Arginine clustering on calix[4]arene macrocycles for improved cell penetration and DNA delivery

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Cell-penetrating peptides are widely used as molecular transporters for the internalization inside cells of various cargo, including proteins and nucleic acids. A special role is played by arginine-rich peptides and oligoarginines covalently linked or simply mixed with the cargo. Here we report cell-penetrating agents in which arginine units are clustered on a macrocyclic scaffold. Instead of using long peptides, four single arginine units were covalently attached to either the upper or lower rim of a calix[4]arene, kept in the cone conformation building a 'parallel' cyclic array. These new macrocyclic carriers show high efficiency in DNA delivery and transfection in a variety of cell lines.
Cell-penetrating peptides (CPPs)\textsuperscript{1–3} are widely used as molecular transporters for the internalization of various molecular and macromolecular cargo inside cells. Among them, arginine-rich peptides, such as HIV-1 TAT fragments\textsuperscript{34,35} and oligoargininies\textsuperscript{5}, have a special role. They can be covalently linked to or, more simply, mixed with the cargo, both strategies showing examples of significant translocation activity\textsuperscript{6}. Because of these features, arginine-rich peptides can also help the penetration of nucleic acids across the cell membranes opening interesting perspectives in gene delivery, which is a necessary prerequisite for gene therapy\textsuperscript{7–11}. In this context, following a non-covalent approach\textsuperscript{12,13}, CPPs are used in formulation, either simply with the nucleic acid filaments or in a ternary assembly, including also a cationic lipid. Alternatively, they can be covalently linked to a lipid structure (Fig. 1a) in which the resulting ratio between arginine units and lipophilic tails is, in general, of several to one \textsuperscript{14–16}. A certain degree of rigidity imposed to the peptide structure, for example by cyclization of the arginine-rich sequences, seems to further enhance the cellular uptake\textsuperscript{17}. We reasoned that the clustering of arginine units on a spatially well-defined macrocyclic scaffold (Fig. 1b) could be exploited to enhance their cell-penetrating properties, and we report here the first example of this strategy, applied to DNA delivery and cell transfection. Considering that cyclodextrins\textsuperscript{18} and calixarenes\textsuperscript{19–25} are non-toxic and have been used as scaffolds for building gene delivery systems, we selected calix[4]arene as the macrocyclic platform and anchored to it single arginine units rather than complex arginine-rich peptides, or linear and long oligoarginines, to limit to a ratio of one to one between amino acids and the lipophilic tails (Fig. 1b). Therefore, we herein describe the synthesis and the DNA binding and condensation properties of two new C-linked \textit{L}-argininocalix[4]arenes and two acyclic Gemini-type analogues. Moreover, taking into account the several examples of polyllysines used for cell transfection\textsuperscript{26}, we also prepared a calixarene adorned at the upper rim with four \textit{L}-lysine units, to verify the differences in activity related to the two types of amino acid. Gene-delivery studies with these compounds showed that the upper-rim arginine derivative 3a, in particular, has exceptional transfection properties and low cytotoxicity, suggesting that the proposed strategy of clustering arginine units on rigid, lipophilic macrocyclic scaffolds could be envisaged as a new approach to improve cell penetration of cargo.

**Results**

**Synthesis.** The upper rim tetraarginino- (3a) and tetra-lysinosinocalix[4]arene (3b), belonging to the class of C-linked peptidocalixarenes\textsuperscript{27,28}, were prepared (Fig. 2) starting from tetramino-tetrahexyloxycalix[4]arene 1, selected on the basis of the previous experimental observation\textsuperscript{19}, such that in the upper rim guanidinocalix[4]arene vectors the presence of hexyl chains at the lower rim allowed cell transfection in the absence of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), with the same efficiency shown in the presence of this helper. The lower rim tetraargininocalix[4]arene 6 was synthesized from the tetrapropylamino precursor 4 (ref. 29; Fig. 2). In the synthetic pathway of both compounds 3a and 6, by using \textit{N}_2-N_boc-\textit{N}_2-tosyl-\textit{L}-arginine or \textit{N}_2-Fmoc-\textit{N}_2-Mtr-\textit{L}-arginine, the unexpected cleavage of the amide bonds between the acid amino units and the calixarene occurred during the deprotection steps. On the contrary, we did not observe this side reaction either when \textit{N}_2-Boc-\textit{N}_2-Pbf-\textit{L}-arginine (Boc-\textit{L}-Arg(Pbf)-OH) and \textit{N}_2-Cbz-\textit{L}-arginine were attached at the upper and at the lower rim of 1 and 4, respectively, or during the removal of Boc groups from the lysine functionalized compound 2b.

The two Gemini-like derivatives (7 and 8 in Fig. 3) were prepared through similar procedures by successfully using Boc-\textit{L}-Arg(Pbf)-OH for both (details in Supplementary Methods). After standard deprotection reactions, the chloride salts of peptido-calixarenes 3a,b and 6, and of non-macro cyclic compounds 7 and 8 were isolated.

All compounds resulted to be water soluble, but although 3a largely tends to form aggregates (Supplementary Fig. S1) due to the presence of aliphatic chains at the lower rim, 6 showed sharp \textsuperscript{1}H NMR signals in D\textsubscript{2}O (Supplementary Fig. S2), ruling out self-assembly phenomena even in the presence of the high concentrations of the inorganic salts (NaCl and MgCl\textsubscript{2}) added to the sample to increase the ionic strength and to reproduce more closely the conditions of the Atomic Force Microscopy (AFM) and transfection experiments. In the latter conditions, 3b, which is monomeric in pure D\textsubscript{2}O, self-assembles in species in slow exchange equilibrium on the NMR time scale with the monomer, whose signals progressively disappear by increasing the salt concentration (Supplementary Fig. S3). This self-assembly activity, although not sufficient, seems an important prerequisite to induce DNA compaction and delivery.

**DNA binding and condensation.** Displacement of ethidium bromide (Supplementary Fig. S4) and electrophoresis mobility...
dimethylaminopropyl)carbodiimide hydrochloride (EDAC), hydroxybenzotriazole (HOBt), Pd(C), HCl, ethanol, rt. Boc: rt, for Boc-L-Lys(Boc)-OH, O methanol, pH 4, rt; (iii) Cbz-L-Arg-OH, N,N dimethylaminopyridine (DMAP), dry CH2Cl2, rt, for 2a, or Boc-L-Lys(Boc)-OH, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIPEA), dry CH2Cl2, rt, for 2b (i, 1) TFA/triisopropylsilane (TIS)/H2O (95/2.5/2.5), rt, for 3a, or TFA/triethylsilane (TES)/CH2Cl2 (10/2.5/87.5) at 0 °C for 3b. (2) dil. HCl in methanol, pH 4, rt; (iii) Cbz-L-Arg-OH, N,N'-dicyclohexylcarbodiimide (DCC), HOBt, dry DMF, rt, SAX (strong anion exchange) sorbent (Cl–); (iv) H2/Pd(C), HCl, ethanol, rt. Boc: t-Butoxycarbonyl, Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, Cbz: carbobenzyloxy.

Figure 2 | Synthesis of peptidocalix[4]arenes 3a,b and 6. Reagents and conditions: (i) Boc-L-Arg(Pbf)-OH (N2BOC-N-0-Pbf-L-arginine), N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC), hydroxybenzotriazole (HOBt), N,N-dimethylaminopyridine (DMAP), dry CH2Cl2, rt, for 2a, or Boc-L-Lys(Boc)-OH, O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N,N-diisopropylethylamine (DIPEA), dry CH2Cl2, rt, for 2b (i, 1) TFA/triisopropylsilane (TIS)/H2O (95/2.5/2.5), rt, for 3a, or TFA/triethylsilane (TES)/CH2Cl2 (10/2.5/87.5) at 0 °C for 3b. (2) dil. HCl in methanol, pH 4, rt; (iii) Cbz-L-Arg-OH, N,N'-dicyclohexylcarbodiimide (DCC), HOBt, dry DMF, rt, SAX (strong anion exchange) sorbent (Cl–); (iv) H2/Pd(C), HCl, ethanol, rt. Boc: t-Butoxycarbonyl, Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonylet-butyloxycarbonyl, Pbf: 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, Cbz: carbobenzyloxy.

Figure 3 | Acyclic ligands. Gemini-type analogues of argininocalixarenes 3a and 6. For synthetic details relative to compounds 7 and 8, and their precursors see Supplementary Information.

shift assays (Supplementary Fig. S5) evidenced the ability of all calixarene (3a,b and 6) and Gemini-like (7 and 8) conjugates to interact with plasmid EGFP-C1 (4731 bp) expressing for green fluorescent protein. AFM experiments in tapping mode on air allowed to visualize the different effects of each of them on the DNA folding (Fig. 4 and Supplementary Fig. S6). The upper rim argininocalixarene 3a induces the DNA condensation to 50–60 nm globular species formed by a single ds-filament (Fig. 4a), and with proper dimension and compaction for cellular uptake. The lower rim arginino derivative 6 causes, on the contrary, the formation of large aggregates involving several filaments (Fig. 4b), partially reduced in size and in compaction degree by a decrease of the ligand concentration (Supplementary Fig. S6a). These multiplasmid aggregates are presumably too large to cross the cell membrane. Large aggregates are also formed by incubation of the plasmid with the lysino derivative 3b (Supplementary Fig. S6b), but the presence of DOPE, at a concentration double the X concentration, does not generate globular condensates even if the interaction ability of the two ligands with the plasmids is unequivocally proved in AFM experiments by the DNA folding (Supplementary Fig. S6c,d), clearly different from the relaxed condition of the ds-filaments (Fig. 4d).

Transfection properties. To verify the ability of these compounds as gene-delivery systems, we selected for transfection the human Rhabdomyosarcoma (RD-4) cells because of their medical relevance and difficulty to be transfected by already established protocols and available formulations. A substantial agreement was found between the effect produced by the different ligands on the plasmid, observed by AFM (Fig. 4 and Supplementary...
Fig. S6), and their ability and efficiency in the transfection process (Fig. 5 and Supplementary Fig. S7). The arginino-calix[4]arene 3a resulted a very poor vector and only in the presence of the adjuvant DOPE was able to deliver pEGFP-C1 DNA (1 nM) in 5% of cells (Fig. 5 and Supplementary Fig. S7). The failure of 6 as a gene-delivery system can be ascribed to the absence of a significant lipophilic region able to counterbalance the polarity of the substituents at the lower rim. Moreover, the shape of this ligand and the volume occupied by the polar heads with respect to the lipophilic portion constituted only by the aromatic cavity is apparently not suitable to induce aggregation phenomena able to support a transfection activity. Its Gemini-type analogue 8 failed in transfection also in the presence of the helper. On the contrary, the activity shown by the upper rim arginino-calix[4]arene 3a is impressive. At 10 μM (cationic/anionic charges ratio N/P = 8.4), it was able to transfect 75% of cells (Fig. 5a,b). Quite relevant is the comparison with its Gemini-type analogue 7 (9% of transfection but with DOPE) and with the commercially available LTX lipofectamine (35%) and polyethyleneimine (PEI; 41%) used as references (Fig. 5b). It is noteworthy the substantial inactivity observed for the lysine containing cluster 3b, whose efficiency is indeed boosted by DOPE to percentages of transfected cells (41%) only slightly lower than those obtained with the arginino-calix[4]arene 3a in presence of the adjuvant (46%). Particularly striking is that the highest transfection efficiency is reached by 3a without the helper lipid DOPE, which otherwise is usually exploited to enhance the vector efficiency and minimize its toxicity. This underlines the per se potency of this vector and, in the specific context of transfection, would allow, in perspective, the use of this macrocyclic cationic surfactant without helpers, thus simplifying the formulations. Remarkably, in the presence of serum the delivery properties of 3a are only slightly reduced with a transfection efficiency over 60%.

The same results were substantially confirmed by experiments with luciferase (Supplementary Fig. S8) again showing the higher efficiency of 3a with respect to the other ligands and LTX. The marked difference between the activity of 3a and that of its non-macrocyclic analogue 7 strengthens the hypothesis of a macrocyclic effect on the transfection process. The rather low cytotoxicity of 3a (Supplementary Fig. S9), which, quite unusually, increases in the presence of DOPE, is also worth noting. In particular, in transfection conditions the level of cell viability in the presence of this arginino-calix[4]arene (Supplementary Fig. S9b) is very close to that observed with LTX.

The comparison between the structurally similar arginine cluster 3a and lysine-cluster 3b was widened by testing their transfection efficiency to other cell lines (Fig. 5c). Besides the C2C12 line to which both ligands resulted inactive and the LTX and PEI also have a very poor efficiency, in all other cases the superiority of the arginino-calix[4]arene 3a over the lysino analogue 3b, in absence of DOPE, appeared unequivocal (Fig. 5c). In fact for 3b, at the best 18% of transfected cells was observed in the case of Hela, while for 3a more than 80% of transfection was obtained in the treatment of this cell line, more than 70% with N2a, 60% with equine adipose-derived stromal cells (EADSCs), and more than 50% with Cos-7 and 40% with Vero cells. Moreover, excluding the cases of HEK cells, where LTX showed a significantly larger efficiency (75% versus ca. 50%), and of the above mentioned C2C12 cells, arginino-calix[4]arene 3a always resulted in a higher or at least comparable transfection activity with those of the references LTX and PEI. With EADSCs cell line, for instance, PEI was completely inefficient, whereas the 60% of transfection was obtained upon treatment with 3a that also almost doubled the LTX (36%).

As in the experiments with RD-4 cells, the presence of DOPE, in general, decreased the transfection efficiency of 3a, drastically in some cases (COS-7, HEK, ISHIKAWA lines), even if a small activity with C2C12 cells was determined, which were not transfected at all in absence of adjuvant. On the other hand, the presence of DOPE transformed lysino-calix[4]arene 3b in a vector (Fig. 5c), which was able to efficiently surpass the arginine cluster 3a in the case of HEK, ISHIKAWA, N2a and Vero cells, resulting in a better transfection efficiency for the latter two cell lines than all the other formulations.

This further supports the idea that simple clustering on the cone calix[4]arene scaffold of a single unit of basic amino acid for lipophilic tail can effectively represent a novel winning strategy to build potent cell-penetrating systems.

Discussion

In conclusion, the clustering of only four units of basic amino acids, such as arginine and lysine, on a rigid macrocyclic scaffold like cone calix[4]arene, displaying two spatially well-defined regions, one apolar at the lower rim and one polar at the upper rim, gives rise to new, potent non-viral vectors for cell transfection. In particular, in the case of the tetraarginino-calix[4]arene 3a, the one to one ratio between amino acid units and lipophilic tails is enough to reach this result, indicating that the circular array provided in this ensemble remarkably boosts the cell penetrating properties that arginine usually shows in linear oligomeric sequences and arginine-rich peptides, such as TAT HIV-1 fragments. The clearly higher efficiency of the arginino cluster 3a compared with analogue 3b bearing four lysine units, in absence of any adjuvant, underlines the superiority of arginine and then of guanidinium group in conferring to the vector its cell penetrating properties. Moreover, the high transfection efficiency shown by the tetr lysino-calix[4]arene 3b when used with DOPE confirms the efficacy of this clustering strategy. The novel amino acid

**Figure 4** | AFM images of plasmid DNA. AFM images in tapping mode on air showing the effects induced on pEGFP-C1 plasmid DNA folding by incubation with (a) 2 μM arginino-calix[4]arene 3a; (b) 1 μM arginino-calix[4]arene 6; (c) 2 μM lysino-calix[4]arene 3b in presence of DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) 4 μM; and (d) image of pEGFP-C1 plasmid DNA 0.5 nM alone. Each image represents a 2 × 2 μm scan.
Figure 5 | Cell transfection experiments. (a) Images by fluorescence microscopy of human Rhabdomyosarcoma cells transfected (in green) upon treatment (at 48 h) with EGFP-C1 plasmid 1 nM formulated with (top) 10 μM calixarene 3a and (bottom) LTX. In histogram b, transfection efficiency (at 48 h) to Rhabdomyosarcoma cells of argininocalix[4]arenes 3a and 6 compared with the non-macrocyclic model 7 (20 μM), the lysinocalixarene 3b, DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), LTX and PEI (polyethyleneimine). Error bars denote s.d. (n > 3). In histogram c, transfection efficiency to other cell lines of argininocalixarene 3a (red bar) compared with argininocalixarene 3a with DOPE (pale red bar), lysinocalixarene 3b (blue bar), lysinocalixarene 3b with DOPE (light blue bar), LTX (grey bar) and PEI (light grey bar). Calixarene derivatives and DOPE were used at 10 and 20 μM, respectively. Error bars denote s.d. (n > 3).

Methods

General. Moisture-sensitive reactions were carried out under a nitrogen atmosphere. Dry solvents were prepared according to standard procedures and stored over molecular sieves. Melting points were determined on an Electrothermal apparatus in capillaries sealed under nitrogen. 1H and 13C NMR spectra were recorded on Bruker AC300, AV300 and AV400 spectrometers (partially deuterated solvents were used as internal standards). For 1H NMR spectra recorded in D2O at values higher than the room temperature, the correction of chemical shifts was performed using the equation \( \delta = 5.060 \times 0.0122 \times T (°C) + (2.11 \times 10^{-7}) \times T2 (°C) \), to determine the resonance frequency of water protons. Electrospray ionization–mass spectrometry (ESI-MS) spectra were recorded on a LTQ Orbitrap XL instrument in ESI mode. Thin-layer chromatography was performed on Merck 60 F254 silica gel and Merck silica gel 60 RP-18. Flash column chromatography was performed on 230–400 mesh Merck 60 silica gel. HPLC purifications were performed on a 1100 Series Liquid Chromatograph Agilent, using an LC Detector. Strong anion-exchange (Cl−) cartridges were used.

5,11,17,23-Tetakis(ω-Amino)-25,26,27,28-tetrakis(n-hexyloxy)calix[4]arene octa-hydrochloride (3a). A solution of calix[4]arene 2a (80 mg, 2.8 × 10^{-2} mmol) in trifluoroacetic acid (TFA)/triisopropylsilane/H2O (95/2.5/2.5, 5 ml) was stirred at room temperature. The progression of the reaction was followed by ESI–MS. After completion, the volatiles were removed under reduced pressure, the solid residue repeatedly suspended in ethyl acetate (3 × 5 ml), and the organic solvent evaporated to help in removing the exceeding TFA. The crude material was then washed with distilled diethyl ether (3 × 5 ml) and removed after sample centrifugation. The trifluoroacetate anion of the resulting TFA-octa-salt was exchanged with chloride by dissolving the solid in a methanol solution of concentrated HCl (pH = 3–4) followed by evaporation (4 ×). Compound 3 was obtained as white solid (37 mg, 2.1 × 10^{-3} mmol, 75%); melting point (mp): 230 °C (dec); 1H NMR (300 MHz, CD3OD): \( \delta = 7.18 \) (s, 4 H), 6.87 (s, 4 H), 4.47 (d, \( J = 12.9 \) Hz, 4 H), 4.06 (bs, 4 H), 3.90 (t, \( J = 7.3 \) Hz, 8 H), 3.40–3.20 (m, 8 H), 3.16 (d, \( J = 12.9 \) Hz, 4 H), 1.98 (bs, 16 H), 1.72 (bs, 8 H), 1.50–1.20 (m, 24 H), 0.94 (t, \( J = 6.3 \) Hz, 12 H); 13C NMR (75 MHz, CD3OD): \( \delta = 167.9, 158.9, 155.3, 136.6, 136.5, 133.2, 122.6, 122.1, 77.5, 54.8, 42.2, 33.7, 32.4, 31.8, 30.2, 27.6, 25.9, 24.3, 14.8 \) (see Supplementary Fig. S10); MS (m/z): [M + 2 + H–8HCl]^2+ calcd. for...
25,26,27,28-Tetraakis-[3-(L-Arg-amino)propanoyl]calix[4]arene octa-hydrochloride (8). Calix[4]arene 5 (80 mg, 4.1 × 10^-14 mmol) was dissolved in MeOH (13 mL) and a catalytic amount of Pd/C (10%) was added. Hydrogenation (1 atm, 24 h) was observed under fluorescence microscope for enhanced green fluorescent protein expression. Each experiment was done at least three times. Statistical differences between treatments were calculated with student’s t-test and multifactorial analysis of variance.

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**Author contributions**

V.B., M.B. and M.L. carried out synthesis, characterization of experiments and binding studies. V.F. carried out electrophoretic mobility shift assay (EMSA), cytotoxicity and transfection studies. G.D. carried out EMSA, cytotoxicity and transfection studies and helped in planning the experiments. F.S. carried out AFM studies, helped in planning experiments and in designing the project, and participated in the preparation of the manuscript. A.C. and R.U. helped in planning experiments and in designing the project and participated in the preparation of the manuscript.

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