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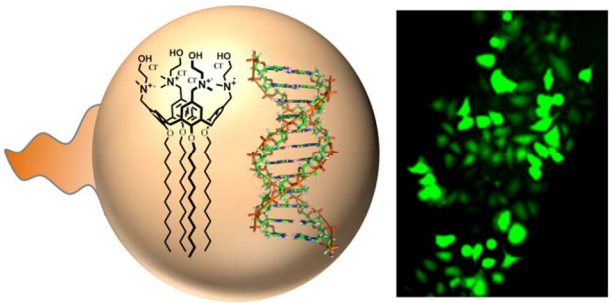

Cationic amphiphilic calixarenes with longer alkyl chains are less cytotoxic and their virus-sized DNA nanoparticles exhibit higher transfection efficiency.

Cationic amphiphilic calixarenes to compact DNA into small nanoparticles for gene delivery

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Abstract

Macrocycles have been attracting increasing attention as scaffolds for preparation of non-viral vectors for gene delivery. Here, following our earlier report, a series of amphiphilic calixarenes bearing cationic choline or N-(2-aminoethyl)-N,N-dimethylammonium groups at the upper rim and alkyl chains at the lower rim were synthesized. The effect of the length of aliphatic chains and the structure of the head group on their self-assembly, interaction with DNA, properties of their DNA complexes, transfection efficiency and cytotoxicity was studied. It was found that longer alkyl chains favor formation of small virus-sized DNA nanoparticles with low polydispersity. Moreover, longer alkyl chains, such as dodecyl groups, significantly improved the transfection efficiency, so that transfection was observed in the presence of fetal bovine serum as well as with or without helper lipid. Finally, we observed that cytotoxicity of these calixarenes clearly decreases with increase of the chain length. On the other hand, the presence of four additional amino groups, which could be protonated at pH<7 affected only the stoichiometry of the complexes with DNA without influencing their transfection efficiency or cytotoxicity. The results obtained provide new insights for designing non-viral vectors based on macrocyclic molecules.

Introduction

Development of nanoscale vehicles to deliver therapeutic genes remains an important challenge in the field of gene therapy.[1, 2] According to *in vitro* and *in vivo* studies, naked DNA and RNA exhibit a very poor capacity to transfect cells, because of a number of barriers and their decomposition by nucleases in biological media.[3-5] Therefore, efficient gene delivery requires specially designed nucleic acid carriers – gene delivery vectors.

Despite the success in the development of truncated viruses, so-called viral vectors,[6] the problem of their safety has stimulated the research on alternative concepts of gene delivery. For many decades significant efforts have been made by chemists to develop artificial DNA carriers, so-called non-viral vectors.[1, 7] Particularly successful were developments of cationic lipids[8-10] and polymers.[11-13] Their complexes with DNA, so-called lipoplexes and polyplexes, respectively show high transfection efficiency *in vitro*, weak immunogenicity and capacity to carry large quantities of genetic information,[1] though their application for gene therapy *in vivo* remains a challenge. One of the key problems of cationic lipids and polymers is the poor structural control of their complexes with DNA,[14] which are generally large infinite and polydisperse structures.[15-17]

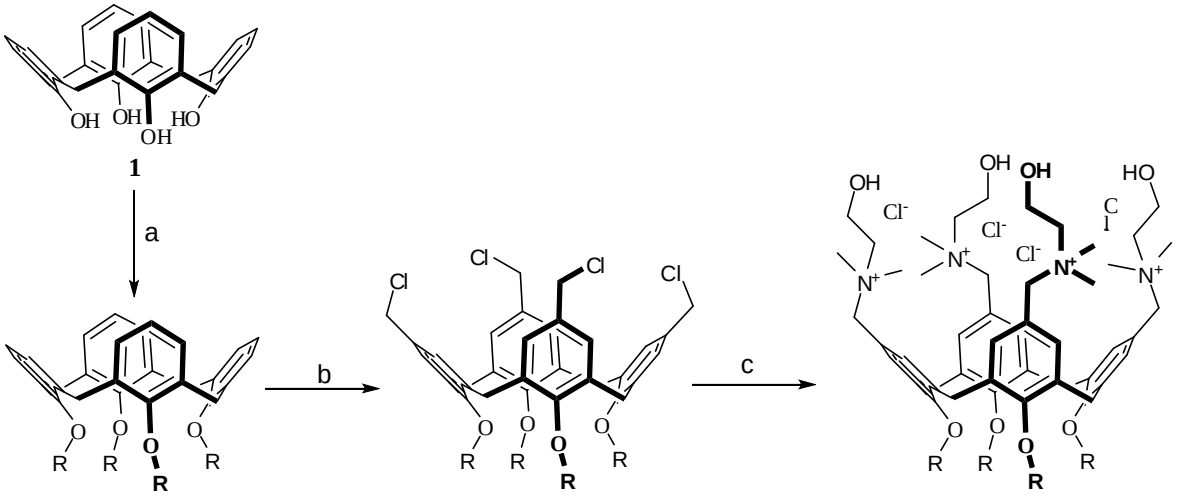
Therefore, the important unsolved problem is to control assembly of non-viral vectors with DNA into small nano-objects of defined structure and architecture that remains stable in biological media.[1, 18-21] A particularly fruitful approach is to employ pre-organized building blocks such as dendrimers,[22-24] gold nanoparticles,[25, 26] silica nanoparticles,[27] carbon nanotubes,[28, 29] and core-shell organic nanoparticles.[21] Among them, fullerenes[30-32] and macrocyclic molecules, such as cyclodextrins,[33-35] calix[4]resorcinarenes,[36-39] and calix[4]arenes[40, 41] are particularly promising because these synthetically available molecules have a well-defined 3D topology due to their persistent shape and capacity to bear multiple groups for interacting with nucleic acids.[42, 43] Recently, we described cationic amphiphiles based on calix[4]arenes and showed that their capacity to form micelles allows preparation of virus-sized DNA nanoparticles with satisfactory transfection efficiency.[44] Moreover recently, Ungaro et al, presented an arginine analogues calix[4]arenes with significantly improved transfection properties,[45] and confirmed that cationic amphiphilic calix[4]arenes are highly fruitful direction for development of nanoscopic non-viral vectors.

Here, we synthesized a series of amphiphilic calix[4]arenes with varied length of the alkyl chains and the nature of the cationic group. Our studies showed that calix[4]arenes derivatives with clear amphiphilic properties are capable to condense DNA into very small nanoparticles. Importantly, the derivatives bearing relatively long chains exhibited higher transfection efficiency together with lower cytotoxicity. Thus, the structure-property relationships were described suggesting new insights for the rational design of non-viral vectors based on macrocycles.

Results and discussion

Design of calix[4]arenes

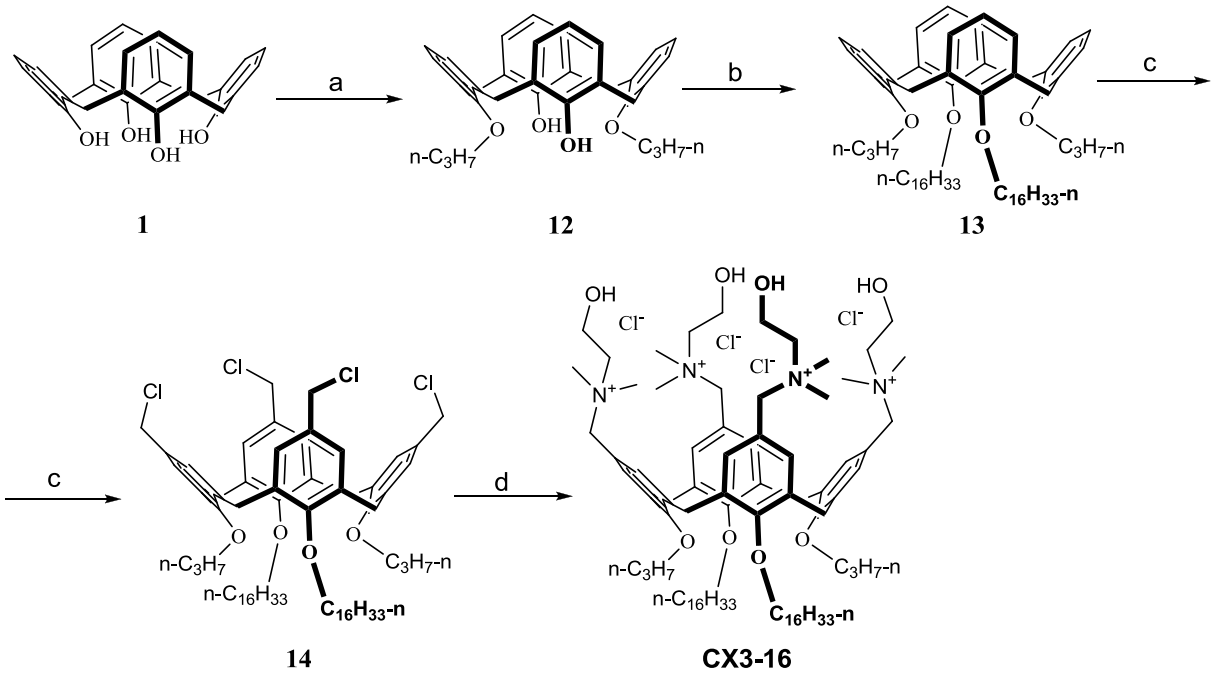
The design is based on our previous work,[44] where the upper and lower rims are functionalized by four cationic groups and aliphatic chains, respectively. A series of new derivatives with aliphatic chains of varied length and with different cationic groups was synthesized. Calixarenes with hexyl, dodecyl and hexadecyl alkyl chains were synthesized as the previously described CX3 and CX8 compounds, according to scheme 1.



a: Alk-Br, DMSO-NaOH; b: CH_3-O-CH_2Cl, $SnCl_4$; c: $(CH_3)_2$N-CH_2-CH_2-OH

Scheme 1. Synthetic route to cationic calixarenes CX3-CX16.

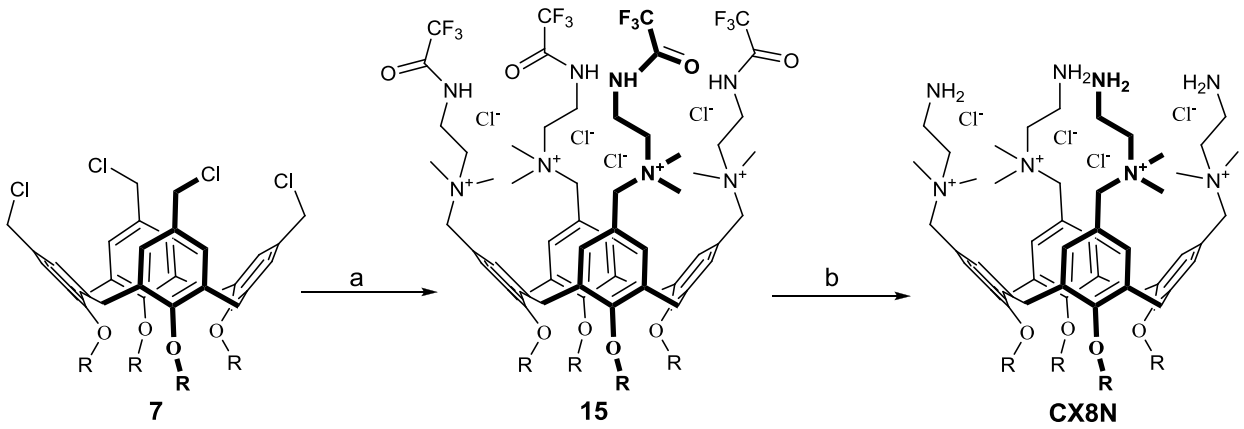
The key step of synthesis is the quaternization reaction, which was done after alkylation of **1** and further chloromethylation. This quaternization was performed in dry THF in all cases. Obtained calixarenes precipitated directly from the reaction mixture despite the presence of highly lipophilic groups. Water solubility of obtained calixarenes was also decreased with increase of alkyl chain length. Thus, CX16 was practically insoluble in water and even in neat DMSO, and was only soluble in methanol and DMSO-chloroform mixtures. To circumvent the solubility problem, we shortened two of the four alkyl chains from hexadecyl to propyl. Calixarene CX3-16 was synthesized according to scheme 2 and required an intermediate step, where two propyl groups were initially introduced into distal position of the lower rim of **1**.



a: n-C_3H_7Br, DMF-K_2CO_3; b: $C_{16}H_{33}$Br, DMSO-NaOH; c: CH_3-O-CH_2Cl, $SnCl_4$; d: $(CH_3)_2$N-CH_2-CH_2-OH

Scheme 2. Synthetic route to calixarene CX3-16.

In our earlier work, we showed that substitution of tetrahedral tetraalkylammonium with planar N-alkyl imidazolium did not influence the transfection efficiency.[44] Therefore, we changed the strategy by replacing the hydroxyethyl group by an aminoethyl group as shown in scheme 3 for calixarene CX8N. These amino groups being in proximity of quaternized ammonium groups should be much less basic than aliphatic amines, so that they could be protonated at the pH of lysosomes, similarly to polyethyleneimine.[46] Introduction of these groups to the macrocycle upper rim was performed by reacting tetrachloromethyl calixarene **7** with trifluoroacetamide of N,N-dimethyl ethylenediamine. The use of unprotected amine in this reaction was unsuccessful, leading to a complex mixture of compounds. Interestingly, addition of a relatively polar amide group to the tetracationic calixarene significantly increased its solubility in mid polar organic solvents. Besides alcohols, DMF and DMSO, calixarene **15** was soluble in THF, chloroform, acetonitrile, but insoluble in diethyl ether. This feature was used for its isolation. Deacylation of **15** was completed in mild conditions by water ammonia. The final product CX8N could not be easily isolated from trifluoroacetate ammonia.



a: $CF_3C(O)NH\text{-}CH_2\text{-}CH_2\text{-}N(CH_3)_2$; b: NH_3, $CH_3OH\text{-}H_2O$

Scheme 3. Synthetic route to calixarene CX8N.

Self-assembly

All synthesized calixarenes (except CX16) showed good water solubility. As these amphiphiles are expected to self-assembly into micelles,[44] we studied their critical micellar concentration (CMC). Pyrene was used as an environmentally-sensitive fluorescent probe molecule.[47] The CMC was determined by plotting the ratio of I_3/I_1 peaks of pyrene (at 373 and 384 nm, respectively) as a function of the calixarene concentration. The typically plot presents sigmoidal shape of the curve, as shown in Figure 1.

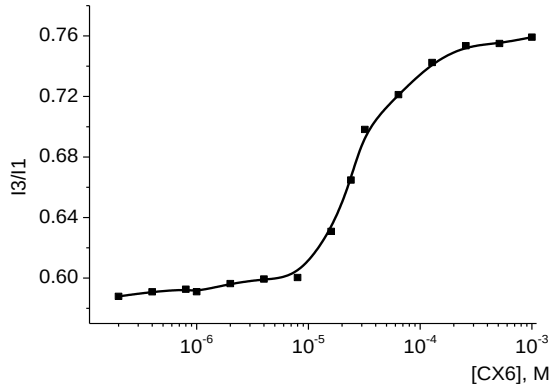


Fig. 1. Intensity ratio I_3/I_1 of pyrene ($1.5 \cdot 10^{-7}$ M) *vs* CX6 concentration in 20 mM Tris buffer, pH 7.4.

The CMC values deduced from the point of growth of the I_3/I_1 ratio were found to decrease with the increase in the chain length (Table 1), as expected. However, for calixarenes with long alkyl chains (CX12, CX16 and CX3-16), the obtained CMC values were not significantly lower than that of CX8 (1-4 µM), and were dependent on the pyrene concentration. We concluded that for these calixarenes, we could not obtain reliable data, because the used pyrene concentration was much higher than their CMC value. As a result, the point of I_3/I_1 ratio growth cannot be easily observed because of the large excess of free pyrene molecules as compared to those bound to micelles. Remarkably, the I_3/I_1 ratio also indicates the lipophilicity level of the pyrene environment in the micelles. According to these data, the least lipophilic binding sites were observed for CX8N micelles ($I_3/I_1 \leq 0.72$), whereas the most lipophilic ones were observed for CX3-16 micelles ($I_3/I_1 \geq 0.83$).

Table 1. CMC and I_3/I_1 ratio values of investigated calixarenes in different media.[a]

	Water		pH 7.4 buffer		pH 7.4 buffer, 150 mM NaCl		pH 5.0 buffer	
	CMC µM	I_3/I_1 Ratio	CMC, µM	I_3/I_1 ratio	CMC, µM	I_3/I_1 ratio	CMC, µM	I_3/I_1 ratio
CX3	390	0.79	68	0.82	64	0.82	–	
CX6	26	0.76	9.8	0.76	4.4	0.81	–	
CX8	19	0.73	4.4	0.75	2.7	0.78	–	
CX8N	17	0.72	3.6	0.69	3.0	0.70	14	0.69
CX3-16	0.75	0.84	1.0	0.83	–	0.83	–	

[a] pH 7.4 referes to 20 mM phosphate buffer (pH 7.4), pH 5.0 referes to 20 mM Acetate buffer (pH 5.0).

Next, the hydrodynamic diameter and polydispersity of the obtained micelles were evaluated by dynamic light scattering (DLS). Obtained values of the hydrodynamic diameter in the range 5-8 nm (Table 2) confirm the micellar nature of the assemblies, as in our earlier work on CX8.[44] Remarkably, the size of the micelles increases with the chain length. Noticeably, CX12 gives smaller particles than CX3-16, though the latter has 38 carbon atoms in their alkyl chains while CX12 has 48 atoms. The micelles of CX8N are larger than those of CX8, probably due to the larger hydration shell of amino groups as compared to hydroxyls. At pH 5, the size of the CX8N micelles was further increased probably due to protonation of the amino groups. The relatively high polydispersity observed in several samples is likely explained by the presence of a small quantity of large particles (1000-5000 nm), which could be observed in the DLS data analysis by scattering

intensity. As DLS is much more sensitive to larger particles (scattering intensity is proportional to the sixth power of the particle size), the detection of very small objects is usually perturbed even by traces of large particles. According to statistics by volume, only the micellar fraction was systematically observed for our calixarenes. In contrast, CX16 showed two populations of particles: a fraction of 30-35 nm size which slowly transforms into larger particles of 300-400 nm size. Thus, CX16 bearing much longer lipophilic chains is unable to form micelles, probably because the volume taken by these chains is comparable or larger to that taken by the polar head groups. This molecular topology is not suitable for micellar assembly and probably favors formation of larger structures.

Table 2. Size and polydispersity of calixarene micelles and calixarene-DNA complexes obtained from DLS data.[a]

Calixarene	Alone		With pCMV-eGFP[b]				With CT-DNA[b]			
			N/P 2		N/P 5		N/P 2		N/P 5	
	d, nm	PdI	*d*, nm	PdI	*d*, nm	PdI	*d*, nm	PdI	*d*, nm	PdI
CX3	2.93	0.42	-	-	-	-	1090	*0.44*	1280	*0.57*
CX6	5.47	0.68	1380	*0.46*	66	*0.12*	1250	*0.64*	90	*0.13*
CX8	5.63	0.25	72	*0.12*	51	*0.11*	115	*0.12*	76	*0.10*
CX8N	6.07	0.34	154	*0.14*	62	*0.10*	1070	*0.50*	112	*0.09*
CX8N pH 5.0	6.56	0.51	-	-	59	*0.25*	103	*0.17*	76	*0.15*
CX12	7.37	0.30	117	*0.13*	55	*0.10*	106	*0.14*	76	*0.04*
CX3-16	8.28	0.17	107	*0.12*	57	*0.10*	102	*0.11*	75	*0.10*

[a] *d* is hydrodynamic diameter, defined by volume statistics; PdI is polydispersity index. All measurements were done in 20 mM Tris buffer at pH 7.4, unless indicated (20 mM acetate buffer for pH 5). [b] 30 μM by phosphate groups.

Interaction with DNA

Gel-electrophoresis was used to study the complexation of calixarenes with DNA. Mixtures of pDNA with calixarenes at N/P ratios 0, 0.2, 0.5, 1, 2 and 5 were prepared in Tris-buffer and tested

after 30 minutes of incubation. For CX8N acetate buffer with pH 5 was also investigated. Signal of free pDNA disappeared at N/P 1 for all studied calixarenes (Fig. 2) indicating the efficient complex formation independently of the calixarene structure. Remarkably, for CX8N at pH 5.0, complex formation is observed already at N/P 0.5 (figure 3B), because full protonation of amino groups of CX8N is expected to increase twice its charge.

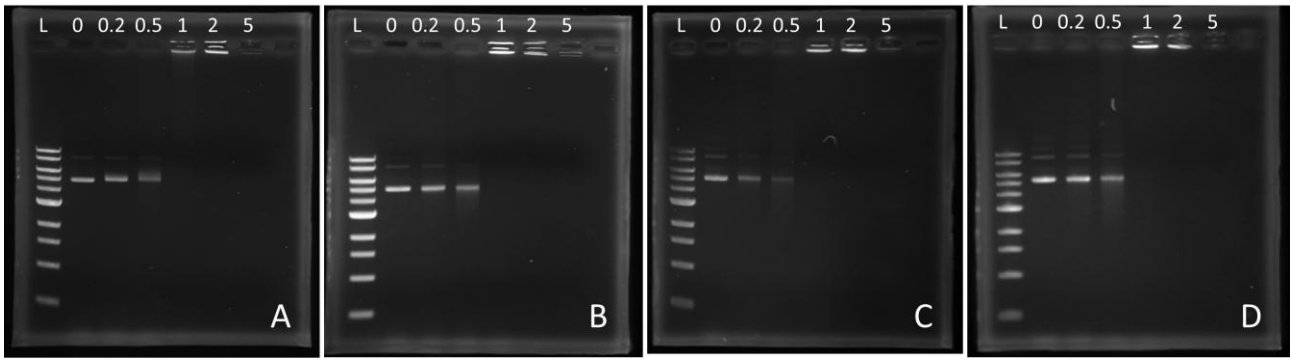


Fig. 2. Gel electrophoresis of pDNA-calixarene complexes at different N/P ratios. A – CX6, B – CX8, C – CX12, D – CX3-16. L is the ladder standard.

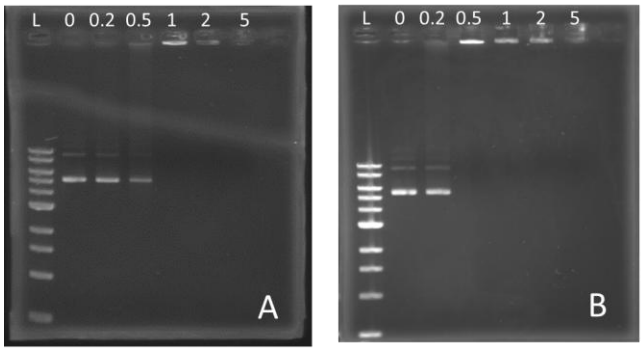


Fig. 3. Gel electrophoresis of pDNA-CX8N complexes at different N/P ratio. (A) pH = 7.4 (20 mM Tris buffer), (B) pH = 5.0 (20 mM acetate buffer). L is the ladder standard.

Interaction of DNA with transfection agents and its further condensation can be monitored using ethidium bromide displacement assay. For all studied calixarenes, a rapid decrease of ethidium bromide fluorescence was observed at N/P ratios around 1 (Fig. 4). Above N/P 2, the fluorescence was nearly constant showing that DNA condensation was completed around N/P 2. The only exception was CX8N, especially at pH 5, where the plateau was reached at N/P 1, confirming the results of gel electrophoresis. The final values of intensity for CX8N were significantly lower than for other calixarenes indicating that the additional positive charges favor stronger DNA condensation.

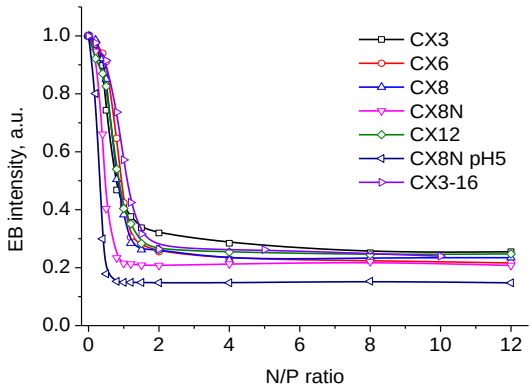


Fig. 4. Exclusion of ethidium bromide (0.4 μM) from CT-DNA (20μM) in the presence of calixarenes. Dependence of ethidium bromide intensity from calixarene concentration expressed in N/P ratio. Concentrations of ethidium bromide and CT-DNA were 0.4 μM and 20 μM, respectively, in 20 mM Tris buffer (pH 7.4) or 20 mM acetate buffer (pH 5.0).

Size of calixarene-DNA complexes

For all calixarenes, except CX3 we observed small, stable, and unimodal particles at N/P ratio 5 (Table 2). At N/P 2, the particle size is larger, especially for CX6 and CX8N, which shows microscopic particles. According to ethidium bromide data, the DNA condensation is completed at N/P > 2. Therefore, the cationic agent should be in excess at higher N/P ratios, which increases the positive charge of the complexes and thus favors their small size due to electrostatic repulsion between particles. Moreover, the size of the complexes is smaller for pDNA compared to CT-DNA, which correlates with the smaller size of the former. The effect of calixarene structure on the size of complexes was observed mainly at N/P ratio 2, where CX6 and CX8N form larger complexes than all other new calixarenes. In this respect, CX6 behaves similarly to CX3, likely as a result of its insufficiently long aliphatic chains. Short chains decrease the capacity of calixarenes to form micelles, which are building blocks for small DNA nanoparticles.[44] Moreover, other reports suggested that analogues of CX3 can form much larger structures, such as vesicles,[48] and thus unable to assemble with DNA into small nanoparticles. In case of CX8N, the additional amino groups may induce additional interactions with nucleic acids through H-bonding, which could be a factor producing aggregation of DNA into large particles.

Transfection study and cytotoxicity

According to our previous data with CX8, the transfection efficiency is significant only in the presence of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). Therefore, we tested the transfection efficiency of all new calixarenes together with CX8 in the presence of DOPE (1 mol eq.) at N/P ratio 2. A plasmid encoding eGFP (pCMV-eGFP) was used for transfection, so that transfected cells were detected by monitoring the expressed GFP using fluorescence microscopy (Fig. 5). Remarkably, the most promising results were observed for CX12, which shows much higher rate of transfected cells (~80%) than the previously reported CX8 (~36%), being comparable to that of jetPEI (~70%), one of the most efficient transfection agents. The rate of transfected cells grows in the following order: CX8N<CX6<CX3-16~CX8<CX12 (Fig. 6). In the presence of serum, only CX12 showed detectable transfection among the studied calixarenes (Fig. 5). Moreover, CX12 was the only calixarene that showed detectable transfection without DOPE. Thus, the long alkyl chains and the large hydrophobic domain of CX12 significantly improve the transfection efficiency.

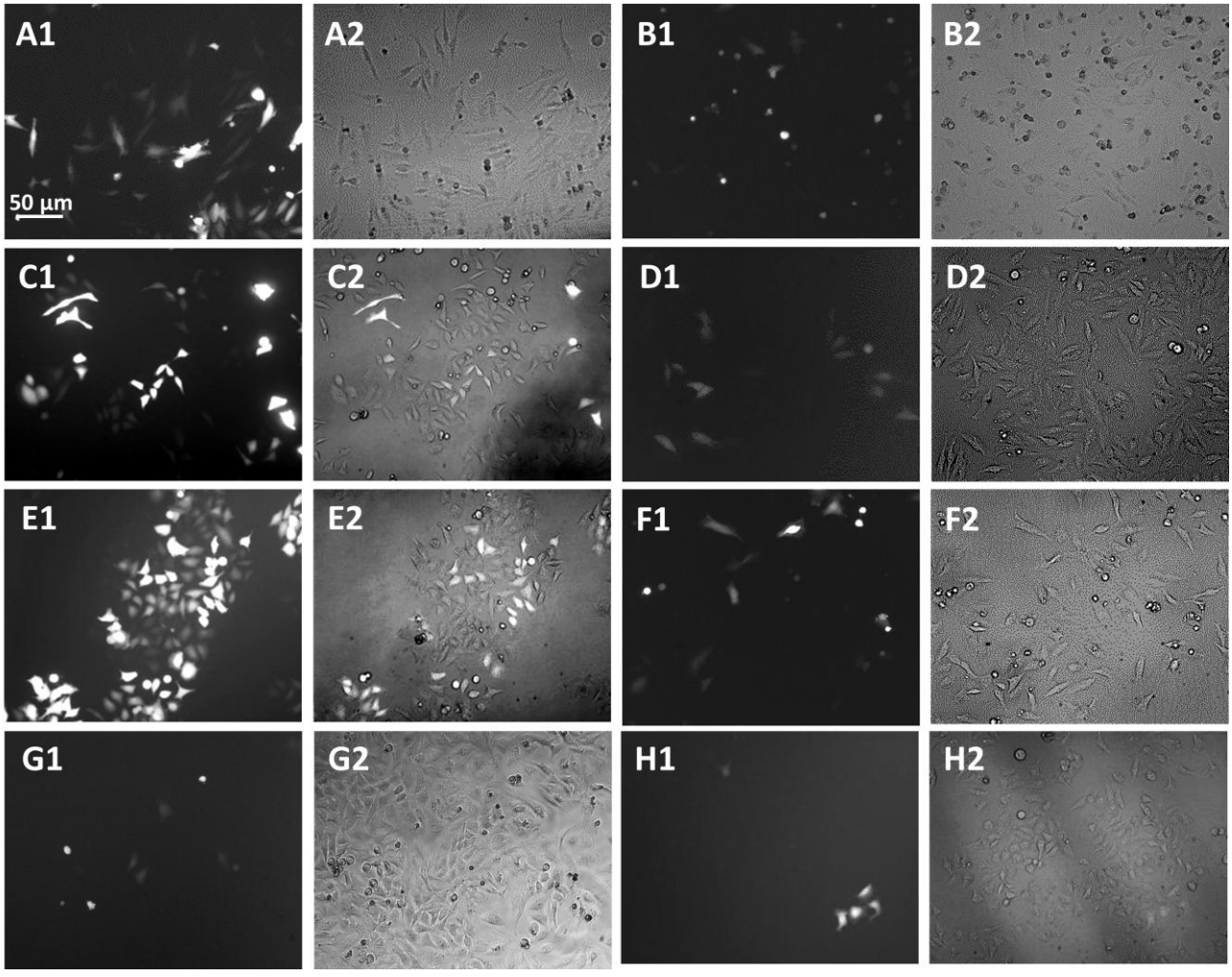


Fig. 5. Fluorescence (A1, B1, C1, D1, E1, F1, G1, H1) and transmission (A2, B2, C2, E2, F2, G2, H2) images of HeLa cells transfected with pCMV-eGFP plasmid complexed with calixarene (N/P 2 with 1eq. of DOPE) or commercial JetPEI. (A) JetPEI; (B) CX6; (C) CX8; (D) CX8N; (E) CX12;

(F) CX3-16; (G) CX12 without DOPE; (H) CX12 in the presence of fetal bovine serum. The images were recorded 48 h after transfection.

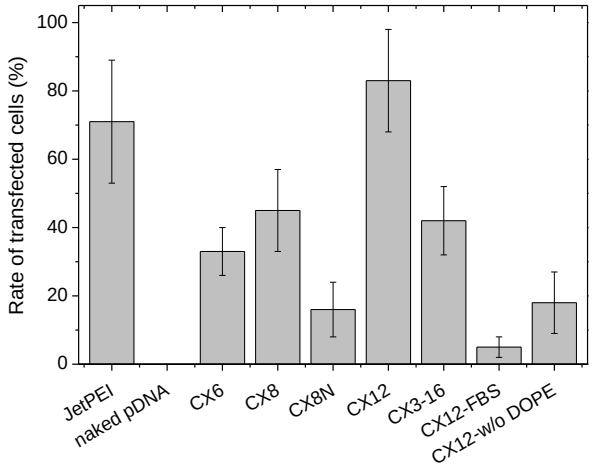


Fig. 6. Rate of transfected cells for calixarenes complexed with pDNA (pCMV-eGFP) in the presence of 1eq. DOPE compared to commercial agent jetPEI and naked pDNA. The complexes or naked DNA were first incubated for 3h with HeLa cells in Opti-MEM without FBS and then for additional 45h with 10% FBS. For CX12, the data with 10% FBS (for all 48h) and the data without DOPE (w/o DOPE) are shown.

Finally, the toxicity of new calixarenes was tested on HeLa cells using MTT assay. The cytotoxicity decreases rapidly with the increase in the length of the aliphatic chains for all concentrations studied, i.e. the following tendency was observed: CX6>CX8~CX8N>CX12>CX3-16. The high cytotoxicity of CX6 is in line with the systematic observation of high cell mortality in the transfection experiments with this calixarene (see Fig. 5B2). However, in these measurements the first 3h of incubation were done in Opti-MEM solution without serum and the remaining 45h with 10% of serum. When the incubation was done in the presence of serum for all 48h, the cytotoxicity of all calixarenes was significantly decreased (Fig. 5B). Nevertheless, the calixarenes with the longest aliphatic chains (CX12 and CX3-16) showed the lowest cytotoxicity. This is the first observation of a clear effect of the aliphatic chain length on the cytotoxicity of calixarenes. Longer aliphatic chains decrease the CMC of CX12 and CX3-16, so that the concentration of free molecules is lower than for calixarenes with shorter chains. Moreover, due to high lipophilicity, CX12 and CX3-16 may stronger interact with the lipid components of the cells, which may prevent them from interacting with other biomolecules, such as proteins and nucleic acids. Thus, CX12, presenting the most hydrophobic domain, exhibited higher transfection efficiency combined with lower cytotoxicity, compared to previously reported CX8.

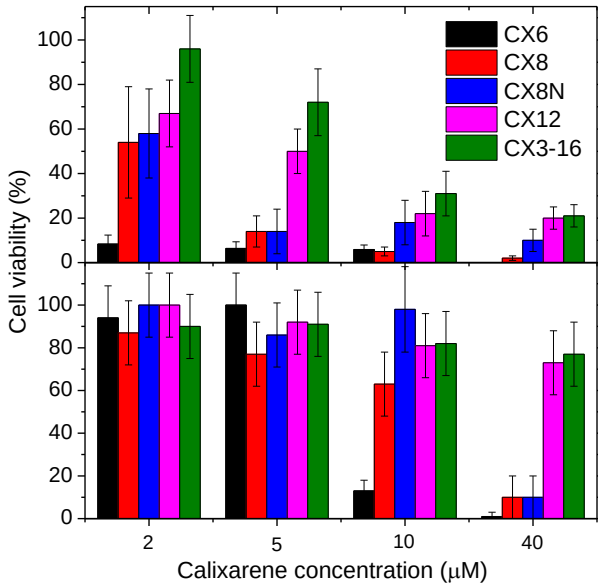


Fig. 7. Cytotoxicity of calixarenes without (A) and with 10% of serum (B) after 3h of incubation. Cell viability was determined by MTT assay after 48 h of incubation.

Conclusions

A series of amphiphilic calixarenes bearing cationic groups at the upper rim and alkyl chains at the lower rim were synthesized. The effect of the length of aliphatic chains and the head group structure on their self-assembly, interaction with DNA, properties of their DNA complexes, transfection efficiency and cytotoxicity was studied. It was found that longer alkyl chains favor formation of smaller particles with DNA, characterized by higher transfection efficiency and lower cytotoxicity. On the other hand, the presence of four additional amino groups, which could be protonated at pH<7 affected only the stoichiometry of the complexes with DNA without affecting their transfection efficiency or cytotoxicity. The results obtained provide new insights for designing new artificial viruses based on macrocyclic molecules.

Experimental

Synthesis

All reactions were carried out in anhydrous solvent under dry atmosphere. NMR spectra were recorded on a spectrometer "Varian VXR-300" using hexamethyldisiloxane and tetramethylsilane as internal standards. Chemical shifts are given in δ scales (^{1}H and ^{19}F). Mass spectra were recorded using Mass Spectrometer Mariner System 5155.

Tetraalkoxy-calixarenes **2**, **4**, **6** were obtained accordingly literature data.[49] Dipropoxycalixarene **12** was synthesized and described earlier.[50] Synthesis of tetrachloromethylcalixarenes **3** and **7** as well as calixarenes CX3 and CX8 was described in literature previously.[51, 52]

General procedure for synthesis of tetraalkoxy-calixarenes 8, 10 and 13. Sodium hydroxide (50% water solution, 3.53 ml, 50.05 mmol NaOH) and then calixarene **1** or **12** (5.40 mmol) were added to DMSO (25 mL). The reaction mixture was stirred for 15 minutes. Alkyl bromide (45.0 mmol) was added in three portions every 30 minutes and the mixture was stirred for 8-12 h at 70–80 °C. After cooling to 20 °C the diluted hydrochloric acid was poured into the reaction mixture. Compounds **8**, **10**, **13** were extracted by chloroform, dried with sodium sulfate. Chloroform was evaporated and the residue was washed with methanol.

25,26,27,28-Tetradodecyloxycalix[4]arene (8). Crude product was dried in *vacuo* (0.5 mm Hg) at 120 °C for 3 hours and crystallized from acetonitrile. Obtained as colorless solid. Yield 96%. M.p. 52 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.60 (d, J = 5.3 Hz, 8H, *m*-ArH), 6.59 (t, J = 5.3 Hz, 4H, *p*-ArH), 4.42 (d, J = 13.2 Hz, 4H, $ArCH_{ax}Ar$), 3.87 (t, J = 7.5 Hz, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_9$-CH_3), 3.17 (d, J = 13.2 Hz, 4H, $ArCH_{eq}Ar$), 1.86 (brm, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_9$-CH_3), 1.44-1.24 (brs, 72H, O-CH_2-CH_2-(C$\underline{H}_2$)$_9$-CH_3), 0.89 (t, J = 6.5 Hz, 12H, O-CH_2-CH_2-$(CH_2)_9$-C$\underline{H}_3$) ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.32, 134.90, 127.81, 121.58, 74.89, 31.72, 30.75, 30.10, 29.77, 29.74, 29.62, 29.58, 29.52, 29.20, 26.14, 22.46, 13.86.

25,26,27,28-Tetrahexadecyloxycalix[4]arene (10). Crude product was recrystallized twice from acetonitrile. Obtained as colorless solid. Yield 83%. M.p. 75-75.5 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.59 (d, J = 5.5 Hz, 8H, *m*-ArH), 6.57 (t, J = 5.5 Hz, 4H, *p*-ArH), 4.42 (d, J = 13.2 Hz, 4H, $ArCH_{ax}Ar$), 3.87 (t, J = 7.3 Hz, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_{13}$-CH_3), 3.16 (d, J = 13.2 Hz, 4H, $ArCH_{eq}Ar$), 1.85 (brm, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.44-1.21 (brs, 104H, O-CH_2-CH_2-(C$\underline{H}_2$)$_{13}$-CH_3) 0.88 (t, J = 6.4 Hz, 12H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$) ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.60, 135.16, 128.07, 121.83, 75.14, 31.96, 31.03, 30.00, 29.89, 29.85, 29.83, 29.81, 29.79, 29.72, 29.41, 26.40, 22.71, 14.12.

25,27-Dihexadecyloxy-26,28-dipropoxycalix[4]arene (13). Macrocycle **13** was precipitated from iso-propanol-ethanol (1:5, 50 ml), then crystallized from this mixture and obtained as pale-yellow solid, which contains near 20% (weight) hexadecylbromide. Yield 39%. M.p. 68 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 8.57 (m, 12H, *m*-ArH and *p*-ArH), 4.42 (d, J = 13.3 Hz, 4H, $ArCH_{ax}Ar$), 3.87 and 3.84 (two t, J = 7.8 Hz, 8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_{13}$-CH_3), 3.16

(d, J = 13.3 Hz, 4H, $ArCH_{eq}Ar$), 1.91 (brm, 4H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.26 (brs, 52H, O-CH_2-CH_2-(C$\underline{H}_2$)$_{13}$-CH_3) 0.98 (t, J = 7.4 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$) 0.88 (t, J = 6.9 Hz, 6H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$) ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.62, 156.56, 135.18, 135.09, 128.10, 128.05, 121.83, 75.09, 70.97, 31.93, 31.00, 30.25, 29.85, 29.79, 29.73, 29.71, 29.68, 29.63, 29.52, 29.37, 26.26, 26.21, 23.29, 22.70, 14.11, 10.39.

General procedure for synthesis of tetrachlorometnyl-calixarenes 5, 9, 11 and 14. To a solution of calixarene **4, 8, 10** (1 mmol) in dry chloroform (40 ml) or **13** in mixture of dry chloroform (40 ml) and dry n-hexane (20 ml) was added methyl-chloromethyl ether (50 mmol) and the resulting solution was cooled to -60 °C under argon atmosphere. Tin tetrachloride (8 mmol) was added dropwise to the mixture under vigorous stirring. Agitation was continued for 30 min at temperatures -60 °C, and then the mixture was allowed to reach a room temperature. Then, distilled water (25 ml) was added and the mixture was stirred to color fading. Organic layer was separated, washed with 3% hydrochloric acid (20 ml), water (20 ml) and brine (20 ml) and then dried overnight by sodium sulfate. Crystallization (from acetonitrile) of yellow solid obtained after solvent removal gave calixerenes **5, 9, 11 or 14.**

5,11,17,23-Tetrachloromethyl-25,26,27,28-tetrahexyloxycalix[4]arene (5). Obtained as pale yellow solid. Yield 73%. M.p. 178-179 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.65 (s, 8H, *m*-ArH), 4.38 (d, J = 13.2 Hz, 4H, $ArCH_{ax}Ar$), 4.30 (s, 8H, Ar-C$\underline{H}_2$Cl), 3.87 (t, J = 7.5 Hz, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_3$-CH_3), 3.16 (d, J = 13.2 Hz, 4H, $ArCH_{eq}Ar$), 1.89 (brm, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_3$-CH_3), 1.44-1.25 (brs, 24H, O-CH_2-CH_2-(C$\underline{H}_2$)$_3$-CH_3) 0.92 (t, J = 6.6 Hz, 12H, O-CH_2-CH_2-$(CH_2)_3$-C$\underline{H}_3$), ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.77, 135.13, 130.86, 128.53, 75.39, 46.62, 32.02, 30.89, 30.19, 25.88, 22.82, 14.06.

5,11,17,23-Tetrachloromethyl-25,26,27,28-tetradodecyloxycalix[4]arene (9). Obtained as pale-yellow solid. Yield 42%. M.p. 69 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.64 (s, 8H, *m*-ArH), 4.37 (d, J = 13.2 Hz, 4H, $ArCH_{ax}Ar$), 4.30 (s, 8H, Ar-C$\underline{H}_2$Cl), 3.86 (t, J = 7.5 Hz, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_9$-CH_3), 3.15 (d, J = 13.2 Hz, 4H, $ArCH_{eq}Ar$), 1.86 (brm, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_9$-CH_3), 1.44-1.12 (brs, 72H, O-CH_2-CH_2-(C$\underline{H}_2$)$_9$-CH_3) 0.88 (t, J = 6.3 Hz, 12H, O-CH_2-CH_2-$(CH_2)_9$-C$\underline{H}_3$) ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.43, 134.77, 130.50, 128.17, 75.04, 46.25, 31.60, 30.53, 29.92, 29.62, 29.58, 29.49, 29.45, 29.39, 29.07, 25.95, 22.34, 13.74.

5,11,17,23-Tetrachloromethyl-25,26,27,28-tetrahexadecyloxycalix[4]arene (11). Obtained as colorless solid. Yield 50%. M.p. 86 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.65 (s, 8H, *m*-ArH), 4.38 (d, J = 13.4 Hz, 4H, $ArCH_{ax}Ar$), 4.30 (s, 8H, Ar-C$\underline{H}_2$Cl), 3.86 (t, J = 7.3 Hz, 8H, O-C$\underline{H}_2$-

CH_2-$(CH_2)_{13}$-CH_3), 3.15 (d, J = 13.4 Hz, 4H, $ArCH_{eq}Ar$), 1.86 (brm, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.41-1.19 (brs, 104H, O-CH_2-CH_2-$(C\underline{H}_2)_{13}$-CH_3) 0.88 (t, J = 6.7 Hz, 12H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$), ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.42, 134.76, 130.51, 128.17, 75.03, 46.24, 31.58, 30.54, 29.92, 29.62, 29.58, 29.49, 29.46, 29.45, 29.42, 29.41, 29.34, 29.03, 25.95, 22.33, 13.74.

5,11,17,23-Tetrachloromethyl-25,27-dihexadecyloxy-26,28-dipropoxycalix[4]arene (14). Obtained as a colorless solid. Yield 93%. M.p. 89 °C. ^{1}H NMR ($CDCl_3$, 299.94 MHz), δ ppm: 6.65 and 6.64 (two s, 8H), 4.38 (d, J = 13.4 Hz, 4H, $ArCH_{ax}Ar$), 4.29 (brs, 8H, Ar-C$\underline{H}_2$Cl), 3.86 and 3.83 (two t, J = 7.5 Hz, 8H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_{13}$-CH_3), 3.16 (d, J = 13.4 Hz, 4H, $ArCH_{eq}Ar$), 1.90 (brm, 4H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.26 (brs, 52H, O-CH_2-CH_2-$(C\underline{H}_2)_{13}$-CH_3) 0.98 (t, J = 7.5 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$), 0.88 (t, J = 6.7 Hz, 6H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$), ^{13}C NMR ($CDCl_3$, 125.73 MHz), δ ppm: 156.77, 156.76, 135.12, 135.10, 130.89, 130.86, 128.56, 75.35, 70.96, 46.60, 31.92, 30.89, 30.16, 29.82, 29.78, 29.76, 29.71, 29.66, 29.61, 29.51, 29.36, 26.20, 26.18, 23.23, 22.69, 14.10, 10.30

General procedure for synthesis of calixarenes CX6, CX12, CX16 and CX3-16. To a solution of tetrachloromethylcalix[4]arene **5, 9, 11** or **14** (1 mmol) in tetrahydrofuran (20 ml) a solution of N,N-dimethylethanolamine (5 mmol) in THF (5 ml) was added under stirring. The reaction mixture was refluxed for 18 hrs. After cooling, the precipitate of calixarene **CX6, CX12, CX16** or **CX3-16** formed was filtered off. The product was dried in vacuum (0.05 mm Hg, 20 °C, 4 hrs) to give a microcrystalline compound.

5,11,17,23-Tetra(N,N-dimethyl-N-hydroxyethylammonium)-methylene-25,26,27,28-tetrahexyloxycalix[4]arene tetrachloride (CX6). Obtained as a pale-yellow microcrystalline hygroscopic compound. Yield 74%. M.p. 270-273 °C (decomp.) ^{1}H NMR ($(CD_3)_2S$=O, 299.94 MHz), δ ppm: 6.99 (s, 8H, *m*-ArH), 5.76 (brs, 4H, OH), 4.54 (s, 8H, Ar-C$\underline{H}_2$-N), 4.34 (d, J = 12.8 Hz, 4H, $ArCH_{ax}Ar$), 3.88 (brt, 16H, O-C$\underline{H}_2$-CH_2-$(CH_2)_3$-CH_3, C$\underline{H}_2$-OH), 3.29 (brs, 12H, $ArCH_{eq}Ar$, N-C$\underline{H}_2$), 2.95 (s, 24H, N-CH_3), 1.90 (m, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_3$-CH_3), 1.45-1.21 (brs, 24H, O-CH_2-CH_2-$(C\underline{H}_2)_3$-CH_3) 0.89 (brt, 12H, O-CH_2-CH_2-$(CH_2)_3$-C$\underline{H}_3$), ^{13}C NMR ($(CD_3)_2S$=O, 125.73 MHz), δ ppm: 157.28, 134.69, 133.23, 121.93, 75.03, 66.78, 64.22, 54.80, 49.76, 31.53, 29.80, 29.75, 25.39, 22.28, 13.85. MS: (m/z) found $[M\text{-}2Cl^-]2^+$ = 1238.8496 (calcd for $C_{72}H_{120}Cl_2N_4O_8^{2+}$; $[M\text{-}2Cl^-]^{2+}$ = 1238.8483).

5,11,17,23-Tetra(N,N-dimethyl-N-hydroxyethylammonium)-methylene-25,26,27,28-tetradodecyloxycalix[4]arene tetrachloride (CX12). Obtained as colorless microcrystalline hygroscopic compound. Yield 39%. M.p. 285-290 °C (decomp.) ^{1}H NMR ($(CD_3)_2S=O$, 299.94 MHz), δ ppm: 6.93 (s, 8H, *m*-ArH), 5.63 (brs, 4H, OH), 4.53 (s, 8H, Ar-C$\underline{H}_2$-N), 4.35 (d, J = 12.8 Hz, 4H, ArCH_{ax}Ar), 3.88 (brt, 16H, O-C$\underline{H}_2$-CH_2-$(CH_2)_9$-CH_3, C$\underline{H}_2$-OH), 3.32 (brs, 12H, ArCH_{eq}Ar, N-C$\underline{H}_2$), 2.97 (s, 24H, N-CH_3), 1.89 (m, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_9$-CH_3), 1.47-1.14 (brs, 72H, O-CH_2-CH_2-(C$\underline{H}_2$)$_9$-CH_3) 0.84 (brt, 12H, O-CH_2-CH_2-$(CH_2)_9$-C$\underline{H}_3$), ^{13}C NMR ($(CD_3)_2S=O$ and 10% $CDCl_3$, 125.73 MHz), δ ppm: 157.24, 134.63, 133.16, 121.78, 75.02, 66.78, 64.14, 54.86, 49.69, 29.85, 29.82, 29.79, 29.55, 29.52, 29.36, 29.27, 29.17, 28.81, 25.89, 22.07, 13.73. MS: (m/z) found $[M\text{-}2Cl^-]^{2+}$ = 1575.2250 (calcd for $C_{96}H_{168}Cl_2N_4O_8^{2+}$; $[M\text{-}2Cl^-]^{2+}$ = 1575.2239).

5,11,17,23-Tetra(N,N-dimethyl-N-hydroxyethylammonium)-methylene-25,26,27,28-tetrahexadecyloxycalix[4]arene tetrachloride (CX16). Obtained as colorless microcrystalline hygroscopic compound. Yield 67%. M.p. 222-225, decomposition 235 °C. ^{1}H NMR (CD_3OD, 500.07 MHz), δ ppm: 7.03 (s, 8H, *m*-ArH), 5.57 (brs, 4H, OH), 4.51 (d, J = 12.9 Hz, 4H, ArCH_{ax}Ar), 4.47 (s, 8H, Ar-C$\underline{H}_2$-N), 4.03 (brs, 16H, O-C$\underline{H}_2$-CH_2-$(CH_2)_{13}$-CH_3, C$\underline{H}_2$-OH), 3.39 (brs, 12H, ArCH_{eq}Ar, N-C$\underline{H}_2$), 2.979 (s, 24H, N-CH_3), 2.01 (m, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.56-1.23 (brs, 104H, O-CH_2-CH_2-(C$\underline{H}_2$)$_{13}$-CH_3) 0.91 (brt, 12H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$). MS: (m/z) found $[M\text{-}4Cl^-]4^+/4$ = 432.64 (calcd for $C_{112}H_{200}N_4O_8^{4+}$; $[M\text{-}4Cl^-]4^+/4$ = 432.70), ^{13}C NMR ($CDCl_3$ + 20%CD_3OD, 125.73 MHz), δ ppm: 158.14, 135.64, 134.05, 121.83, 76.26, 68.09, 65.59, 56.00, 50.36, 31.25, 30.69, 30.29, 30.14, 30.09, 30.08, 30.07, 30.03, 30.01, 29.98, 29.92, 29.91, 29.60, 26.50, 22.89, 14.23. MS: (m/z) found $[M\text{-}3Cl^-]^{3+}$ = 1764.5095 (calcd for $C_{112}H_{200}ClN_4O_8^{3+}$; $[M\text{-}3Cl^-]^{3+}$ = 1764.5055).

5,11,17,23-Tetra(N,N-dimethyl-N-hydroxyethylammonium)-methylene-25,27-dihexadecyloxy-26,28-dipropoxycalix[4]arene tetrachloride (CX3-16). Obtained as colorless microcrystalline hygroscopic compound. Yield 75%. M.p. 260-265 °C (decomp.) ^{1}H NMR ($(CD_3)_2S=O$, 299.94 MHz), δ ppm: 7.09 and 6.85 (two s, 8H, p*m*-ArH), 5.70 (brs, 4H, OH), 4.57 and 4.49 (two s, 8H, Ar-C$\underline{H}_2$-N), 4.35 (d, J = 13.3 Hz, 4H, ArCH_{ax}Ar), 3.91-3.83 (brm, 16H, O-C$\underline{H}_2$-CH_2-CH_3, O-C$\underline{H}_2$-CH_2-$(CH_2)_{13}$-CH_3, C$\underline{H}_2$-OH), 3.35 (brs, 8H, ArCH_{eq}Ar, N-C$\underline{H}_2$), 3.35 (brs, 4H, N-C$\underline{H}_2$), 3.00 and 2.90 (two s, 24H, N-CH_3), 1.89 (m, 4H, O-CH_2-C$\underline{H}_2$-CH_3, O-CH_2-C$\underline{H}_2$-$(CH_2)_{13}$-CH_3), 1.24 (brs, 52H, O-CH_2-CH_2-(C$\underline{H}_2$)$_{13}$-CH_3) 1.01 (t, J = 7.4 Hz, 6H, O-CH_2-CH_2-C$\underline{H}_3$) 0.85 (t, J = 6.9 Hz, 6H, O-CH_2-CH_2-$(CH_2)_{13}$-C$\underline{H}_3$) ^{13}C NMR (CD_3OD, 125.73 MHz), δ ppm: 160.02, 159.46, 137.40, 136.62, 135.31, 134.84, 123.23, 123.07, 78.70, 77.01, 70.27, 66.74, 64.87, 57.00, 51.26, 51.04, 33.24, 31.66, 31.51, 31.17, 31.00, 30.97, 30.94, 30.64, 27.51, 25.43, 24.78, 23.90, 14.66, 11.26.

5,11,17,23-Tetra(N-trifluoroacetamido-N',N'-dimethyl-ethylenediamino)-methylene-25,26,27,28-tetraoctyloxycalix[4]arene tetrachloride (15). To a solution of tetrachloromethylcalix[4]arene **7** (1 mmol) in tetrahydrofuran (15 ml) a solution of trifluoroacetamide of N,N-dimethylethylendiamine (4.5 mmol) in THF (5 ml) was added under stirring. The reaction mixture was refluxed for 12 hrs. After cooling, 30 ml of diethyl ether was added. The pale-yellow precipitate (calixarene **15**) formed was filtered off. The product was washed by THF then redissolved in methanol (10 ml). Obtained solution was evaporated under reduced pressure, resulted solid then re-suspended and sonificated in diethyl ether (15 ml). The ether was evaporated under reduced pressure at room temperature to give the calixarene **15**. Obtained as a pale-yellow microcrystalline hygroscopic compound. Yield 72%. M.p. 280-285 °C (decomp.) ^{1}H NMR ($(CD_3)_2S$=O, 299.94 MHz), δ ppm:10.17 (s, 4H, NH-C(O)CF_3), 6.93 (s, 8H, *m*-ArH), 4.62 (s, 8H, Ar-C$\underline{H}_2$-N), 4.33 (d, J = 12.5 Hz, 4H, ArCH_{ax}Ar), 3.88 (brt, 8H, C$\underline{H}_2$-NH-C(O)), 3.74 (brt, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.42 (brs, 12H, ArCH_{eq}Ar, N-C$\underline{H}_2$), 3.01 (s, 24H, N-CH_3), 1.86 (m, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.50-1.19 (brs, 40H, O-CH_2-CH_2-(C$\underline{H}_2$)$_5$-CH_3) 0.85 (brt, 12H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$). ^{19}F NMR ($(CD_3)_2S$=O, 110.4 MHz), δ ppm: -74.42 (s, CF_3C(O)), ^{13}C NMR ($(CD_3)_2S$=O, 125.73 MHz), δ ppm: 157.62, 134.95, 133.26, 121.65, 116.94, 114.65, 75.06, 66.30, 60.07, 49.56, 33.83, 31.56, 29.95, 29.56, 29.21, 26.01, 22.25, 13.90.

5,11,17,23-Tetra(N,N-dimethyl-ethylenediamino)-methylene-25,26,27,28-tetraoctyloxycalix[4]arene tetrachloride (CX8N). To tetra(trifluoro)acetamido-calixarene **15** (0.8 mmol) was added water (20 ml), methanol (20 ml) and ammonia solution (35%, 20 ml). The reaction mixture was refluxed under vigorous stirring for 24 h. After cooling the reaction mixture was evaporated from the 1l flask. Precipitate was washed by diethyl ether (100 ml), solvent was decanted and solid was dried by in *vacuum* at room temperature. The calixarene **CX8N** is pale yellow microcrystalline compound was obtained as mixture with ammonium trifluoroacetate. Yield 90%. M.p. 290-295 °C (decomp.) ^{1}H NMR ($(CD_3)_2S$=O, 299.94 MHz), δ ppm: 6.83 (s, 8H, *m*-ArH), 4.61 (s, 8H, Ar-C$\underline{H}_2$-N), 4.32 (d, J = 12.5 Hz, 4H, ArCH_{ax}Ar), 3.87 (brt, 8H, C$\underline{H}_2$-NH_2), 3.56 (brt, 8H, O-C$\underline{H}_2$-CH_2-$(CH_2)_5$-CH_3), 3.36 (brs, 12H, ArCH_{eq}Ar, N-C$\underline{H}_2$), 3.07 (s, 24H, N-CH_3), 1.83 (m, 4H, O-CH_2-C$\underline{H}_2$-$(CH_2)_5$-CH_3), 1.59-1.14 (brs, 40H, O-CH_2-CH_2-(C$\underline{H}_2$)$_5$-CH_3) 0.85 (brt, 12H, O-CH_2-CH_2-$(CH_2)_5$-C$\underline{H}_3$). ^{19}F NMR ($(CD_3)_2S$=O, 110.4 MHz), δ ppm: -73.61 (s, CF_3COO^-), ^{13}C NMR ($(CD_3)_2S$=O, 125.73 MHz), δ ppm: 157.62, 134.95, 133.26, 121.65, 116.94, 114.65, 75.06, 66.30, 60.07, 49.56, 33.83, 31.56, 29.95, 29.56, 29.21, 26.01, 22.25, 13.90.

General methods

Dynamic light scattering measurements. The complexes of calixarenes with CT-DNA (Sigma, D 1501, highly polymerized DNA from calf thymus, with molecular weight between 10×10^6 and

15×10^6 daltons, verified by gel electrophoresis on agarose) or pDNA (pCMV-eGFP plasmid) were prepared by mixing equal volumes of DNA and calixarene in 20 mM Tris (pH 7.4) buffer. The final DNA (phosphate) concentration was 30 μM, while the calixarene concentration was varied to provide the final N/P. The N/P ratio between calixarene and DNA was expressed as the molar ratio between all the charged Nitrogen atoms of the calixarene and the phosphate groups of the DNA. DLS measurements were done after 30 min of incubation at room temperature. The average size of the complexes was determined with a Zetamaster 3000 (Malvern Instruments, Paris, France) with the following specifications: sampling time, 30 s; medium viscosity, 1.054 cP; refractive index (RI) medium, 1.34; RI particle, 1.45; scattering angle, 90°; temperature, 25 °C.

Gel electrophoresis. The calixarene/pDNA complexes were prepared as for DLS measurements, but at 30 μM pDNA concentration. Gel electrophoresis was performed on a 1% agarose gel in 0.5×TAE (Tris-acetate-ethylenediaminetetraacetic acid) buffer at a constant voltage of 100 mV. After electrophoresis, the gel was incubated in a 1 μg/mL solution of EtBr for 30 min and then visualized by an UV transilluminator.

Fluorescence measurements. Absorption spectra were recorded on a Cary 4000 spectrophotometer (Varian) and fluorescence spectra on a FluoroMax 3.0 or Fluorolog (Jobin Yvon, Horiba) spectrofluorometer. All the spectra were corrected for the fluorescence of the blank (corresponding solution without the fluorescence dye). For CMC measurements with pyrene, a 20 mM Tris buffer (pH 7.4) solution, containing 150 (for CX3-CX8) or 30 nM (for CX8N and CX3-16) of pyrene was titrated with increasing quantities of calixarenes, added from the stock solution in water. The spectra were recorded 1 min after each addition. In the ethidium bromide exclusion assay, an aliquot of CT-DNA (20 μM final concentration of DNA phosphate) was added to the solution of ethidium bromide (0.4 μM). After 2 min, increasing quantities of calixarenes were added from the stock solution. Fluorescence intensity at 600 nm, excited at 550 nm, was recorded 2 min after each calixarene addition and then plotted as a function of the corresponding N/P ratio.

Transfection and cytotoxicity. HeLa cells (ATCC CCL-2) were grown in Dulbecco's modified Eagle medium (DMEM, Gibco-Invitrogen), supplemented with 10% fetal bovine serum (FBS, Lonza) and 1% antibiotic solution (penicillin-streptomycin, Gibco-Invitrogen) at 37° C in humidified atmosphere containing 5% CO_2. Cells were seeded onto 24-well plate at a density of 2×10^4 cells/well 24h before the transfection. The transfection was done by using calixarene/pDNA (eGFP-Luc plasmid, 1 μg/well) complexes at different N/P ratios in serum-free Opti-MEM (Gibco-Intvitogen), followed by 3 h incubation at 37 °C. Calixarene/pDNA complexes were obtained as for DLS measurements, but in MES (pH 7.0) buffer. In some formulations, DOPE (1:1 molar ratio with respect to calixarene) was used. For this purpose, a mixture of DOPE and calixarene in ethanol was

evaporated in a round bottom flask to obtain a film. Then, Tris buffer (pH 7.4) was added and the samples were hydrated overnight at rt. Then, the samples were vortexed vigorously for 1 min and further sonicated in an ultrasonic bath for 15 min. The obtained suspensions were mixed with an equal volume of pDNA in Tris buffer to obtain a desired N/P ratio. All formulations were added to cells 30 min after incubation at rt. After 3 h, 10% of FBS was added and cells were harvested for another 45 h. In case of incubation with serum, the complexes were added to cells in DMEM medium containing 20% FBS and incubated for 48h. Then, the fluorescence images were recorded using BioRad epifluorescence microscope.

Cytotoxicity of calixarenes was evaluated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. HeLa cells were seeded and after 24 h supplemented with calixarenes at different concentrations in serum-free Opti-MEM and further incubated for 3 h at 37 °C. Then, 10% of FBS was added and cells were harvested for another 45 h. Alternatively, calixarenes were added to the cells in DMEM medium with 10% FBS and incubated for 48h. Then, cells were washed with Phosphate Buffer Saline (PBS) and incubated with serum free medium containing 0.5 mg/mL MTT for 3 h at 37°C. Media was discarded and formazan crystals were re-suspended in 0.5 mL MTT solvent (Sigma-Aldrich). Absorbance of formazan solution was measured at 570 nm with respect to the background at 690 nm, using a Cary 4000 spectrophotometer. Viability was expressed as relative absorbance (%) of the sample vs. control non-treated cells.

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Electronic supplementary information (ESI) available: original FT-IR, ^{1}H NMR and ^{13}C NMR spectra are presented for all novel compounds.

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