₂O₂₈S₄: C 60.87, H 7.48, N 13.85, S 3.96. Found: C 60.80, H 7.43, N 13.89, S 3.82.

5,11,17,23-Tetra-(4-(L-arginine-N-amidomethyl)-1H-1,2,3triazol-1-methyl)-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene octahydrochloride (2e). A solution of 14 (20 mg, 6.18 µmol) in TFA-TIS-H₂O (95:2.5:2.5, 2 mL) was stirred at rt for 1 h. The solvent was removed under reduced pressure and the residue was washed with EtOAc (15 mL). Then the solid was dissolved in 0.1 M HCl solution followed by evaporation under reduced pressure to obtain the product as hydrochloride. Yield: 13 mg (99%). $[\alpha]_D = +21.4$ (c 1.0 in MeOH); ¹H NMR (300 MHz, CD₃OD): δ 8.21 (bs, 4 H, CH_{triazole}), 6.71 (bs, 8 H, Ar), 5.39 (bs, 8 H, CH_2 NHCO amide), 4.75–4.50 (m, 8 H, $ArCH_2$), 4.44 (d, J =13.2 Hz, ArC H_{ax} Ar), 4.00 (t, J = 7.39 Hz, 4 H, CH-2-Arg), 3.85 (t, J = 7.4 Hz, 8 H, CH_2 - 1_{Hex}), 3.27–3.08 (m, 12 H, CH_2 -5-Arg, $ArCH_{eq}Ar$,), 2.08–1.82 (m, 16 H, CH_2 -3-Arg, CH_2 -4-Arg), 1.78-1.61 (m, 8 H, CH₂-2_{Hex}), 1.53-1.33 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.00-0.90 (m, 12 H, CH₃-6_{Hex}); ¹³C NMR (75 MHz, CD₃OD): δ 170.2 (CO amide), 158.6 (CN guanidine), 158.2, 137.0 (Ar), 136.8 (C-4_{triazole}), 130.0, 129.8 (Ar), 129.7 (C-5_{triazole}), 76.6 (C-1_{Hex}), 55.6 (CH-2-Arg), 54.1 (CH₂NHCO), 41.8 (CH-5-Arg), 35.5 (CH₂Ar), 33.3 (C-3_{Hex}), 31.6 (ArCH₂Ar), 31.5 (C-2_{Hex}), 29.6 (CH₂-3-Arg), 27.3 (C-4_{Hex}), 25.4 (CH-4-Arg), 24.0 (C-5_{Hex}), 14.5 (C-6_{Hex}); ESI-MS: m/z 610.0 [M + 3H]³⁺, 914.4 $[M + 2H]^{2+}$, 936.3 $[M + 2Na]^{2+}$, 952.3 $[M + 2K]^{2+}$; Anal. calcd for $C_{92}H_{152}Cl_8N_{32}O_8\cdot 4H_2O$: C 50.45, H 7.36, N 20.47. Found: C 50.48, H 7.34, N 20.51.

5,11,17,23-Tetraaminomethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene tetrahydrochloride (20). To a solution of 18 (300 mg, 0.306 mmol) in EtOAc-EtOH (1:1, 50 mL), a catalytic amount of Pd/C (30 mg) and 1 M HCl (4 mL) were added. Hydrogenation was carried out at 2 atm for 48 h. Then, the catalyst was filtered and the solvent was removed under reduced pressure. Yield: 313 mg (99%); ¹H NMR (400 MHz, CD₃OD): δ 6.87 (s, 8 H, Ar), 4.49 (d, J = 13.2 Hz, 4 H, ArC H_{ax} Ar), 3.92 (t, J = 7.2 Hz, 8 H, CH_2-1_{Hex}), 3.84 (s, 8 H, CH_2NH_2), 3.31 (m, 4 H, ArC H_{eq} Ar), 2.00–1.85 (m, 8 H, C H_2 - 2_{Hex}), 1.55–1.30 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.00-0.85 (m, 12 H, CH₃-6_{Hex}); 13 C NMR (100.6 MHz, CD₃OD): δ 157.0–126.7 (Ar),

75.2 (C-1_{Hex}), 42.6 (CH_2NH_2), 31.9 (C-3_{Hex}), 30.3 (Ar CH_2Ar), 30.1 (C-2_{Hex}), 25.8 (C-4_{Hex}), 22.5 (C-5_{Hex}), 13.1 (C-6_{Hex}); ESI-MS: m/z 877.6 [M + H]⁺, 899.8 [M + Na]⁺; Anal. calcd for C₅₆H₈₈Cl₄N₄O₄: C 65.74, H 8.67, N 5.48. Found: C 66.91, H 8.37, N 5.62.

Heptakis[6-(2-(di-tert-butoxycarbonylguanidino)ethylthio)-2,3di-O-hexanoyl]cyclomaltoheptaose (21). To a solution of heptakis[2,3-di-O-hexanoyl-6-(2-aminoethylthio)]-cyclomaltoheptaose heptahydrochloride (8, 50 mg, 15 µmol) in dry DCM (5 mL), under an Ar atmosphere, Et₃N (61 µL, 440 µmol) and N-N'-ditert-butoxycarbonyl-N"-triflylguanidine (86 mg, 220 µmol) were added. The reaction mixture was stirred overnight. The reaction mixture was washed with an aqueous solution of 2 M KHSO₄. The organic layer was washed with a saturated aqueous solution of NaHCO3, dried (MgSO4), filtered and concentrated. The residue was purified by column chromatography (DCM \rightarrow 1:4 EtOAc-cyclohexane). Yield: 67 mg (99%); $R_{\rm f} = 0.75 \ (1:3 \ \text{EtOAc-cyclohexane}); \ [\alpha]_{\rm D} = +30.7 \ (c \ 1.0 \ \text{in}$ MeOH); ¹H NMR (300 MHz, CD₃OD, 313 K): 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), 4.26-4.15 (m, 7 H, H-5), 3.93 (t, 7 H, H-4), 3.78-3.53 (m, 14 H, H-6a, H-6b), 3.16 (bs, 14 H, CH₂NH), 2.98-2.85 (m, 14 H, CH₂S), 2.51-2.14 (m, 28 H, CH₂-2_{Hex}), 1.74-1.56 (m, 28 H, CH₂-3_{Hex}), 1.57-1.43 (bs, 126 H, CMe₃), 1.45-1.24 (m, 56 H, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.02-0.85 (m, 42 H, CH₃-6_{Hex}); ¹³C NMR (75 MHz, CD₃OD, 313 K): δ 174.5, 173.4 (CO ester), 164.6 (CN), 157.3, 154.2 (CO carbamate), 98.6 (C-1), 84.5 (C-4), 80.4 (CMe₃), 73.0 (C-5), 71.9 (C-3), 71.5 (C-2), 41.4 (C-6), 35.2, 35.0 (C-2_{Hex}, CH₂NH), 34.1 (CH₂S), 32.6, 32.5 (C-4_{Hex}), 28.9, 28.6 (CMe_3) , 25.6 $(C-3_{Hex})$, 23.6, 23.5 $(C-5_{Hex})$, 14.5 $(C-6_{Hex})$; ESI-MS: m/z 2332.2 [M + 2Na]²⁺, 1562.7 [M + 3Na]³⁺; Anal. calcd for C₂₁₇H₃₇₁N₂₁O₇₀S₇: C 56.43, H 8.10, N 6.37, S 4.86. Found: C 56.26, H 8.00, N 6.12, S 4.51.

Heptakis[6-(2-guanidinoethylthio)-2,3-di-O-hexanoyl]cyclomaltoheptaose heptahydrochloride (1f). A solution of 16 (60 mg, 13 μmol) in DCM-TFA (1:1, 2 mL) was stirred at rt for 3 h. The solvent was eliminated under reduced pressure and coevaporated several times with water. The residue was dissolved in a 0.1 M HCl solution and freeze-dried to yield the product as hydrochloride. Yield: 45 mg (99%); $[\alpha]_D = +68.3$ (c 1.0 in DMF); ¹H NMR (300 MHz, CD₃OD): δ 5.39 (t, 7 H, $J_{2,3}$ = $J_{3,4} = 9.4 \text{ Hz}, \text{ H-3}$, 5.18 (d, $J_{1,2} = 3.51 \text{ Hz}, 7 \text{ H}, \text{ H-1}$), 4.86 (m, 7 H, H-2), 4.16-4.07 (m, 7 H, H-5), 3.96 (t, 7 H, H-4), 3.48 (t, 14 H, CH₂NH), 3.20–3.11 (m, 14 H. H-6a, H-6b), 2.97–2.85 (m, 14 H, CH_2S), 2.55–2.21 (m, 28 H, CH_2 -2_{Hex}), 1.72–1.55 (m, 28 H, CH₂-3_{Hex}), 1.41-1.26 (m, 56 H, CH₂-4_{Hex}, CH₂-5_{Hex}), 0.99-0.87 (m, 42 H, CH₃-6_{Hex}); 13 C NMR (125.7 MHz, DMSO-d₆, 323 K): δ 172.2, 171.2 (CO), 156.8 (CN), 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 (CH₂NH), 33.1, 32.9 (C-2_{Hex},), 32.1 (CH₂S), 30.6, 30.4 (C-4_{Hex}), 23.6, 23.5 (C-3_{Hex}), 21.5 (C-5_{Hex}), 13.3, 13.2 (C-6_{Hex}); ESI-MS: m/z 1609.6 [M + $2H^{2+}$, 1073.0 [M + 3H]³⁺, 805.1 [M + 4H]⁴⁺; Anal. calcd for C₁₄₇H₂₆₆Cl₇N₂₁O₄₂S₇·7H₂O: C 49.06, H 7.84, N 8.17, S 6.24. Found: C 48.91, H 7.78, N 8.03, S 6.15.

5,11,17,23-Tetra-[*N*,*N*'-di-(*tert*-butoxycarbonyl)]-guanidinomethyl-25,26,27,28-tetrakis(*n*-hexyloxy)calix[4]arene (22). To

a solution of 20 (100 mg, 0.098 mmol) in dry DCM (5 mL), Et₃N (137 μL, 0.98 mmol) was added. Then, a solution of N-N'di-tert-butoxycarbonyl-N"-triflylguanidine (192 mg, 0.49 mmol) in dry DCM (1 mL) was added. The mixture was stirred at rt, under N2 atm, 48 h. The reaction mixture was washed with an aqueous solution of 2 M KHSO₄. The organic layer was washed with a saturated aqueous solution of NaHCO3, dried (MgSO₄), filtered and concentrated. The residue was purified by column chromatography (2:1 DCM-cyclohexane \rightarrow 1:5 EtOAc-cyclohexane). Yield: 165 mg (91%); $R_f = 0.24$ (1:6 EtOAc-cyclohexane); 1 H NMR (300 MHz, CDCl₃): δ 7.74 (bs, 4 H, NH), 7.55 (bs, 4 H, NH), 6.60 (s, 8 H, Ar), 4.38 (d, J = 12.8Hz, 4 H, ArC H_{ax} Ar), 4.27 (d, J = 4.4 Hz, 8 H, C H_2 NH), 3.84 (t, J =7.6 Hz, 8 H, CH_2-1_{Hex}), 3.10 (d, J = 13.2 Hz, 4 H, $ArCH_{eq}Ar$), 1.98-1.85 (m, 8 H, CH₂-2_{Hex}), 1.52-1.28 (m, 96 H, CH₂-3_{Hex}, CH_2 -4_{Hex}, CH_2 -5_{Hex}, CMe_3), 0.90 (t, J = 6.8 Hz, 12 H, CH_3 -6_{Hex}); ¹³C NMR (75 MHz, CDCl₃): δ 163.6 (CN), 156.1 (Ar), 155.7, 153.1 (CO carbamate), 135.1-127.9 (Ar), 86.0, 82.9 (CMe₃), 75.4 (C-1_{Hex}), 44.9 (CH₂NH), 32.2, 31.6 (C-3_{Hex}), 31.1 (ArCH₂Ar), 30.3 $(C-2_{Hex})$, 28.3, 27.1, 30.9 (CMe_3) , 26.0 $(C-4_{Hex})$, 22.9, 22.6 $(C-5_{Hex})$, 14.1 $(C-6_{Hex})$; ESI-MS: m/z 1846.7 $[M + H]^+$, 1868.7 $[M + Na]^+$, 1885.7 $[M + K]^+$; Anal. calcd for $C_{100}H_{156}N_{12}O_{20}$: C 65.05, H 8.52, N 9.10. Found: C 65.23, H 8.64, N 9.03.

5,11,17,23-Tetraguanidinomethyl-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene tetrahydrochloride (2f). To a solution of 17 (69 mg, 0.037 mmol) in dry dioxane (7 mL), TES (59 μL, 0.37 mmol) and 37% HCl (500 µL) were added. The mixture was stirred for 24 h at rt. Then, the solvents were evaporated under reduced pressure. The product was precipitated with Et₂O (6 mL) and dried under reduced pressure. Yield: 44 mg (99%); 1 H NMR (400 MHz, CD₃OD): δ 6.75 (s, 8 H, Ar), 4.49 (d, J = 13.2 Hz, 4 H, ArC H_{ax} Ar), 4.15 (s, 8 H, C H_2 NH), 3.93 (t, J =7.2 Hz, 8 H, CH_2 - 1_{Hex}), 3.23 (d, J = 13.2 Hz, 4 H, $ArCH_{eq}Ar$), 2.05-1.95 (m, 8 H, CH₂-2_{Hex}), 1.55-1.31 (m, 24 H, CH₂-3_{Hex}, CH₂-4_{Hex}, CH₂-5_{Hex}), 1.01-0.93 (m, 12 H, CH₃-6_{Hex}); ¹³C NMR (100.6 MHz, CD₃OD): δ 158.3 (CN), 157.6–129.0 (Ar), 76.5 (C-1_{Hex}), 45.9 (CH₂NH), 33.6 (C-3_{Hex}), 31.9 (ArCH₂Ar), 31.5 $(C-2_{Hex})$, 27.3 $(C-4_{Hex})$, 24.0 $(C-5_{Hex})$, 14.5 $(C-6_{Hex})$, ESI-MS: m/z1045.89 $[M + H]^+$; Anal. calcd for $C_{60}H_{96}Cl_4N_{12}O_4$: C 60.49, H 8.12, N 14.11. Found: C 60.13, H 7.95, N 13.89.

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