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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 *1,3-alternate* conformation functionalized with guanidinium groups show strong dependence of aggregation properties on the ratio of guanidinium/*n*-decyl fragments attached to phenolic groups. Increasing the amount of guanidinium fragments improves solubility of the macrocycles in water as well as sorption capacity toward polynucleotide molecules. Synthesized thiacalixarenes show relatively high toxicity comparable with that for similar receptors based on classical calixarene.

Introduction

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

On the other hand design of synthetic receptors for anionic and polyanionic substrates is a challenge due to 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 regards design of preorganized receptors which can be assembled into nanosized colloid structures may help in solving this complex task.

Among all types of functional groups applying in receptors for polyanionic substrates, guanidinium fragment provides the greatest affinity[11] due to geometrical and charge complementarity to carboxylate and phosphate groups (Fig. 1).[1]

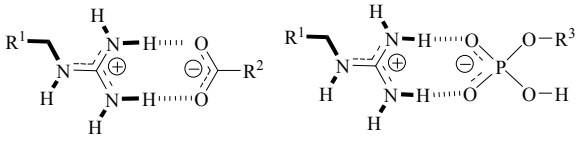

Fig.1. The complementarity of guanidinium moiety to a carboxylate and phosphate groups.

Applying molecular platforms like calixarenes and thiacalixarenes allows to construct molecules combining different types of functional groups to adjust affinity of colloid receptors toward anionic and polyanionic substrates.[9,10] In the *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.[11,12] That allows us to design conformationally more flexible receptors compared with conventional calix[4]arene[13,14] for more effective recognition of biological anionic substrates.[15]

Herein we continue to develop our approach to synthesis of preorganized guanidinium receptors based on stepwise functionalization of the lower rim of *p-tert*-butylthiacalix[4]arene platform.[16,17] We study aggregation and cytotoxicity of cationic thiacalixarenes functionalized with guanidinium fragments as well as their binding affinity toward 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 all target compounds **1**-**3** based on differences of reactivity N-(3-Bromopropyl)phthalimide and N-(2-Bromoethyl)phthalimide during interaction with *p-tert*-butylthiacalix[4]arene in presence of carbonates of alkali metals.[19] We described synthesis of compounds **1** and **2** in our previous studies.[16,17] Scheme 1 shows the synthetic route to compound **3**.

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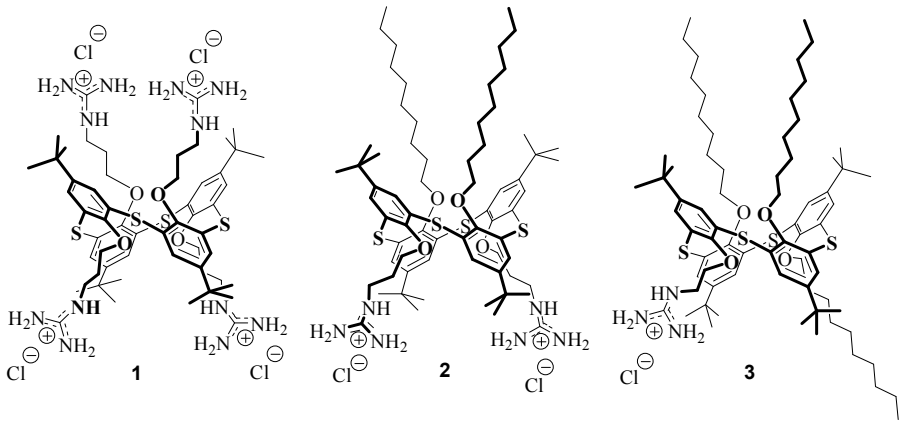
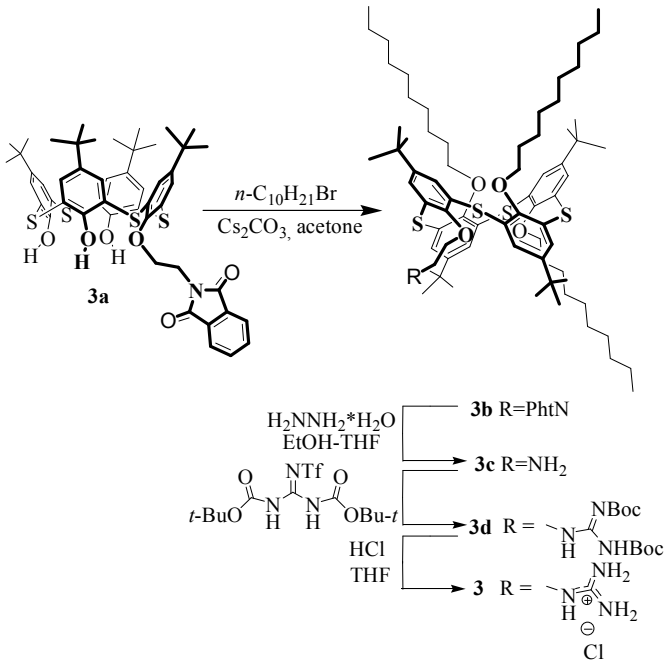


Fig.2. Molecular structures of compounds **1**-**3**.



Scheme 1. Synthetic route to **3**.

Compound **1** is water-soluble and forms nanoaggregates which dissociate during interaction with DNA.[17] Solubilization of amphiphilic compound **2** via formation of solid lipid nanoparticles (SLNs) was described earlier[16] so we used the same nanoprecipitation technique for water insoluble compound **3**.[20-22] Size and morphology of the obtained SLNs were characterized by scanning electron microscopy (SEM), results are presented in Figure 3. Figure 3A shows that **2**-based SLNs are localized on the surface as raspberry-like aggregates with an average diameter 102 nm. The average diameter of **3**-based SLNs is equal to 94 nm. The surfaces of **3**-based SLNs are much smoother (Figure 3B) compared with aggregates based on compound **2**, so we can assume that **3**-based SLNs try to minimize their surface area and they are not so stable as **2**. This assumption was proved by further self-precipitation of **3**-based SLNs from the solution after several days of staying, hence all further studies were performed only for compounds **1** and **2**. Figure 4 summarizes aggregation behaviour of compounds **1**-**3**.

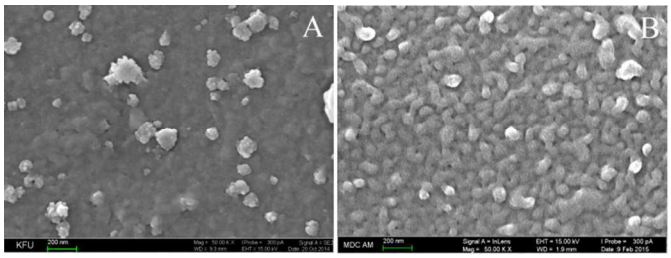


Fig.3. SEM images of **2**-based (A) and **3**-based (B) SLNs (scale bars are 200 nm).

We attribute differences in aggregation behaviour of initial compounds **1**-**3** to the differences in their structures. Stepwise replacement of hydrophilic 3-guanidiniumpropyl fragments in compound **1** to hydrophobic *n*-decyl groups leads to insolubility of compound **2** and **3** in water. Moreover presence of three *n*-decyl groups in compound **3** results in low stability of **3**-based SLNs compared with **2**-based SLNs.

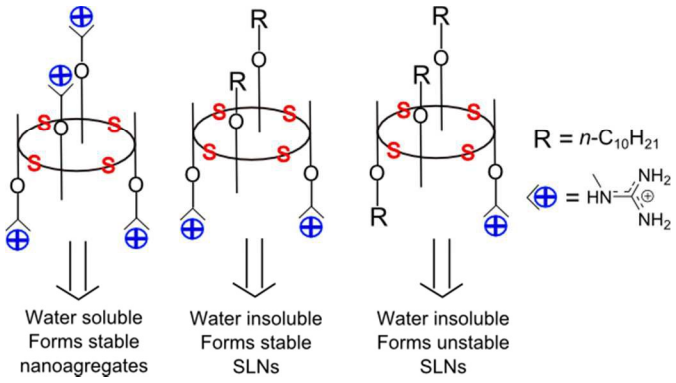


Fig.4. Aggregation behaviour of synthesized compounds **1**-**3**.

Interaction between aggregates based on compound **1** and **2** with polynucleotide (phMGFP) was examined by gel electrophoresis method (Fig.5.). The analysis of the gel shows the total binding of pDNA occurs up to 15.4 and 1060 μM for compounds **1** and **2** consequently. Thus macrocycle **1** possess higher DNA sorption capacity than **2** (69 times); we associate it with the difference in number of charged guanidinium fragments, and with the fact that significant amount of compound **2** is located inside the SLNs and is not able to interact with polynucleotides.

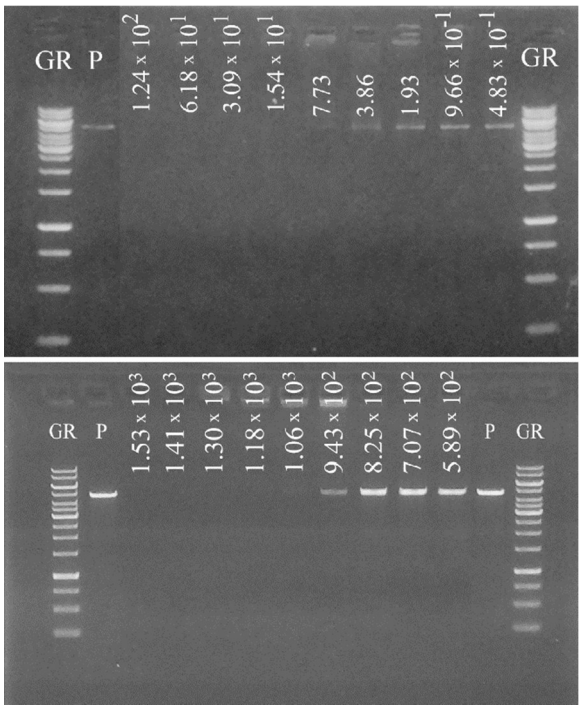


Fig.5. Agarose gel electrophoresis of pDNA (25 μg/ml) incubated with increasing amount of compounds **1** (top) and **2** (bottom). [Values are expressed in μM, P - pure plasmid DNA, GR - GeneRuler 1 kb DNA Ladder].

In order 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 obtained results.

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

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

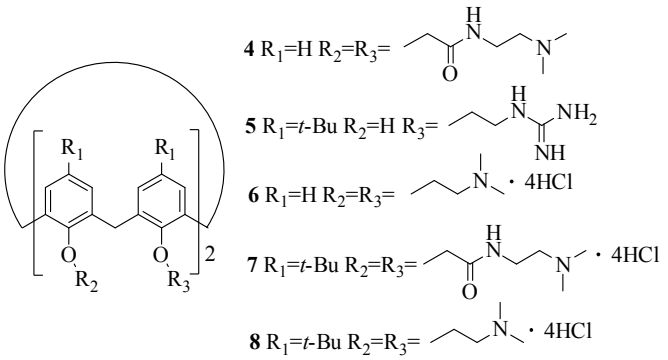


Fig.6. Molecular structures of compounds **4**-**8** based on calix[4]arene.

We compared cytotoxicity of synthesized compounds **1** and **2** with 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.

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

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 different nature of the cell lines. But as a first-order approximation we can conclude that synthesized macrocycles **1** and **2** possess similar cytotoxicity as the most toxic compounds **6** and **8** based on calixarene platform.

Conclusions

Our research shows aggregation behaviour of synthesized thiacalix[4]arenes functionalized with guanidinium groups strongly depends on the ratio of hydrophilic/hydrophobic fragments in receptors' structures. Increasing the amount of guanidinium fragments improves solubility of the receptors in water as well as their sorption capacity toward polynucleotide molecules. *in vitro* cytotoxicity assay shows high toxicity of

synthesized compounds comparable with that for similar receptors based on 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) was taken from the collection of State Science Institution National Research Institute of Veterinary Virology and Microbiology of Russian Academy of Agricultural Sciences. N,N'-bis(*tert*-butoxycarbonyl)-N''-triflyl-guanidine has been obtained as described.[24]

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

FT-IR spectroscopy. IR spectra were recorded using Fourier Transform Spectrum 400 IR spectrometer (Perkin Elmer).

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

MALDI MS. Mass spectra were recorded 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 bottom flask equipped with a magnetic stirrer, 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 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 ^{1}H-^{1}H 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 the compound **3b** and 1 ml (20 mmol) of hydrazine hydrate was refluxed in a mixture of 30 ml THF and 30 ml ethanol for 20 hrs. Then solvent was evaporated, 60 ml of water was added and white powder was filtered off. Obtained powder was dried in dessicator 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**). The 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 the 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 3Å, and then the dichloromethane was evaporated under reduced pressure. Obtained white powder was dried in dessicator 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 ^{1}H-^{1}H 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 the solution of 0.50 g of the compound **3d** in 40 ml of tetrahydrofuran. The reaction mixture was stirred for 24 hours. Then solvent was evaporated under vacuum and 40 ml of water were added to the reaction mixture. The precipitate was filtered off and washed with water. The obtained white powder was dried in dessicator

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 stirring 50 ml of ultrapure water was added and the solution was stirred 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 final concentration of 3 mg/ml (2.4 mM).

Scanning electron microscopy. Measuring was carried out by using field-emission high-resolution scanning electron microscope Merlin Carl Zeiss. Observation photo of morphology surface apply at accelerating voltage of incident electron 15 kV and current probe 300 pA in order to minimum modify sample.

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

Cytotoxicity assay. All cell types were seeded at a concentration of 11 000 cells per well 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 routine microscopes Eclipse TS100 (Nikon), and CKX31 (Olympus), as well as Fluorescence Microscope Olympus IX70 were used to assess cell viability relative to untreated cells. All measurements were done in triplicate, and the experiments were done at least three times.

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Notes and references

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†Electronic Supplementary Information (ESI) available: Full details on charactirization of synthesized compounds are available. See DOI: 10.1039/c000000x/

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P-tert-butylthiacalix[4]arenes equipped with guanidinium fragments: aggregation, cytotoxicity, and DNA binding abilities

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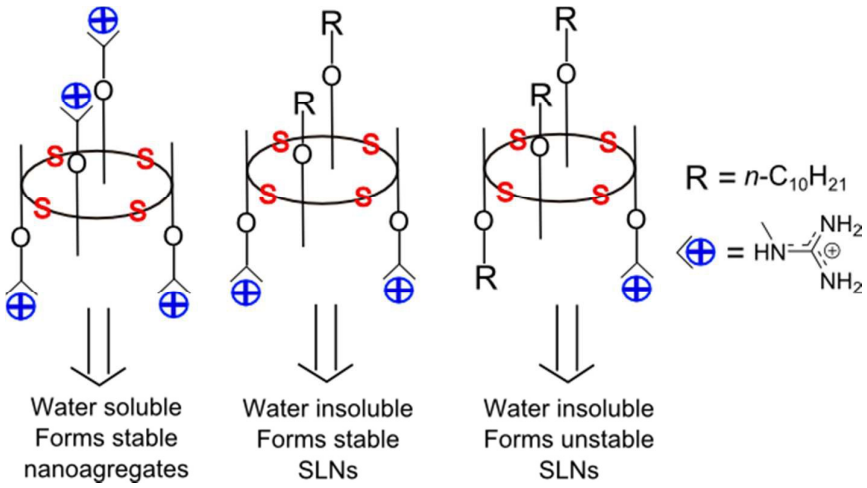

Abstract

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