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Macrocyclic Nonviral Vectors: High Cell Transfection Efficiency and Low Toxicity in a Lower Rim Guanidinium Calix[4]arene

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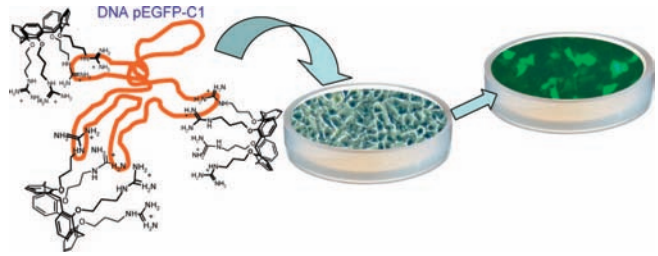
ABSTRACT



New multivalent cationic lipids, one of them showing high efficiency and low toxicity in cell transfection, have been obtained by attaching guanidinium groups at the lower rim of calix[4]arenes.

Since the first report in 1987,[1] cell transfection mediated by cationic lipids (Lipofection) has become a very useful methodology for inserting therapeutic DNA into cells, which is an essential step in Gene Therapy.[2] Among the numerous variants of this procedure reported in the literature, the most promising for both in vitro and in vivo applications is considered the combination of a cationic lipid (cytofectin) with a neutral, biologically available colipid (called "helper lipid") since it allows a good balance between efficiency and toxicity.[3] Several scaffolds have been used for the synthesis of cationic lipids, and they include polymers,[4] dendrimers,[5] nanoparticles,[6] Gemini surfactants,[7] and, more recently, macrocycles.[8] It is well-known that oligoguanidinium compounds (polyarginines and their mimics, guanidinium-modified aminoglycosides, etc.) efficiently penetrate cells,

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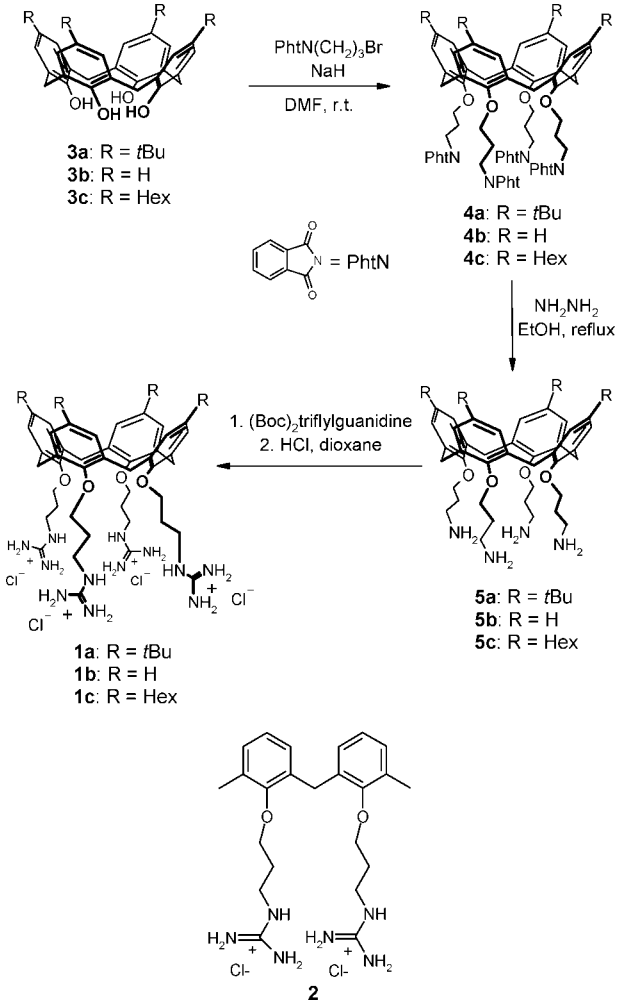
delivering a large variety of cargos.[2b,9] We have previously reported[8c] that calix[*n*]arenes bearing guanidinium groups directly attached on the aromatic nuclei (upper rim) are able to condense plasmid and linear DNA and perform cell transfection in a way which is strongly dependent on the macrocycle size, lipophilicity, and conformation. Unfortunately, these compounds are characterized by low transfection efficiency and high cytotoxicity especially at the vector concentration required for observing cell transfection (10−20 μM), even in the presence of the helper lipid DOPE (dioleoylphosphatidylethanolamine).[2c,10]

Interestingly, we have now found that attaching guanidinium moieties at the phenolic OH groups (lower rim) of the calix[4]arene through a three carbon atom spacer results in a new class of cytofectins. One member of this family (**1b**), when formulated with DOPE, performs cell transfection quite efficiently and with very low toxicity, surpassing a commercial lipofectin widely used for gene delivery. We report in this communication the basic features of this new class of cationic lipids, in comparison with a nonmacrocyclic (Gemini-type) model compound (**2**).

Scheme 1. Synthesis of the Lower Rim Guanidinium Calix[4]arenes **1a**−**c** and Molecular Formula of the Nonmacrocyclic Compound **2**



We synthesized (Scheme 1 in the article and Schemes S1 and S2 in the Supporting Information) three new lower rim guanidinium calix[4]arenes **1a**−**c** in the fixed cone conformation and compound **2**, which is a linear model of compound **1b**. All compounds are water soluble in the range 1×10^{-3}−7×10^{-2} M, **1c** being the least and Gemini **2** the most soluble. The ^{1}H NMR spectra of compounds **1a**,**b** and **2** in D_2O (see Figures S1, S3 and S7 in Supporting Information) at room temperature are sharp, whereas broadening occurs for **1c** (see Figure S5 in Supporting Information), indicating in the latter case significant aggregation in water. However, at the very low concentration ($\leq 10^{-5}$ M) used in the following studies, no evidence for aggregation was obtained for all the investigated compounds.

The ability of compounds **1a**−**c** and **2** to bind plasmid DNA pEGFP-C1 (4731 bp) was assessed through gel electrophoresis (Figure 1) and ethidium bromide displacement assays[11] (Figure S9 in Supporting Information). Both experiments evidenced that the macrocyclic derivatives **1a**−**c** bind to plasmid more efficiently than Gemini **2**. In particular, the electrophoretic data revealed that the DNA mobility is strongly affected by the macrocyclic compounds **1a**,**c** already at 50 μM. The highly condensed plasmids remain in the well and are not visible in the gel because the staining reagent (ethidium bromide) can not penetrate them. On the contrary, no change is observed with compound **2** even at 200 μM.

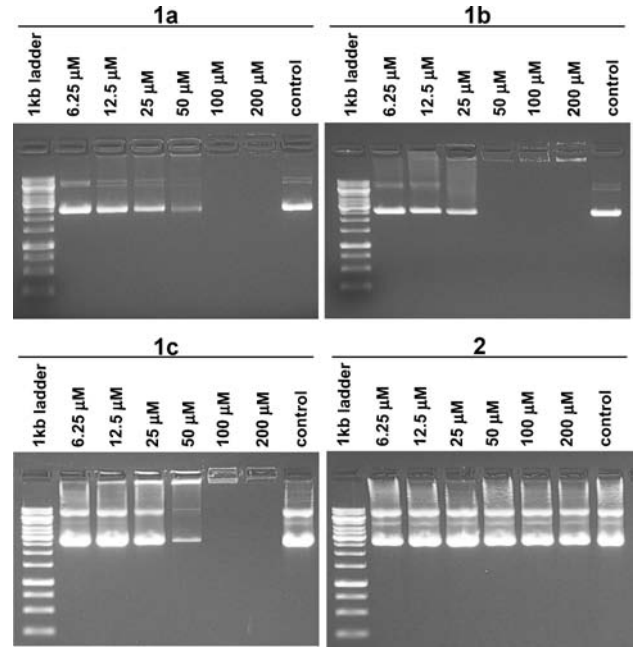


Figure 1. Electrophoresis mobility shift assays performed with plasmid DNA (pEGFP-C1) (25 nM) incubated with guanidinium calix[4]arenes **1a**, **1b**, and **1c** and compound **2** at increasing concentration (indicated above the gel). The control is plasmid without ligand.

Interesting information on the DNA condensation properties of our ligands was obtained by atomic force microscopy (AFM) studies performed in the tapping mode (Figure 2). A

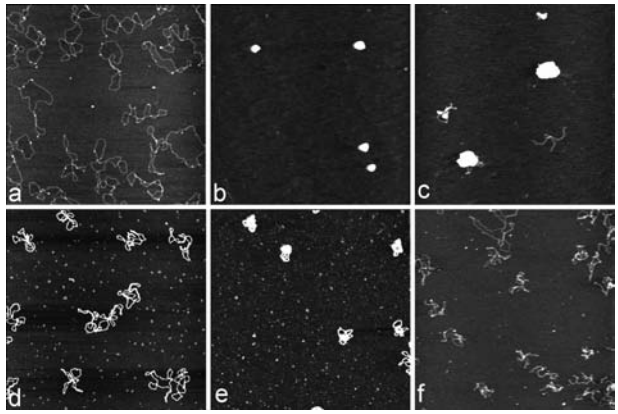


Figure 2. AFM images showing the effects induced on plasmid DNA by guanidinium ligands **1a**−**c** and **2**. All images were obtained with the microscope operating in tapping mode in air and with supercoiled pEGFP-C1 plasmid deposited onto mica at a concentration of 0.5 nM. (a) Plasmid without guanidinium derivative. Plasmid incubated with (b) **1a** 0.6 μM; (c) **1c** 2.5 μM; (d) **1b** 1.8 μM; (e) **1b** 1.8 μM + 10% EtOH; and (f) **2** 50 μM. Each image represents a 2 × 2 μm scan.

rather intriguing and subtle dependence of the DNA condensation ability of compounds **1a**−**c** on the alkyl substituent at the upper rim was observed. The *p-tert*-butyl derivative **1a** forms highly condensed, nanometric condensates of single DNA plectonemes even at 0.6 μM concentration (Figure 2b) with N/P = 0.5 (N/P = guanidinium/phosphate ratio), whereas the hexyl derivative **1c** condenses DNA only at concentration of 2.5 μM (N/P = 2) and forms condensates constituted by more filaments (Figure 2c). On the contrary, the upper rim unsubstituted compound **1b** does not form tight condensates, although the single plectonemes are much more constrained (Figure 2d) with respect to their relaxed state (Figure 2a), due to their interaction with the charged guanidinium groups. At concentrations of **1b** higher than 5 μM (N/P ≥ 4), no DNA results deposited on the mica surface. Evidently, in these conditions, **1b** causes the complete masking of the DNA negative charges preventing its deposition.

The compact condensates formed by **1a** and **1c** are partially relaxed (Figure S10 in Supporting Information) upon addition of ethanol (10% in the buffer solution), while in the case of the *para*- unsubstituted **1b** at 1.8 μM (N/P = 1.5), the presence of the alcohol favors the plasmid condensation (Figure 2e). This indicates that in *para*-alkyl substituted macrocycles **1a** and **1c** the primary electrostatic interaction between the guanidinium groups and the DNA phosphate anions is followed by hydrophobic interactions between the alkyl chains. The latter interactions, which induce the formation of condensates, are partially lost in hydroalcoholic solution. In the case of **1b**, which lacks alkyl chains at the upper rim, only charge−charge interactions, which are strengthened in the ethanol/water mixture, control the DNA condensation process. Overall, the former "double interaction" mechanism in DNA condensation is more efficient than the latter "single interaction" pathway, and this explains the AFM results. No DNA condensation at all was observed with Gemini **2** (up to 50 μM, N/P = 20) with or without ethanol (Figure 2f).

Encouraged by the biophysical evidence of DNA binding and condensation, we performed transfection experiments using plasmid DNA pEGFP-C1 (1 nM), which expresses a green fluorescent protein detectable in the cell by fluorescence microscopy, and RD-4 human rhabdomyosarcoma cells. This cell line was chosen because, beyond the medical relevance, it is easy to grow and not very well transfectable by traditional methods compared to other cell lines like HEK 293 cells and therefore useful to judge the real transfection capability of our compounds. No transfection occurs when either DOPE (Figure S11 in Supporting Information) or ligands **1a**−**c** are used alone, whereas the formulation ligand/DOPE (1/2 molar ratio) especially at ligand 10 μM (N/P = 4) is quite effective. In these conditions, we were pleased to see (Figures 3 and 4 in the article and Figure S12 in Supporting Information) that compound **1b** is a very efficient transfectant for RD-4 human rhabdomyosarcoma cells. The amount of transfected cells (48%, Figure 4) is higher than that achieved by the commercially available lipofectamine[12] LTX (30%, Figures 3 and 4) and by the previously investigated upper rim tetraguanidinium calix[4]arenes (less than 20%, Figure 4). Moreover, little transfection activity is observed for compounds **1a** (3−4%) and **1c** (6−7%) and for **2** (ca. 6% at 20 μM).

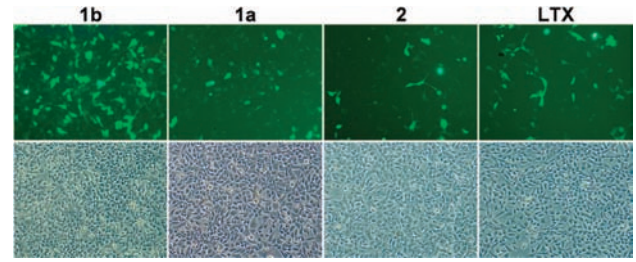


Figure 3. Transfection experiments performed with pEGFP-C1 plasmid 1 nM, guanidinium calixarene/DOPE (1/2 molar ratio, 10/20 μM), **2**/DOPE (1/1 molar ratio), and lipofectamine LTX formulations to rhabdomyosarcoma cells. Transfected cells are visualized with fluorescence microscopy (upper row, in light green because they express the enhanced green fluorescence protein EGFP) and phase contrast microscopy (lower row).

Quite rewarding was the finding that the most active compound **1b** has a very low cytotoxicity (Figure 5) showing

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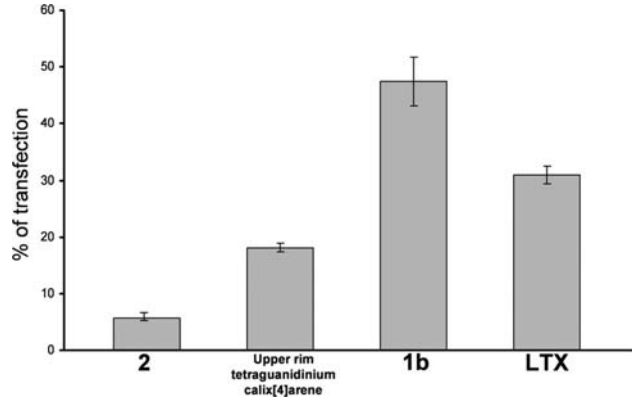

Figure 4. In vitro transfection efficiency as percentage of transfected cells observed upon treatment of RD-4 human rhabdomyosarcoma cells with guanidinium derivative/DOPE formulations and lipofectamine LTX.

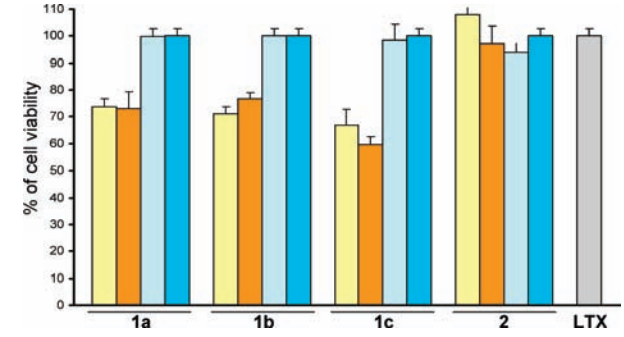

Figure 5. Post-transfection cell viability in the presence of ligand alone (yellow bars), ligand/DOPE formulation (orange bars), DOPE alone (light blue bars), without any treatment (blue bars), and in the presence of LTX (gray bar).

75−80% of cell viability at 48 h from transfection. Similar results were obtained with **1a**, while **1c** is more toxic (60% of cell viability).

It is not surprising that we did not find a strict correlation between the transfection efficiency of the **1a**−**c**/DOPE formulations and the ability of the ligands to condense DNA as disclosed by AFM studies. In fact, cell transfection achieved with cytofectin/DOPE formulations is a complex supramolecular function, and many important steps outside and inside the cell must be positively affected to achieve the goal.[13]

Since it is well-known in the field of synthetic nonviral vectors that transfection efficiency may depend on the type of cell used, we tested our lead compound **1b** in a varying cell line setting using again LTX as a reference (see Figure S13 in Supporting Information). Comparable results with the two formulations are obtained with AUBEK and BoMAK cells, whereas LTX is definitely more active in the case of hMSC and N2a cell lines. The reverse is true for Vero cells which are not transfected at all by the commercial product LTX, while **1b**/DOPE gives transfection (ca. 12%).

In summary, we have found that attaching guanidinium groups at the phenolic oxygen atoms (lower rim) of calix[4]arenes discloses the possibility to significantly enhance the cell transfection ability of these synthetic cationic lipids formulated with DOPE and reduce their toxicity to cells, if compared to the same macrocycles with the charged groups directly linked to the aromatic nuclei (upper rim). The DNA binding and condensation properties, cell transfection ability, and toxicity shown by the macrocyclic cytofectins **1a**−**c** strongly depend on the substituents at the upper rim of the calixarene. Comparison of the DNA condensation and cell transfection efficiency of the most active compound **1b** with its open chain analogue **2**, having a very similar lipophilic/hydrophilic ratio, suggests a possible positive role of the macrocyclic scaffold on gene delivery to cell, which needs to be investigated in more detail. Notably, the transfection efficiency of the little toxic **1b**/DOPE formulation is even higher than that of the commercially available LTX in the case of RD-4 human rhabdomyosarcoma and Vero cell lines. The results obtained warrant further studies which are ongoing in our laboratory aimed at elucidating the mechanism of cellular uptake and intracellular trafficking of this novel DNA delivery system and its cargo to establish a structure−activity relationship.

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Supporting Information Available: Experimental section and characterization for all new intermediates and compounds **1a**−**c** and **2**, ^{1}H and ^{13}C NMR spectra of **1a**−**c** and **2**, Schemes S1 and S2, and Figures S1−S13. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) The first additions of **1a**−**c** to the ethidium bromide/DNA mixture result in a drastic and regular fluorescence quenching (Fig. S9 in Supporting Information). However, at higher ligand concentrations (at N/P ratio of 4.2 for **1a**, 8.5 for **1b**, and 5 for **1c**), a marked change in the slope of the titration curve occurs. This is probably due to the interaction between ethidium bromide and the DNA−calixarene complex and prevents a quantitative evaluation of the ligand affinity for plasmid.

(12) Lipofectamine LTX Reagent is a cationic lipid-based, animal-origin free formulation for the transfection of DNA into eukaryotic cells with low cytotoxicity. See: http://www.invitrogen.com/downloads/F-069843-LpofctLTX_FHR.pdf.

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