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Investigation of DNA binding abilities of solid lipid nanoparticles based on *p-tert*-butylthiacalix[4]-arene platform†

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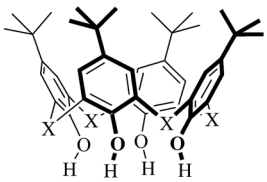
An amphiphilic thiacalix[4]arene derivative functionalized with guanidinium groups forms stable solid lipid nanoparticles (SLNs) with high ζ-potential in water. Applying gel electrophoresis and fluorescent spectroscopy methods shows that the SLNs have high binding affinity to double-stranded DNA, but despite this fact, we have not observed any significant transfection activity toward three different mammalian cell lines. A UV-spectroscopic study reveals that interaction between the SLNs and the polynucleotide leads to partial denaturation of the DNA located on the surface of the nanoparticles that can hinder transfection.

Introduction

The molecular design of synthetic receptors that can effectively interact with anionic and polyanionic substrates is an important task for supramolecular chemistry.[1] One of the most promising scientific areas applying such receptors is gene therapy. The success of gene therapy is related to the development of carrier systems able to efficiently interact and transport nucleic acids to the targeted nuclei through the cell membrane.[2] Despite the high efficiency of viral carriers, their application is limited by their toxicity and immunogenicity, therefore the investigation of other potential carriers, including cationic lipids,[3] polymers,[4] dendrimers,[5] and peptides,[6] has attracted considerable attention from researchers.[2]

Solid lipid nanoparticles (SLNs) are important among the existing lipid-based systems, because of the simplicity of their preparation and low toxicity.[7] In addition to the conventional lipids used for producing this type of carrier, a new class of SLNs based on self-assembled amphiphilic calix[4]arenes was developed by P. Shahgaldian and A. Coleman *et al.*[8–10] The affinity of the cationic SLNs toward polyanionic biomacromolecules as well as their ability to transfect mammalian cells were also demonstrated.[11]



X=CH_2 *p-tert*-butylcalix[4]arene

X=S *p-tert*-butylthiacalix[4]arene

Functionalization of both an upper and a lower rims of the *p-tert*-butylcalix[4]arene by hydrophilic and hydrophobic fragments is a common approach (Scheme 1) to obtain such amphiphilic molecules.[12–14] We suggest alternative way based on our previous study[15] consisting of stepwise functionalization of the lower rim of *p-tert*-butylthiacalix[4]arene platform by hydrophilic and hydrophobic moieties, spatial separation of which is provided by using 1,3-*alternate* conformation of the molecular platform (Scheme 1). For *p-tert*-butylthiacalix[4]arene the bond length between the aromatic residue and bridging group is 15% larger than the one in methylene bridged *p-tert*-butylcalix[4]arene,[16,17] that provides weaker circular hydrogen bond and as a result greater conformational flexibility of the molecular platform that makes *p-tert*-butylthiacalix[4]arene an ideal candidate for design such amphiphilic molecules (Scheme 2).

The aim of this study was to create amphiphilic *p-tert*-butylthiacalix[4]arene in 1,3-*alternate* conformation equipped with guanidinium groups, examine its ability to form aggregates and also investigate binding affinity of this aggregates toward polynucleotide molecules.

Results and discussion

The target compound **3** was synthesized by a two-step synthesis.[18] First, amine **1** reacted with *N*,*N*′-di-(*tert*-butoxycarbonyl)-*N*″-triflyl

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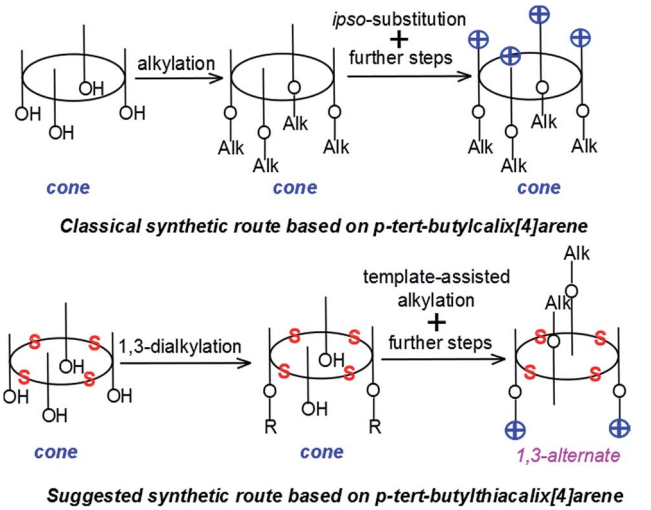
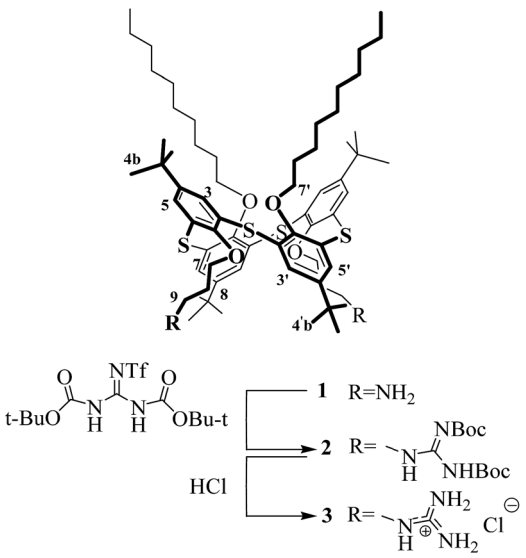
† Electronic supplementary information (ESI) available: Full details of the syntheses and biological analysis, DLS hydrodynamic diameter and SEM measurements, ζ-potential measurements. See DOI: 10.1039/c5ra03814f



Scheme 1 Comparison of classical and suggested approaches to synthesis of amphiphilic molecules.



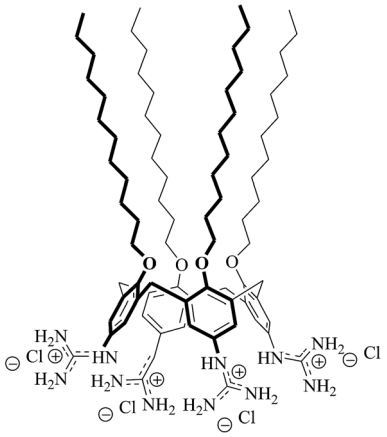
Scheme 2 Synthetic route to 3.

guanidine and then the resulting product was treated with hydrochloric acid to remove the *tert*-butoxycarbonyl protecting groups (Scheme 1). The amine **1** was synthesized as previously described.[15]

It turns out that compound **3** is insoluble in water therefore we used nanoprecipitation technique to solubilize it *via* formation of solid lipid nanoparticles (SLNs).[8–10] The obtained SLNs were characterized by dynamic light scattering (DLS) and electrophoretic mobility measurements. The results showed, that an average hydrodynamic diameter is 132 nm (PDI = 0.10) and a ζ-potential of is +61 ± 3 mV. The changes in these parameters after repeated measurements during 10 days were negligible. It should be noted that hydrodynamic diameter of the SLNs didn't change during dilution of the initial solution (3 mg mL^{-1}) up to 30 μg mL^{-1}. The positive value of the ζ-potential confirms that the guanidinium groups of compound **3** were partially directed outward of the SLNs.

It is interesting to compare properties of the synthesized **3**-based SLNs with previously described SLNs based on macrocycle **4**.[19] Despite the four cationic guanidinium fragments in the compound **4** the ζ-potential of the **4**-based SLNs (+31 mV) is half as much as the **3**-based SLNs.

We explain higher ζ-potential of the obtained in this work SLNs by the lower acidity of the propylguanidinium fragments (pK_a = 12.7, 20 °C, water) in compound **3** compared with the phenylguanidinium fragments (pK_a = 10.9, 20 °C, water)[20] in compound **4**. Lower acidity increases the stability of the cationic form as well as ζ-potential of the colloid particles.[21]



We used scanning electron microscopy (SEM) to examine morphology of obtained nanoparticles, the result is presented in Fig. 1. It could be seen that the **3**-based SLNs are localized at the surface as roughly spherical aggregates and measurement reveals a diameter 102 ± 36 nm that are in good agreement with the results obtained with DLS. From the image, it can be clearly observed that the particles have solid structure resistant to the high vacuum used in SEM.

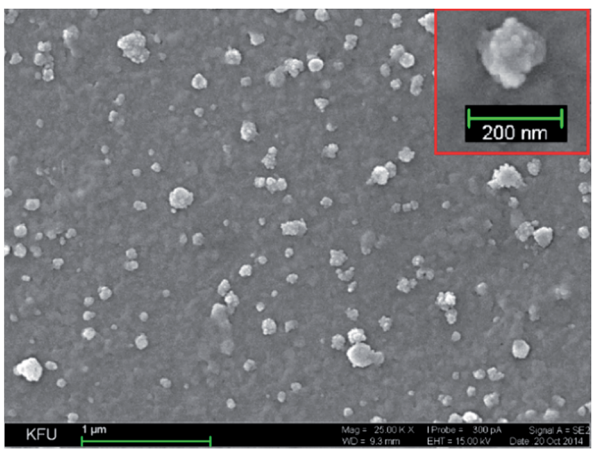


Fig. 1 SEM images of **3**-based SLNs (scale bars 1 μm and 200 nm (inset)).

The affinity of the 3-based SLNs to the model polynucleotide (salmon sperm DNA) was evaluated using fluorescence spectroscopy study of the displacement of the fluorescent probe (ethidium bromide – EB) from DNA.[22] It was found that SLNs can effectively interact with the polyanionic surface of the biopolymer resulting in a decrease in the fluorescence of the DNA–EB complex (Fig. 2).

Interaction between SLNs and pDNA (phMGFP) was observed by gel electrophoresis method (Fig. 3). The analysis of the gel shows that at concentrations of SLNs from 1.35 to 1.95 mg mL^{-1} no visible band for the free pDNA is detectable. This result suggests that the DNA is adsorbed at the surface of the SLNs. For concentrations of SLNs below than 1.35 mg mL^{-1}, the band corresponding to the plasmid DNA is visible on the gel, which mean that a part of the pDNA does not adsorb on the surface of the SLNs and remains free in solution.

In order to assess the possibility of transfecting mammalian cells using 3-based SLNs they have been incubated with three different cell lines: CV-1 (monkey kidney cell line), saiga kidney cell line and L – mouse fibroblast cell line.† It turns out that despite the high binding ability of SLNs toward the polynucleotides no any significant transfection activity was observed.

To reveal the reason of such behavior we applied UV-spectroscopy to examine the solutions with different DNA/SLNs ratios. From the spectra obtained we found that interaction between SLNs and DNA was manifested in a hyperchromicity of the absorption band at 260 nm (Δ_{260} in Table 1). This fact can be explained by the unstacking between complementary bases of DNA.[23]

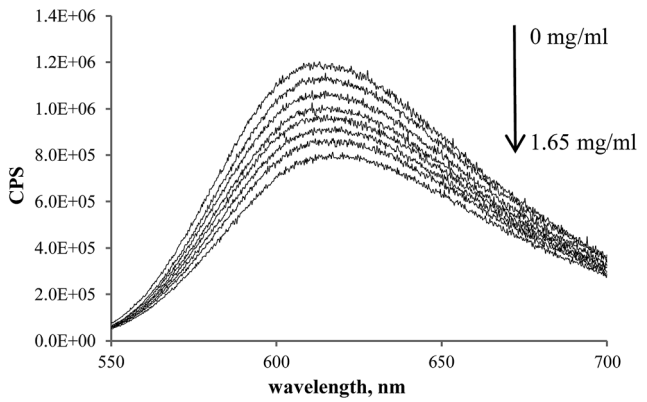


Fig. 2 Emission spectra of the DNA–EB system in the presence of the increasing amounts of the 3-based SLNs.

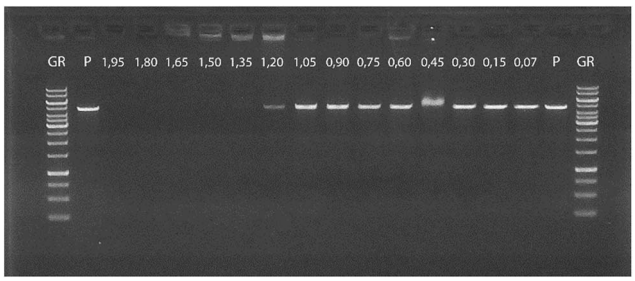


Fig. 3 Agarose gel electrophoresis of pDNA (25 μg mL^{-1}) incubated with increasing amount of 3-based SLNs. [values are expressed in mg mL^{-1}, GR – GeneRuler 1 kb DNA Ladder, P – pure plasmid DNA].

Table 1 UV spectroscopy data of mixtures of DNA (concentration is 20 μg mL^{-1}) with increasing amount of 3-based SLNs

	Absorbance at 260 nm				
SLNs (mg mL^{-1})	A_{SLNs}	A_{DNA}	A_{mix}	Δ_{260}	DoD
0.60	0.192	0.398	0.642	0.052	35
1.20	0.351	0.398	0.850	0.101	68
1.80	0.529	0.398	1.054	0.127	86

It is also known that the ratio of absorbance of single stranded DNA to double stranded DNA at 260 nm is equal to 1.37,[24] so the degree of denaturation (DoD in Table 1) of DNA can be evaluated. From the Table 1, it can clearly be seen that addition of increasing amounts of the SLNs to the DNA solution increases the degree of denaturation of DNA. It can be assumed that the high ζ-potential of the 3-based SLNs provides strong SLN–DNA interaction, which leads to distortions of a structure in the DNA located on the surface of nanoparticles and can hinder transfection.

Conclusions

Synthesized amphiphilic thiacalix[4]arene functionalized with guanidinium groups forms stable solid lipid nanoparticles in water. Applying of the flexible thiacalix[4]arene platform instead of classic methylene bridged calix[4]arene allows us to use the 1,3-*alternate* conformation for spatial separation of the hydrophobic and cationic domains. Using the macrocycle with cationic groups not conjugated with aromatic fragments provides the high ζ-potential (+61 mV) and stability of the nanoparticles. Applying gel electrophoresis and fluorescent spectroscopy methods reveals that 3-based SLNs have high binding affinity to double-stranded DNA, but despite this fact, we have not observed any significant transfection activity toward three different cell lines. UV-spectroscopy study reveals that interaction between the SLNs and polynucleotide leads to partial denaturation of the DNA located on the surface of nanoparticles that can hinder transfection.

Experimental

General

Salmon sperm DNA (≤5% protein, $A_{260/280} \sim 1.4$) and Tris–HCl were purchased from Sigma Aldrich. 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. Ethidium bromide was purchased from Acros Organics. N,N′-Bis(tert-

butoxycarbonyl)-N″-triflylguanidine has been obtained as described in this work.[25]

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-25,27-didecyl-26,28-bis[3-(bis-*tert*-butoxycarbonyl-guanidine)propoxy]-2,8,14,20-tetrathiacalix[4]arene (1,3-*alternate*) (2)

The stoichiometric amount of *N*,*N*′-di-(*tert*-butoxycarbonyl)-*N*″-triflyl guanidine in 20 mL of dichloromethane was added to the solution of 1.00 g of the compound **1** in 40 mL of dichloromethane at room temperature. 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 desiccator under reduced pressure. Yield 80%. Mp: 110–111 °C. Found: C, 65.81; H, 8.51; N, 5.11; S, 7.84. $C_{88}H_{138}N_6O_{12}S_4$ requires C, 66.05; H, 8.69; N, 5.25; S, 8.01%. MS (MALDI-TOF): calculated $[M^+]$ m/z = 1600.3, found $[M + H]^+$ m/z = 1601.5, [M + K] m/z = 1638.8. ν_{max}/cm^{-1} 1265 (COC); 1638 (N–CO); 1616, 1638 (C═N); 1718 (C═O) and 3332 (NH). δ_H (400 MHz; $CDCl_3$): 0.80–1.40 (42H, br.m, $(CH_2)_8CH_3$, CH_2–CH_2–CH_2), 1.25 (18H, s, $(CH_3)_3C$), 1.29 (18H, s, $(CH_3)_3C$), 1.51 (18H, s, $(CH_3)_3C$–O–CO–N), 1.51 (18H, s, $(CH_3)_3C$–O–CO–NH), 3.30 (4H, m, CH_2NH), 3.83 (4H, t, $^{3}J_{HH}$ = 7.9 Hz, CH_2O), 3.97 (4H, t, $^{3}J_{HH}$ = 7.9 Hz, CH_2O), 7.32 (4H, s, ArH), 7.39 (4H, s, ArH), 8.35 (2H, br.t., $^{3}J_{HH}$ = 4.6 Hz, $HNCH_2$), 11.51 (2H, s, NHCO). δ_C (125 MHz; $CDCl_3$): 14.1, 22.7, 25.8, 27.8, 28.0, 28.3, 28.8, 29.3, 29.6, 29.7, 30.0 31.3, 31.9, 34.38, 34.40, 37.7, 65.3, 68.5, 79.3, 83.2, 127.1, 127.3, 128.0, 128.2, 145.93, 145.96, 153.2, 156.3, 156.6, 156.9, 163.4. Spectrum ^{1}H–^{1}H NOESY (the most important cross-peaks): $H^{4b}/H^{7'}$, $H^{4'b}/H^{7}$, $H^{3'}/H^{7}$, $H^{3}/H^{7'}$.

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

2 mL of concentrated hydrochloric acid were added to the solution of 0.50 g of the compound **2** 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 desiccator under reduced pressure. Yield 98%. Found: C, 67.75; H, 8.88; N, 6.85; S, 10.46. $C_{68}H_{108}Cl_2N_6O_4S_4$. requires C, 64.17; H, 8.55; N, 6.60; S, 10.08%. MS (MALDI-TOF): calculated $[M^+]$ m/z = 1272.8, found $[M - HCl - Cl]^+$ m/z = 1200.0. ν_{max}/cm^{-1} 1266 (COC); 1665 (C═N); 3330 (NH). δ_H (400 MHz; $CDCl_3$): 0.80–1.40 (38H, m, $(CH_2)_8CH_3$), 1.24 (18H, s, $(CH_3)_3C$), 1.30 (18H, s, $(CH_3)_3C$), 1.82 (4H, m, $CH_2CH_2CH_2$), 3.10 (4H, br.m., NH_2CH_2), 3.81 (4H, br.m., CH_2N), 4.12 (4H, br.m., CH_2O), 7.34 (4H, s, ArH), 7.46 (4H, s, ArH). δ_C (125 MHz; $CDCl_3$): 30.9, 31.6, 33.7, 34.2, 34.3, 39.0, 73.8, 122.1, 129.0, 133.3, 134.4, 142.8, 148.2, 156.0, 156.4. Spectrum ^{1}H–^{1}H NOESY (the most important cross-peaks): $H^{4'b}/H^{3'}$, H^{4b}/H^{3}, $H^{4b}/H^{3'}$, $H^{4'b}/H^{5}$, H^{7}/H^{8}, H^{8}/H^{9}.

SLNs preparation

The SLNs suspensions were prepared by dissolving 150 mg 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^{-1}.

DLS

The size distribution of **3**-based SLNs was determined by dynamic light scattering on the nanoparticle size analyzer Zetasizer Nano ZS (Malvern) using polystyrene cuvettes. The analyzer is equipped with a 4 mW He–Ne laser operating at a wavelength of 633 nm. Measurements were carried out at an angle of 173° with automatic determination of position of measurement inside the cuvettes. Results were processed using the DTS Nano software.

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.

Ethidium bromide displacement assays

Fluorescence spectra were recorded on the fluorescence spectrometer Fluorolog 3 (Horiba Jobin Yvon). Excitation wavelength of 500 nm and a scanning range for emission wavelengths from 550–700 nm was chosen. Excitation and emission slits were 3 nm. Quartz cuvettes with optical path length of 2 mm were used. Emission spectra were automatically

adjustment by the program Fluorescence. Ethidium bromide (0.5 equivalents per base pair) was incubated with DNA (0.38 mg mL^{-1}) in a buffer solution (5 mM Tris–HCl, pH 7.5) during 20 min prior to titration by SLNs. Increasing volumes of the **3**-based SLNs solution (300 μg mL^{-1}) in a buffer solution (5 mM Tris–HCl, pH 7.5) were mixed with the premixed DNA–EB solution (final concentration of polynucleotide 0.15 mg mL^{-1}) 30 min prior to measurement. The experiment was carried out at 25 °C.

Agarose gel electrophoresis

Gel electrophoresis was conducted according to a common technique.[26]

Transfection assays

CV-1 (monkey kidney cell line), saiga kidney cell line and L – mouse fibroblast cell line were grown in Dulbecco's Modified Eagle Medium. One day before transfection, cells were plated in the appropriate amount of medium so that they reached 80–90% confluence on the day of the transfection experiment. Transfections were performed in 24-well plates, when cells were confluent (approximately 5×10^4 cells). Transfection positive controls were obtained using TurboFect Transfection Reagent ("Thermo Scientific", USA) following the manufacturer's procedure. Negative controls were obtained by adding the pure plasmid DNA. Fluorescence microscopy was used to count the number of green cells while the total amount of cells was evaluated using bright field phase contrast microscopy.

UV spectra measurements

Absorption spectra were recorded on the Shimadzu UV-3600 UV-spectrometer. Quartz cuvettes with optical path length of 10 mm were used. Increasing volumes of the **3**-based SLNs solution (3 mg mL^{-1}) was incubated with DNA (0.38 mg mL^{-1}) in a buffer solution (5 mM Tris–HCl, pH 7.5) during 30 min prior to measurement (final concentration of polynucleotide 0.015 mg mL^{-1}). The experiment was carried out at 25 °C.

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