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p-tert-Butylthiacalix[4]arenes equipped with guanidinium fragments: aggregation, cytotoxicity, and DNA binding abilities†

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Mono-, di- and tetracationic thiacalix[4]arenes in a 1,3-*alternate* conformation functionalized with guanidinium groups showed a strong dependence of the aggregation properties with the ratio of guanidinium/*n*-decyl fragments attached to phenolic groups. Increasing the amount of guanidinium fragments improved the macrocycles solubility in water as well as the sorption capacity towards polynucleotide molecules. The synthesized thiacalixarenes showed relatively high toxicity comparable with that for similar receptors based on the classical calixarene.

Introduction

The molecular design of synthetic receptors that can effectively recognize anionic and polyanionic substrates is an important task of supramolecular chemistry.[1] Growing interest in anion recognition is caused by wide spreading of anionic "guests" in biological systems: DNA, RNA, most enzyme substrates, and cofactors are anionic molecules.[2]

On the other hand, design of synthetic receptors for anionic and polyanionic substrates is a challenge due to the wide variety of their geometric forms, sensitivity to pH values, and strong solvation in polar media.[3] Applying various types of colloid receptors, including cationic lipids, polymers, dendrimers, and peptides, which are able to effectively interact with polyanionic surfaces of biomacromolecules, is one of the most promising approaches in supramolecular chemistry.[4–8] In this regard design of preorganized receptors, which can be assembled into nanosized colloid structures, may help in solving this complex task.

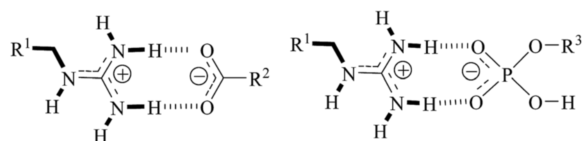

Fig. 1 The complementarity of the guanidinium moiety to a carboxylate and phosphate groups.

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Among all types of functional groups applied in receptors for polyanionic substrates, the guanidinium fragment provides the greatest affinity[9] due to geometrical and charge complementarity to carboxylate and phosphate groups (Fig. 1).[1]

Applying molecular platforms like calixarenes and thiacalixarenes allows to construct molecules that combine different types of functional groups to adjust the affinity of colloid receptors towards anionic and polyanionic substrates.[10,11] In *p-tert*-butylthiacalix[4]arene, the bond length between the aromatic residue and bridging group is 15% larger than that in methylene bridged *p-tert*-butylcalix[4]arene,[12,13] which allows for the design of conformationally more flexible receptors compared with conventional calix[4]arene[14,15] for more effective recognition of biological anionic substrates.[16]

Herein, we continue to develop our approach towards synthesis of preorganized guanidinium receptors based on stepwise functionalization of the lower rim of the *p-tert*-butylthiacalix[4]arene platform.[17,18] We studied aggregation and cytotoxicity of cationic thiacalixarenes functionalized with guanidinium fragments, as well as their binding affinity towards polynucleotide molecules.

Results and discussion

The three cationic thiacalix[4]arenes in 1,3-*alternate* conformation, differing in the ratio of guanidinium/*n*-decyl fragments attached to phenolic groups, were included in this study (Fig. 2). Synthesis of the target compounds **1–3** based on differences in the reactivity of *N*-(3-bromopropyl)phthalimide and *N*-(2-bromoethyl)phthalimide during interaction with *p-tert*-butylthiacalix[4]arene in the presence of alkali metal carbonates.[19] The synthesis of compounds **1** and **2** was carried out in our previous studies.[17,18] Scheme 1 shows the synthetic route to compound **3.**

Compound **1** is water-soluble and forms nanoaggregates, which dissociate during interaction with DNA.[17] Solubilization

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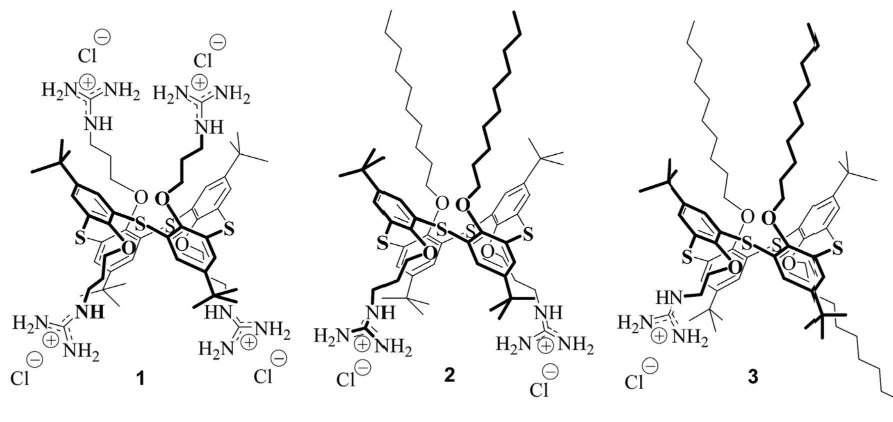
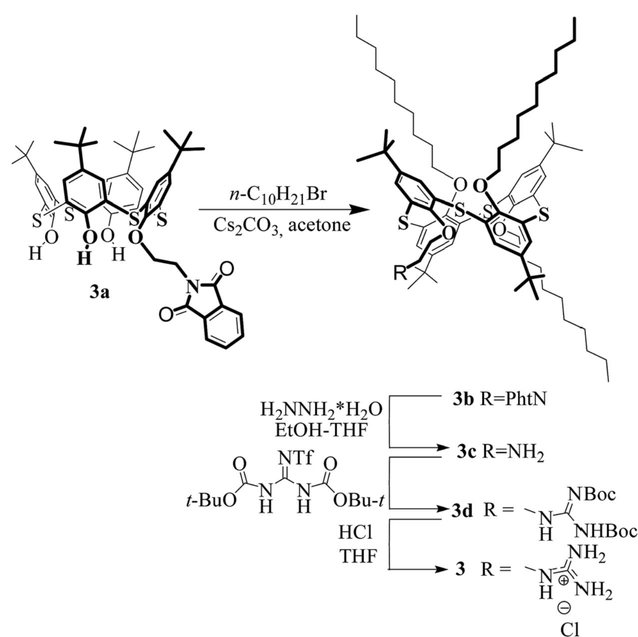


Fig. 2 Molecular structures of compounds **1–3**.



Scheme 1 Synthetic route to **3**.

of amphiphilic compound **2** *via* formation of solid lipid nanoparticles (SLNs) was described earlier[16] so the same nanoprecipitation technique was used for water insoluble compound **3**.[20–22] The size and morphology of the obtained SLNs were characterized by scanning electron microscopy (SEM), and the results are presented in Fig. 3. Fig. 3A shows that **2**-based SLNs were localized on the surface as raspberry-like aggregates with an average 102 nm diameter. The average diameter of **3**-based SLNs was 94 nm. The surfaces of **3**-based SLNs were much smoother (Fig. 3B) compared with aggregates based on compound **2**, so it is assumed that the **3**-based SLNs tried to minimize their surface area and were not as stable as **2**. This assumption was proven by further self-precipitation of **3**-based SLNs by a slow precipitation procedure and hence all further studies were performed with compounds **1** and **2**. The aggregation behaviour of compounds **1–3** is summarized in Fig. 4.

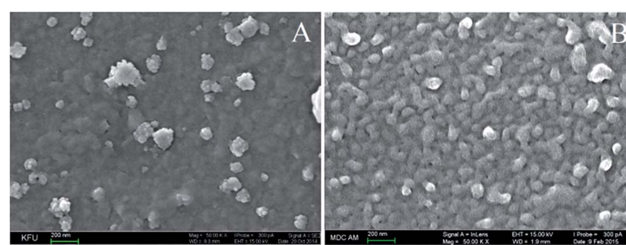


Fig. 3 SEM images of **2**-based (A) and **3**-based (B) SLNs (scale bars are 200 nm).

The differences in aggregation behaviour of compounds **1–3** is attributed to the differences in their structures. Stepwise replacement of hydrophilic 3-guanidiniumpropyl fragments in compound **1** to hydrophobic *n*-decyl groups led to insolubility of compound **2** and **3** in water. Moreover, the presence of three *n*-decyl groups in compound **3** resulted in low stability of **3**-based SLNs compared with **2**-based SLNs.

Interaction between aggregates based on compound **1** and **2** with polynucleotide (phMGFP) was examined by the gel electrophoresis method (Fig. 5). The gel analysis showed the total binding of pDNA occurs at up to 15.4 and 1060 μM for compounds **1** and **2**, respectively. Thus macrocycle **1** possesses higher DNA sorption capacity than **2** (69 times); we associate

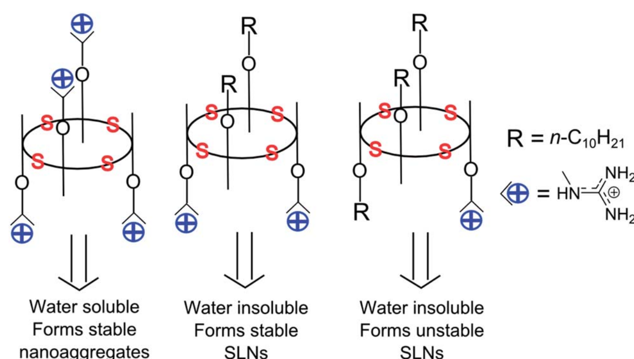


Fig. 4 Aggregation behaviour of synthesized compounds **1–3**.

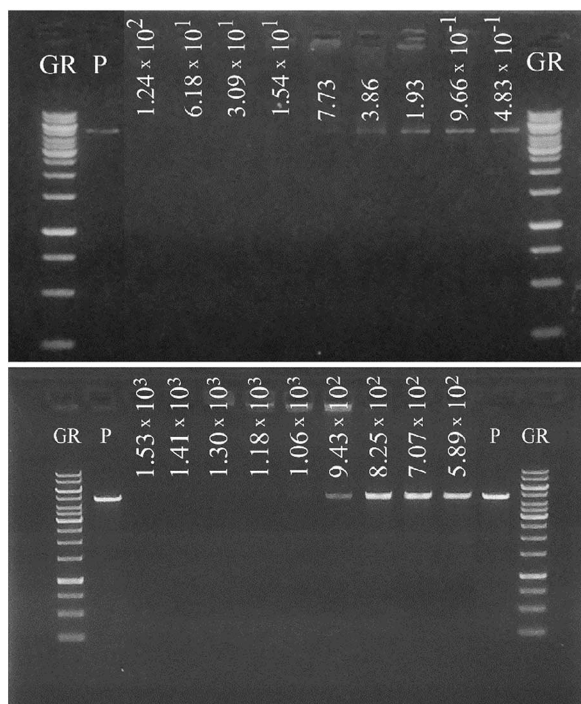


Fig. 5 Agarose gel electrophoresis of pDNA (25 μg mL^{-1}) incubated with increasing amounts of compounds **1** (top) and **2** (bottom). [Values are expressed in μM, P – pure plasmid DNA, GR – GeneRuler 1 kb DNA Ladder].

Table 1 IC_{50} and IC_{100} values (μM) of compounds **1** and **2** on cell viability

	1		**2**	
	IC_{50}	IC_{100}	IC_{50}	IC_{100}
CV-1	1.2	2.5	1.8	7.4
SK	1.2	5.0	3.7	7.4
MF	0.6	2.5	1.8	7.4

this with the difference in the number of charged guanidinium fragments, and there being a significant amount of compound **2** located inside the SLNs that is not able to interact with polynucleotides.

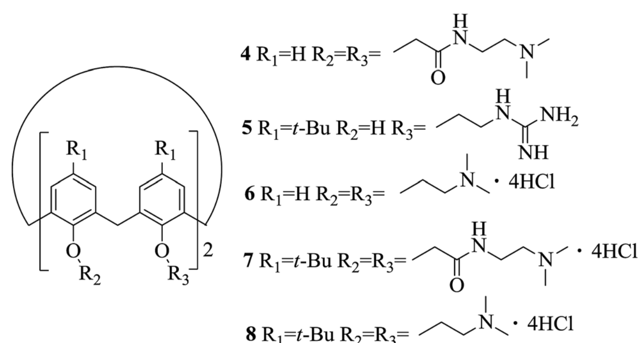


Fig. 6 Molecular structures of compounds **4–8** based on calix[4] arene.

Table 2 IC_{50} values (μM) of compounds **4–8** on a tumor cells viability[23]

	4	**5**	**6**	**7**	**8**
HUVEC	3	3	2	8	2
2H11	>100	80	0.7	15	4
Fibroblasts	35	5	0.2	6	1
FSAII	>20	10	0.7	10	2
MA148	0.5	40	1.5	15	3
A549	2	8	0.8	6	1
SCK	100	4	0.7	9	0.7
B16F10	80	100	1	8	2

To assess *in vitro* cytotoxicity of compounds **1** and **2**, increasing amounts of them were incubated with samples of three different cell lines: CV-1 (monkey kidney cell line), saiga kidney cell line (SK) and L-mouse fibroblast cell line (MF). Table 1 shows the obtained results.

It turns out that despite the significant difference in DNA sorption capacity, the compounds possessed similar cytotoxicity toward the chosen cell lines.

The cytotoxicity of synthesized compounds **1** and **2** was compared with the cytotoxicity of previously described calix[4] arene derivatives **4–8** (Fig. 6).[23] Table 2 groups the IC_{50} values of compounds **4–8** for tumor cell lines.

It should be kept in mind the direct comparison of the IC_{50} values of our compounds and compounds listed in Table 2 is not strictly correct due to the different nature of the cell lines. However, as a first-order approximation it can be concluded that synthesized macrocycles **1** and **2** possess similar cytotoxicity as the most toxic compounds **6** and **8**, based on the calixarene platform.

Conclusions

This study showed aggregation behaviour of synthesized thiacalix[4]arenes functionalized with guanidinium groups strongly depended on the ratio of hydrophilic/hydrophobic fragments in the receptor structures. Increasing the amount of guanidinium fragments improved the receptor solubility in water as well as their sorption capacity towards polynucleotide molecules. The *in vitro* cytotoxicity assay showed high toxicity of synthesized compounds comparable with that for similar receptors based on the classical calixarene.

Experimental

General

Plasmid DNA phMGFP was purchased from Promega. Cell cultures (CV-1 (monkey kidney cell line), saiga kidney cell line and L-mouse fibroblast cell line) were taken from the collection of State Science Institution National Research Institute of Veterinary Virology and Microbiology of the Russian Academy of Agricultural Sciences. *N*,*N*′-bis(*tert*-butoxycarbonyl)-*N*″-triflylguanidine was obtained as described.[24]

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NMR spectroscopy

The ^1H, ^{13}C, 2D ^1H–^1H NOESY NMR spectra were obtained on a Bruker Avance-400 spectrometer. Chemical shifts were determined relative to the signals of residual protons of the deuterated solvent ($CDCl_3$).

FT-IR spectroscopy

IR spectra were obtained using a Fourier Transform Spectrum 400 IR spectrometer (Perkin Elmer).

Elemental analysis

Elemental analysis was performed with a Perkin Elmer 2400 Series II instrument.

MALDI MS

Mass spectra were obtained with the MALDI-TOF Dynamo Finnigan mass analyzer using *p*-nitroaniline as a matrix.

Synthesis of 5,11,17,23-tetra-*tert*-butyl-25,26,27-tridecyl-28-[2-phthalimidoethoxy]-2,8,14,20-tetrathiacalix[4]arene (1,3-*alternate*) (3b)

In a round bottomed flask equipped with a magnetic stirrer and reflux condenser, a mixture of 1.50 g (1.14 mmol) of the compound **3a**, 1.51 g (6.84 mmol) 1-bromodecane, 2.23 g (6.84 mmol) of freshly powdered cesium carbonate and 60 mL of acetone was refluxed for 48 hours. After cooling the reaction mixture, the precipitate was filtered off, the solvent from the filtrate was distilled off under reduced pressure and the residue was recrystallized from methanol. Yield 82%. Found: C, 72.81; H, 8.51; N, 1.05; S, 9.64. $C_{80}H_{115}NO_6S_4$ requires C, 73.07; H, 8.81; N, 1.07; S, 9.75. MS (MALDI-TOF): calculated $[M^+]$ $m/z = 1313.8$, found $[M + H]^+$ $m/z = 1314.6$, $[M + Na]^+$ $m/z = 1336.5$, $[M + K]^+$ $m/z = 1352.6$. ν_{max}/cm^{-1} 1267 (COC); 1715, 1775 (C=O) δ_H (400 MHz; $CDCl_3$): 0.80–1.37 (57H, br.m, $(CH_2)_8CH_3$), 1.28 (9H, s, $(CH_3)_3C$), 1.29 (9H, s, $(CH_3)_3C$), 1.34 (18H, s, $(CH_3)_3C$), 3.60 (2H, m, CH_2N), 3.86 (6H, m, CH_2O), 4.13 (2H, m, CH_2O), 7.32 (2H, s, ArH), 7.37 (4H, m, ArH), 7.70 (2H, d, $J = 2.4$ Hz, ArH), 7.73 (2H, m, Pht), 7.88 (2H, m, Pht). δ_C (125 MHz; $CDCl_3$): 14.2, 14.3, 22.8, 22.9, 25.94, 25.97, 28.8, 29.1, 29.4, 29.5, 29.69, 29.73, 29.8, 29.9, 30.0, 31.5, 32.0, 32.1, 34.34, 34.37, 34.5, 36.4, 64.4, 68.5, 69.0, 123.4, 127.6, 128.1, 128.2, 128.47, 128.50, 128.52, 128.7, 132.3, 134.0, 145.4, 146.0, 157.0, 157.2, 167.9. Spectrum ^1H–^1H NOESY (the most important cross-peaks): $H^{4b}/H^{7'}$, $H^{4+b}/H^{7'}$.

Synthesis of 5,11,17,23-tetra-*tert*-butyl-25,26,27-tridecyl-28-[2-aminoethoxy]-2,8,14,20-tetrathiacalix[4]arene (3c)

A mixture of 1.00 g of compound **3b** and 1 mL (20 mmol) of hydrazine hydrate was refluxed in a 30 mL THF and 30 mL ethanol mixture for 20 h. Then, the solvent was evaporated, 60 mL of water was added and white powder was filtered off. The obtained powder was dried in a desiccator under reduced pressure. Yield 93%. Found: C, 72.62; H, 9.51; N, 1.11; S, 10.64. $C_{72}H_{113}NO_4S_4$ requires C, 72.98; H, 9.61; N, 1.18; S, 10.82. MS (MALDI-TOF): calculated $[M^+]$ $m/z = 1183.8$, found $[M + H]^+$ $m/z = 1184.6$. ν_{max}/cm^{-1} 1266 (COC); 3362 (NH_2). δ_H (400 MHz; $CDCl_3$): 0.85–1.35 (57H, br.m, $(CH_2)_8CH_3$), 1.28 (18H, s, $(CH_3)_3C$), 1.29 (18H, s, $(CH_3)_3C$), 2.45 (2H, t, $J = 5.1$ Hz, CH_2N), 3.82 (4H, m, CH_2O), 3.90 (2H, m, CH_2O), 3.95 (2H, t, $J = 5.0$ Hz, CH_2O), 7.32 (2H, d, $J = 2.5$ Hz, ArH), 7.35 (2H, d, $J = 2.4$ Hz, ArH), 7.35 (2H, s, ArH), 7.36 (2H, s, ArH). δ_C (125 MHz; $CDCl_3$): 14.3, 22.8, 25.8, 25.9, 28.9, 29.2, 29.5, 29.7, 29.8, 29.9, 30.0, 30.2, 31.47, 31.49, 31.51, 32.1, 34.35, 34.38, 34.45, 68.8, 127.1, 127.6, 127.8, 128.0, 128.3, 128.7, 146.0, 157.0.

Synthesis of 5,11,17,23-tetra-*tert*-butyl-25,26,27-tridecyl-28-[2-(bis-*tert*-butoxycarbonyl-guanidine)ethoxy]-2,8,14,20-tetrathiacalix[4]arene (1,3-*alternate*) (3d)

A stoichiometric amount of *N*,*N*′-di-(*tert*-butoxycarbonyl)-*N*″-triflyl guanidine in 20 mL of dichloromethane was added to the ice cooled solution of 1.00 g of compound **3c** in 40 mL of dichloromethane. After 24 hours, the mixture was washed with 2 M aqueous sodium bisulfate (10 mL) and saturated sodium bicarbonate (10 mL). Each aqueous layer was extracted with dichloromethane (2 × 10 mL). The combined organic phases were washed with brine (10 mL), dried by molecular sieves 3 Å and then the dichloromethane was evaporated under reduced pressure. Obtained white powder was dried in a desiccator under reduced pressure. Yield 61%. Found: C, 69.71; H, 9.13; N, 2.81; S, 8.82. $C_{83}H_{131}N_3O_8S_4$ requires C, 69.85; H, 9.25; N, 2.94; S, 8.99. MS (MALDI-TOF): calculated $[M^+]$ $m/z = 1425.88$, found $[M - 2Boc + H]^+$ $m/z = 1227.8$. ν_{max}/cm^{-1} 1263 (COC); 1637 (N–CO); 1616, 1637 (C=N); 1718 (C=O) and 3332 (NH). δ_H (400 MHz; $CDCl_3$): 0.85–1.33 (57H, br.m, $(CH_2)_8CH_3$), 1.26 (9H, s, $(CH_3)_3C$), 1.27 (9H, s, $(CH_3)_3C$), 1.27 (18H, s, $(CH_3)_3C$), 1.49 (9H, s, Boc), 1.50 (9H, s, Boc), 3.10 (2H, m, CH_2N), 3.84 (6H, m, CH_2O), 4.05 (2H, t, $J = 7.0$ Hz, CH_2O), 7.31 (2H, s, ArH), 7.32 (2H, d, $J = 2.4$ Hz, ArH), 7.34 (2H, s, ArH), 7.57 (2H, d, $J = 2.4$ Hz, ArH), 8.30 (1H, t, $J = 5.6$ Hz, $NHCH_2$), 11.37 (1H, s, NHBoc). δ_C (125 MHz; $CDCl_3$): 14.3, 22.9, 25.9, 26.0, 28.2, 28.5, 29.1, 29.2, 29.4, 29.5, 29.7, 29.8, 29.9, 30.0, 30.1, 31.48, 31.53, 32.0, 32.1, 34.3, 34.4, 40.1, 66.6, 68.9, 69.2, 78.8, 82.9, 128.1, 128.18, 128.20, 128.3, 128.58, 128.61, 128.9, 145.4, 145.5, 145.8, 153.1, 156.2, 156.7, 157.2, 157.4, 163.8. Spectrum ^1H–^1H NOESY (the most important cross-peaks): $H^{4'b}/H^{Boc}$, $H^{3'}/H^{Boc}$.

Synthesis of 5,11,17,23-tetra-*tert*-butyl-25,26,27-tridecyl-28-[2-guanidiniumethoxy]-2,8,14,20-tetrathiacalix[4]arene chloride (1,3-*alternate*) (3)

2 mL of concentrated hydrochloric acid were added to a solution of 0.50 g of the compound **3d** in 40 mL of tetrahydrofuran. The reaction mixture was stirred for 24 hours. Then, the solvent was evaporated under vacuum and 40 mL of water was added to the reaction mixture. The precipitate was filtered off and washed with water. The obtained white powder was dried in a desiccator under reduced pressure. Yield 65%. Found: C, 68.86; H, 9.16; N, 3.23; S, 9.86. $C_{73}H_{116}ClN_3O_4S_4$. requires C, 69.40; H, 9.25; N, 3.30; S, 10.15. MS (MALDI-TOF): calculated $[M^+]$ $m/z = 1261.8$, found $[M - Cl]^+$ $m/z = 1226.8$. ν_{max}/cm^{-1} 1265 (COC); 1663 (C=N); 3333 (NH). δ_H (400 MHz; $CDCl_3$): 0.85–1.67 (57H, br.m, $(CH_2)_8CH_3$), 1.28 (36H, s, $(CH_3)_3C$), 3.40 (2H, m,

CH_2N), 3.77 (4H, br.t, $J = 8.5$ Hz, CH_2O), 4.05 (2H, br.t, $J = 7.6$ Hz, CH_2O), 4.14 (2H, br.m, CH_2O), 7.34 (4H, s, ArH), 7.40 (2H, s, ArH), 7.50 (2H, s, ArH), 8.93 (1H, br.m, $NHCH_2$). δ_C (125 MHz; $CDCl_3$): 14.3, 22.9, 25.83, 25.90, 28.6, 29.4, 29.6, 26.72, 29.76, 30.2, 31.4, 31.5, 32.0, 34.4, 34.7, 69.2, 127.9, 128.0, 128.1, 128.2, 129.4, 129.5, 130.1, 131.4, 147.5, 147.6, 156.4.

SLNs preparation

The SLNs suspensions were prepared by dissolving 150 mg (0.119 mmol) of **3** in 5 mL THF. After 5 min of stirring, 50 mL of ultrapure water was added and the solution was stirred for one more minute. The tetrahydrofuran was subsequently evaporated under reduced pressure at 40 °C. The remaining solution was adjusted to 50 mL with ultrapure water to obtain a 3 mg mL^{-1} (2.4 mM) final concentration.

Scanning electron microscopy

Measurements were carried out by using a field-emission high-resolution scanning electron microscope by Merlin Carl Zeiss. Images were acquired to observe the surface morphology with a 15 kV incident accelerating voltage and 300 pA probe current to minimize the sample being altered by the imaging.

Agarose gel electrophoresis

Gel electrophoresis was conducted according to a common technique.[25]

Cytotoxicity assay

All cell types were seeded at an 11 000 cells per well concentration and allowed to adhere for 24 h at 37 °C in 5% CO_2/95% air before treatments were initiated. The cells were then exposed to various concentrations of thiacalix[4]arenes for 72 h. Inverted optical microscopes Eclipse TS100 (Nikon), and CKX31 (Olympus), as well as a Fluorescence Microscope Olympus IX70, were used to assess cell viability relative to untreated cells. All measurements were carried out in triplicate and the experiments were carried out at least three times.

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