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Multivalent and multifunctional calixarenes in bionanotechnology

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Abstract: The key features of calixarene derivatives as multivalent ligands for biomacromolecules and as multifunctional catalysts are herein critically reviewed. The ease of functionalization and the possibility to control the regio- and stereochemical disposition of multiple ligating units around a central core allow to obtain ligands with high affinity and selectivity especially for proteins and nucleic acids. The hydrophilic/lipophilic character can also be finely tuned allowing to obtain monomeric hybrid derivatives or amphiphiles able to self-assemble alone or in co-formulation with lipids to give nanoparticles and liposomes that incorporate calixarenes. The knowledge acquired up to now sheds therefore light on the future applications of calixarenes in bionanotechnology and nanomedicine.

1. Introduction: from Molecular Receptors to Multivalent Ligands

From the dawn of Supramolecular Chemistry, the cyclic oligomers made-up of phenol and formaldehyde (Figure 1, left), are progressively assuming increasing importance. At the end of the 1970's, C. D. Gutsche, beside establishing the ideal reaction conditions to isolate pure tetramers, hexamers or octamers, also proposed to name them "calixarenes" because of their similarity in shape to a "calix" (Latin name of a vase).[1,2] Like a vase these meta-cyclophanes promptly proved to be able to host in their lipophilic cavities small apolar organic molecules, as was shown by Andreetti, Ungaro and Pochini who published the first X-ray crystal structure of an inclusion complex, the adduct between p-tert-butylcalix[4]arene and toluene.[3] The popularity of these macrocycles rose rapidly in Supramolecular Chemistry during the following years not only because they can be prepared with high purity even in hundreds of kilo scale, but also because of their ease of functionalization both at the upper and lower rim. Well reproducible regio- and stereoselective methodologies[4] are nowadays available for the preparation of an incredibly wide range of calixarene derivatives. A rapid screening on SciFinder® allows to quote that roughly more than 30,000 different calixarene derivatives, as single compounds, were prepared in the latest 50 years. Interestingly, especially for what concerns the smaller calix[4]arenes, the introduction of groups equal or bulkier than propyl on the phenolic oxygen atoms conformationally blocks the macrocycle in one of the four limiting structures: cone, partial cone, 1,2-alternate or 1,3-alternate (Figure 1, right).

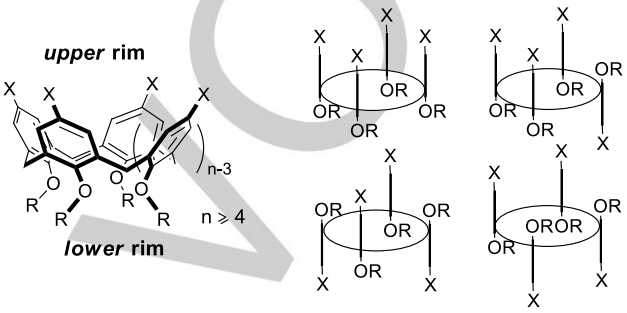
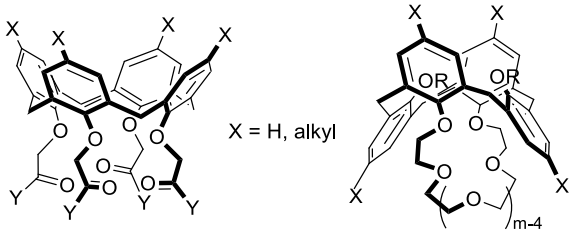


Figure 1. The general formula of calix[n]arenes (left) and the four limiting conformations for the smaller calix[4]arene: cone, partial cone, 1,2-alternate and 1,3-alternate (displayed in clockwise order from the upper left corner).

This allows to mould the calixarene scaffold, giving rise to stereoisomers that possess quite different properties. At the end of the last century, a series of extremely efficient and selective ligands for spherical metal ions were thus developed by introducing chelating units at the lower rim of calix[4]arenes.[5,6] The introduction of acetic ester or amide groups (**1**) on calix[4]arenes fixed in the cone structure, in fact, resulted in efficient ligands for Na^+ and trivalent lanthanide cations,[7] while the preparation of 1,3-dialkoxy-calix[4]arene-crown-5 (**2**) and crown-6 (**3**) derivatives gave rise to some of the most selective ionophores for K^+ and Cs^+ known so far. The smaller calix-crown-5 derivatives **2** in the 1,3-alternate structure have a K^+/Na^+ selectivity higher than that of the natural occurring valinomycin.[8] The calix-crown-6 family[9] **3** shows a Cs^+ selectivity so striking to be applied in the separation of long-lived cesium radioisotopes from spent nuclear fuel. The industrial process developed by DOE in the U.S., and currently using 2 kilos of one of these derivatives,[10] probably represents one of the most important applications of Supramolecular Chemistry to modern pressing social issues. A plethora of examples of receptors for metal ions were reported in the following years, including ionophores selective for hard metal ions (divalent alkaline earth or trivalent lanthanide and actinides) or soft transition metal ions,[5,6] depending on the nature of the donating atoms present on the ligating units introduced on the macrocycles. Several of these ionophores were included in ion-selective electrodes and ion-selective field-effect transistors or chromophoric and fluorophoric versions of these ligands were also proposed to develop optical devices.[11]



1: Y = OR', NR''R'''

2: m = 5, R = alkyl
3: m = 6, R = alkyl

At the turn of the millennium, following a biomimetic approach, our group found that macrobicyclic calixarenes functionalized with amino acids or oligopeptides have an antimicrobial activity towards gram-positive bacterial thanks to the binding (Figure 2, top left) to the terminal D-Ala-D-Ala part of peptidoglycan through electrostatic, H-bonding and possibly CH-π interactions.[12,13] Similarly, macrobicyclic peptidocalixarenes are able to complex small polar organic anions in DMSO via the interaction with their carboxylate residues (Figure 2, top right).[14] Anionic macrobicyclic calixarenes, on the other side, complex 1-alkoxyglucosides and -galactosides in apolar solvents, with a slight preference for the β-anomers (Figure 2, bottom).[15]

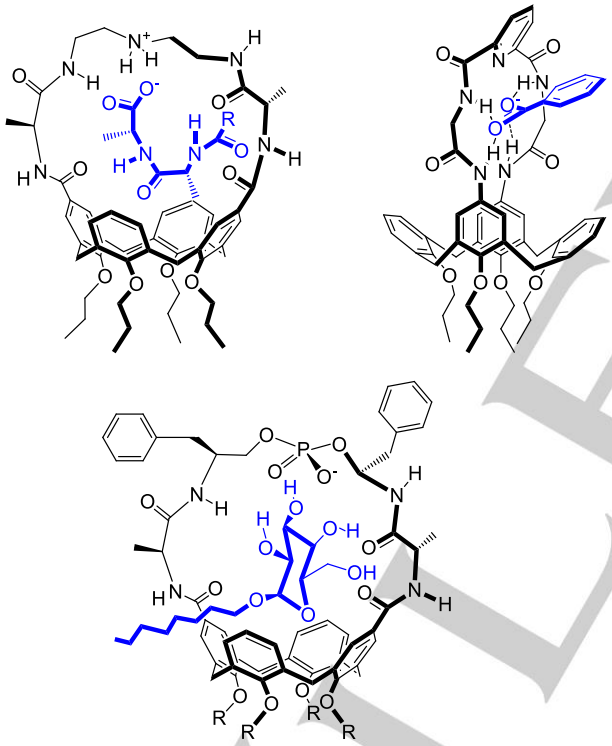


Figure 2. Proposed structures for the complexes between the macrobicyclic peptidocalixarenes and N-Ac-D-Ala-D-Ala (top, left), benzoate (top, right) and a β-glucoside (bottom).

In the latest twenty years, we and others started to use calixarenes no more as building blocks for the construction of preorganized endo-receptors for ions or small organic molecules but as platforms for the synthesis of multivalent ligands,[16,17] with the aim to bind large biomacromolecules and to modify their biological activities.[18] Multivalency[19,20] is a particularly important instrument used by Nature and adopted in bioorganic and supramolecular chemistry to increase efficiency and specificity of ligands for large biomacromolecules. Nature uses multivalency especially in carbohydrate-protein interactions which, taken singularly, are usually considered weak for the biological world. By exposing multiple copies of carbohydrates on its exterior, in the region called glycocalyx (Figure 3, right), a cell is able to communicate and interact with the protein receptors present on a neighbouring cell via multiple carbohydrate-protein interactions. This is the *Glycoside Cluster Effect*, responsible of a wide range of physiologic and pathologic events throughout the cell life.[21] When a positive multivalent effect is observed, the binding free energy originated by the interactions of two entities through "n" interactions is much higher than the sum of the "n" binding free energies of each single interaction thanks to a highly favourable entropic contribution.[21] Chemists started to mimic the multivalent effects observed in Nature also in the design of novel ligands for proteins[22,23] or in nanotechnology to improve binding between components of molecular materials and machines.[22]

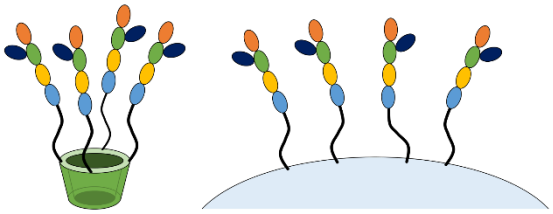


Figure 3. A multivalent calixarene, glycocalixarene when adorned with saccharide units (left), and a portion of the multivalent surface (glycocalyx) of a cell (right) expose multiple copies of the same oligosaccharide used for the efficient binding of biomacromolecules.

Calixarenes are ideal cores for the construction of multivalent ligands (Figure 3, left), as their chemistry is nowadays well consolidated and allows the easy introduction of ligating units selectively at one of the two rims. The resulting valency can moreover be easily set to 4, 5, 6, 8 or multiples by using, respectively, calix[4]-, -[5]-, -[6]-, -[8]arenes which are the most easily accessible macrocycles. Another important advantage lies in the fact that the hydrophilic/lipophilic character can be modulated at will, by introducing appropriate polar or lipophilic groups at the rim opposite to that carrying the ligating units. The introduction of polar or even charged ligating units often makes the multivalent ligand water soluble, a clearly important feature for biologically active compounds. For other particular purposes, such as the incorporation of the multivalent ligand into vesicles, liposomes or lipophilic layers, on the other side, it is important to have amphiphilic multivalent ligands and this can be achieved by the introduction of polar ligating units at one rim and lipophilic chains at the other rim.[18] Especially for the smaller calix[4]arenes, where the structure can be blocked into the cone, partial cone or 1,2- and 1,3-alternate geometries, the multivalent ligand can be easily moulded as desired. Quite common are the examples of multivalent calix[4]arene ligands blocked in the cone structure since it allows to have the ligating units projected in one direction and preorganized to interact with the surface of the target biomacromolecule. Moreover, when polar groups are linked at the upper rim, the combination of electrostatic, H-bonding and dipole-dipole interactions with the CH-π and hydrophobic interactions offered by the calixarene cavity may

give rise to synergistic effects that enhance the binding and the selectivity.

In the present review we will give a critical overview of some of the most relevant examples of the use of multivalent calixarenes to bind proteins, nucleic acids and other biopolymers with perspectives for their application in bionanotechnology and nanomedicine.[24] The light will be especially put on those examples where the target of binding is clearly identified and the mechanism of action at least hypothesised.

2. Ligands for Proteins

Proteins perform their biochemical functions such as catalysis, signalling and transport by way of specific recognition events. Different strategies were used in these years to bind proteins and modify their biological activity by using calixarene ligands (Figure 4).

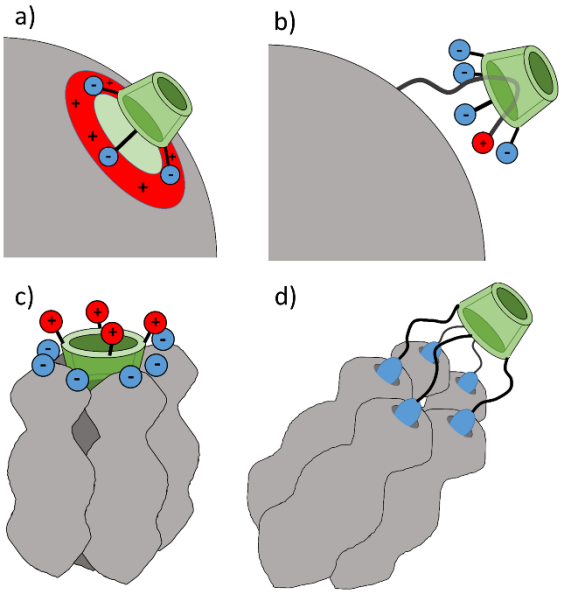
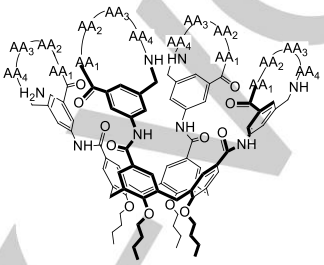


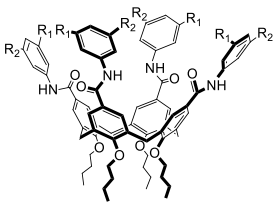
Figure 4. The four different strategies used to target proteins with calixarene ligands: a) large surface area, hot spot, recognition (Sect. 2.1), b) single amino acid residue binding (Sect. 2.2), c) stoppering of lipophilic pockets (Sect. 2.3), d) binding to multiple recognition sites (Sect. 2.4).

2.1. Large Surface Area Recognition

The use of calixarenes as synthetic agents to target protein surfaces was pioneered by Hamilton around 20 years ago as a mean to disrupt protein-protein interactions (PPIs).[25] PPIs are involved in the regulation of several important cellular processes, among which DNA replication, transcription and translation, cell growth and differentiation, signal transduction and intercellular communication. Cancer, Alzheimer's and Parkinson's diseases and a number of other pathological conditions are produced by pathways involving misregulated PPIs. PPIs are driven by a combination of hydrophobic effects, electrostatic interactions and hydrogen bonds[26] and generally involve quite large surface areas (750-1500 Å^2 of each protein is buried in the complex), often referred as hot-spots, that are almost flat or scarcely convex and lack well-defined binding pockets.[27] Despite the intrinsic difficulties related to the design of small synthetic ligands for protein surfaces, Hamilton synthesized a library[28] of proteomimetic calix[4]arene derivatives (**4**) able to bind efficiently to different proteins and disrupt important PPIs also in vivo. The library is based on a calix[4]arene in the cone structure functionalised at the upper rim with four identical peptide loops formed by four natural amino acids and a 3-(aminomethyl)benzoic acid derivative that connects the loop to the calixarene through an amide bond. This design provides two main advantages in terms of affinity and selectivity: (i) the central hydrophobic region is surrounded by a variable periphery whose recognition features can be easily varied by changing the amino acids of the loops; (ii) the fairly large surface area of the molecule allows the burial of a sizeable region of the protein surface.



4a: $AA_1 = AA_3$ = Gly; $AA_2 = AA_4$ = Asp
4b: $AA_1 = AA_3$ = Gly; AA_2 = Asp; AA_4 = Tyr
4c: $AA_1 = AA_3$ = Gly; $AA_2 = AA_4$ = Lys



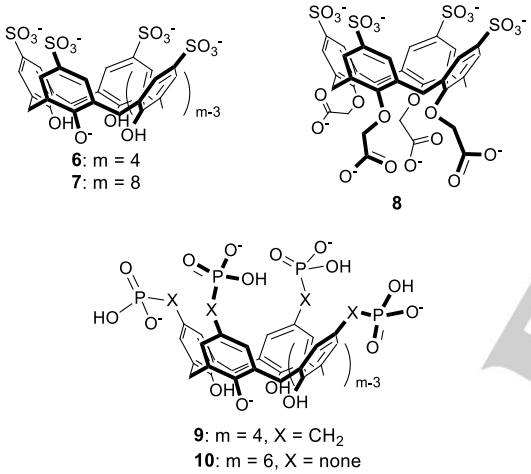
5a: R_1 = COOH; R_2 = $COOCH_2Ph$
5b: $R_1 = R_2$ = COOH

The first member of the library, ligand **4a**, containing the negatively charged Gly-Asp-Gly-Asp loops, is complementary to the region of the surface of cyt c that is buried when cyt c forms a complex with cyt c peroxidase consisting of a hydrophobic patch including the heme edge surrounded by positively charged Lys residues (Figure 4a).[29] **4a** binds strongly to this region thanks to a combination of hydrophobic effect and electrostatic interactions and inhibits the reduction of heme Fe(III) by ascorbate,[29] the binding of the natural protein partner cyt c peroxidase and the apoptosis protease activating factor-1 Apaf-1.[25] A second member of the library (**4b**), with peptide sequence Gly-Asp-Gly-Tyr was shown to strongly bind to platelet derived growth factor (PDGF) by recognizing the region of the PDGF surface rich in hydrophobic and cationic residues involved in the binding to its cognate receptor (PDGFR) and, consequently, hinder the PDGF-PDGFR interaction.[30] Similarly, a third member (**4c**, with Gly-Lys-Gly-Lys loops) targets vascular endothelial growth factor (VEGF) and is able to disrupt the binding to its receptor.[31] In both cases, the inhibition of the binding of the growth factors to their receptors resulted in the disruption of the signal transduction pathways triggered by these PPIs (mainly cell growth and angiogenesis) and, eventually, in the inhibition of tumour growth and angiogenesis both in vitro and in vivo. From a second-generation library having acyclic substituents on the isophthalic moiety, two other calixarene derivatives (**5a** and **5b**) showed very promising biological activity. **5a** is able to bind both PDGF and VEGF, inhibits the binding of both growth factors to their receptors and suppresses the signalling pathways.[32] This resulted in angiogenesis and tumorigenesis inhibition in mice. A subsequent structure-activity study clarified that three isophthalic derivatives are sufficient to ensure activity and that the linker between the calixarene and the substituent plays a role in influencing the cell activity.[33] **5b**, functionalised with 8 carboxylic acid groups, displayed a significant antiviral activity against HIV.[34] The target of **5b** is

gp120, the viral protein that starts the process of HIV viral entry into the cell by interacting with the CD4 receptor on the cell surface. By binding to gp120, **5b** blocks HIV entry and inhibits its replication. To identify the region on gp20 involved in the interaction with **5b**, antibody competition experiments and docking studies were performed. The results point to a region where a central hydrophobic patch is surrounded by peripheral basic amino acids, but further experiments are needed to confirm this hypothesis. Very interestingly, **5b** is active also against viral strains resistant to existing drug candidates, and this could be due to multi-site binding of **5b** on gp120.

2.2. Amino Acid Residue Recognition

An alternative approach to bind protein surfaces with calixarenes consists in the recognition of single amino acid residues (Figure 4b). In the latest years, Crowley published a number of works were p-sulfonato and p-phosphonatocalix[4]-,[35–38] -[6]-[39] and -[8]arenes[40,41] (**6**-**10**) are shown by NMR studies and, for the first time, by X-ray crystal structures to bind the Lys and Arg side chains on cyt c and other proteins through electrostatic, cation-π and CH-π interactions or via hydrophobic effects.



A common feature observed in all of these complexes is the binding of Arg or Lys ammonium groups via interaction with the negatively charged head groups at the upper rim of the calixarenes. This binding is assisted by the inclusion of the aliphatic chains of these amino acids in the calixarene cavity, and increased by CH-π and cation-π interactions (see Figures 5a as an example). Several other interactions are also important to stabilize the complexes and take place between the calixarene and the peptide backbone or the neighbouring lateral residues of the protein, sometimes also mediated by water molecules. Once bound on the protein surface, the calixarene is able to modify the interaction properties working as a supramolecular glue to facilitate protein crystallization,[35–37,39] to obtain distinct crystalline architectures at the solid state[38,40] and well-defined protein assemblies in solution.[39,41] At the solid state, the crystal packing and porosity is deeply dependent on the calixarene size, substituents at the lower rim and rigidity of the macrocyclic structure.

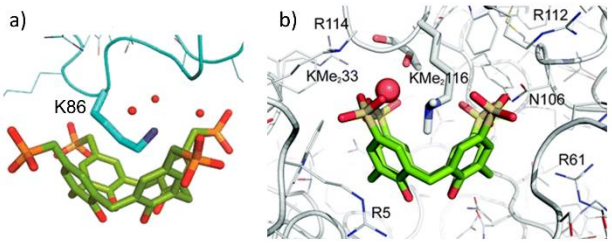
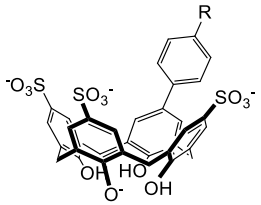
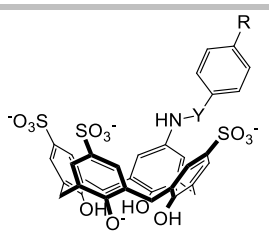


Figure 5. X-ray crystal structure of a) **9** with lysine residue of cyt *c* (adapted with permission from J. M. Alex et al., *Cryst. Growth Des.* **2018**, *18*, 2467−2473. Copyright 2018 American Chemical Society) and b) **6** with dimethyl lysine lateral chain of lysozyme-KMe_2 (adapted from R. E. McGovern et al., *Chem. Sci.*, **2015**, *6*, 442-449 - Published by The Royal Society of Chemistry).

In parallel, binding to specific amino acid side chains on the protein surface was shown by Hof to be a valuable strategy to modulate PPIs and in particular PPIs involved in pathological conditions such as those involving methylated histones.[42] Methylation of the lateral amine group of Lys residues present in the histone tails is a post-translational modification which affects gene regulation and is associated with cancer. Lys residues methylated by methyltransferase enzymes represent an epigenetic marker specifically recognized by various reader proteins. The recognition motif consists of a well-characterized "aromatic cage" formed by a cluster of aromatic amino acid side chains (Trp and Tyr) that give cation-π interactions, flanked by carboxylate side chains for additional electrostatic contacts. Thanks to its aromatic cavity surrounded by negative charges, p-sulfonatocalix[4]arene **6** is a proteomimetic of the "aromatic cage" with a high affinity for trimethylated Lys ($LysMe_3$).[43] After observation that **6** is able to recognize the $LysMe_3$ contained in peptides representing the unstructured different histone tails and disrupt the $LysMe_3$-dependent PPI with the chromodomain CBX7,[44] Hof improved the ligand design by substituting one of the sulfonate groups with para-substituted aromatic rings differently spaced from the calixarene scaffold.[45] This modification was meant to increase the selectivity of the ligand and one member of the small library (**11a**) indeed displayed the highest selectivity for $LysMe_3$ over Lys observed for the sulfonatocalixarene class. Different members of the library (e.g. **11a-d**) subsequently proved superior to **6** in the binding of histone 3 trimethylated on Lys27 and in the disruption of its interaction with the chromobox homolog 7 (CBX7) in vitro.[46] Some members of the library (**12a-c**) were also shown to inhibit the interaction of histone 3 trimethylated on Lys9 with the CHD4 PHD2 finger also in vivo, without significantly disrupting the interaction of this protein with the unmethylated histone 3.[42] The structural characterization of **6** bound to methylated Lys on a protein surface was obtained in collaboration with Crowley, who solved the crystal structure of the complex between methylated lysozyme and **6**. In the structure (Figure 5b), the $LysMe_2$ side chain is inserted in the aromatic cavity of the calixarene and the binding is driven by cation-π interactions supported by water-mediated salt bridges and hydrophobic effect, mimicking very well the "aromatic cage" of chromodomains.[47]



11a: R = H
11b: R = CN
11c: R = CH_3
11d: R = OCH_3

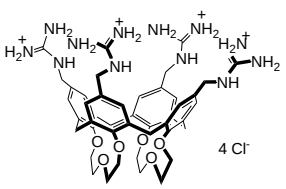


12a: Y = SO_2, R = COO^-
12b: Y = SO_2, R = Br
12c: Y = CO, R = Br

The inclusion of Lys and Arg side chains was recently exploited also by Guo, Ravoo and coll. to verify the ability of calixarene derivatives in preventing protein fibrillation. An amphiphilic calix[5]arene, functionalized at the upper rim with carboxylic acid units and at the lower one with dodecyl chains, co-assembles with an amphiphilic cyclodextrin, forming aggregates which expose multiple inclusion cavities on their surface. The ability of the calixarenes in hosting the Lys side chain, in synergy with the cyclodextrins that include tyrosine side chains, not only hampers the aggregation in fibrils of amyloid-β peptides $Aβ_{42}$, but even dissolves preformed mature fibrils.[48] With a similar approach, the amphiphilic p-tetrasulfonatocalix[4]arene bearing dodecyl chains at the lower rim was shown[49] to form micelles that, as multivalent assemblies, strongly bind Arg and Lys side chains exposed on insulin through multiple host-guest inclusion complexes. The resulting inhibition of insulin fibrillation was demonstrated to be a peculiarity of this particular calixarene amphiphile since inhibition was obtained only to a much lower extent with the hydrophilic tetrahydroxy-p-sulfonatocalix[4]arene **6** or with p-dodecylbenzenesulfonate as monomeric model of the macrocycle. The remarkable performance of the calixarene containing micelles was attributed not only to their multicavity nature but also to the possibility of insertion into the "fatty" micellar core of the liphophilic side chains of Phe, Leu and Val present in the protein via hydrophobic effect.

2.3. Binding to Lipophilic Pockets

Besides disrupting protein complexes, calixarene binding of protein surfaces can also serve to repair malfunctioning PPIs. De Mendoza showed that a positively charged tetra(guanidinomethyl)calixarene (**13**) is able to structurally recover a damaged PPI in a mutated form of p53 found in many human cancers.[50] p53 is a tetrameric protein that in presence of DNA damages induces the expression of the DNA repair machinery or triggers apoptosis if the damage is irreversible, thus protecting the organism from tumours. In mutated p53 the tetrameric assembly is destabilized by a missing ion pair interaction. **13** is able to recover the wild-type tetrameric structure by the simultaneous interaction of the four guanidinium groups with four Glu carboxylates of the protein and by the fitting of the cone scaffold in the hydrophobic clefts between the monomers (Figure 4c). Subsequently, the design of the ligand was improved by replacing the rigid short bis-crown loops at the lower rim with propyl chains that allow an increased flexibility to the calixarene scaffold (**14**). This enhanced flexibility resulted in a stronger interaction of the ligand with the floppy hydrophobic cleft of the mutant form and allowed the guanidinium groups to reach the optimal position for H bonding and ion pairing with the carboxylates of the protein.[51]



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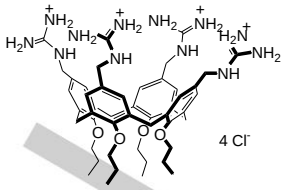

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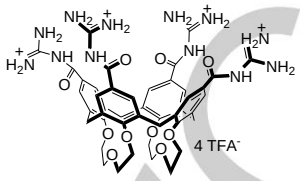

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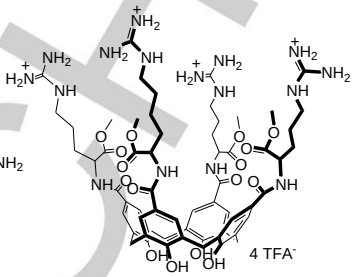


16

Calixarenes **15** and **16**, with guanidinium or arginine moieties at the upper rim and a rigid scaffold, were shown by the same group to bind to the surface of voltage-dependent potassium channels (Kv1.x) in a reversible manner.[52] The binding is promoted by electrostatic interactions with four negatively charged Asp residues exposed near the conical pore entrance to the channel while the conical scaffold inserts at the entrance of the pore.

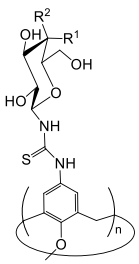
Another interesting example of calixarene derivatives being able to fit the hydrophobic pocket of proteins was provided by us with a series of amphiphilic calix[4]arenes, in the cone structure, and bearing the guanidinium group directly linked at the upper rim. Especially p-guanidino calixarene **23a** and **23b** potently inhibit the LPS-stimulated Toll-Like receptor-4 (TLR4) activation and the subsequent production of inflammatory cytokines in human and mouse cells.[53] Docking studies showed that these amphiphilic calixarenes form complexes with both CD14 and TLR4/MD-2 heterodimers. The lower rim alkyl chains and the lipophilic external walls of the calixarene scaffold are buried into the CD14 or MD-2 hydrophobic pockets, while the guanidinium groups establish attractive contacts with polar amino acid residues at the portal of the pocket. The binding to the two co-receptors CD-14 or MD-2 suggests a direct antagonist effect of such calixarenes and the possibility to modulate the TLR4 signalling preventing excessive and deregulated TLR4 activation which may lead to acute sepsis and septic shocks.

2.4. Binding to Multimeric Proteins

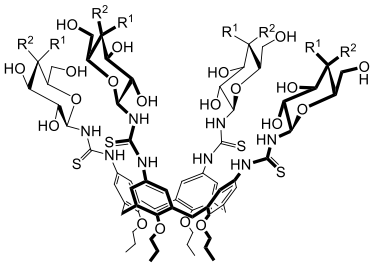
Since 1995, when we reported[54] in collaboration with the group of Dondoni and Marra the first synthesis of glycocalixarenes, calixarenes functionalised with saccharide units, these derivatives rapidly gained popularity and also other research groups[55] contributed to widen the procedures for the introduction of sugar residues onto these macrocycles. In 1996, an important article by Roy and Meunier,[56] reporting on the interaction of polysialylated calixarenes with tetrameric wheat germ agglutinin (WGA), marked the beginning of the use of these macrocycles as platform for the design of multivalent lectin ligands. Lectins represent an important class of proteins that, lacking enzymatic and immunological functions, mediate fundamental biological processes, both physiological and pathological, through the recognition of multiple copies of saccharide residues. Generally, these recognition processes are characterized by the occurrence of the glycoside cluster

effect.[21,57] The relevance of calixarenes in this field is especially related to their structural properties, which offer the opportunity of designing and testing series of potential multivalent ligands with rather subtly different stereochemical dispositions of carbohydrates with improved affinity and selectivity.

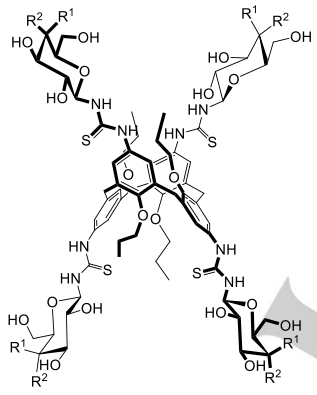
In this context, we achieved important results in the inhibition of medically relevant human galectins with lactosyl-thioureidocalixarenes (**17**-**19**).[58,59] These derivatives not only displayed very high multivalent effects in the inhibition of the binding of lectins to tumour cells, but evidences were collected on the fundamental role played by the different structural features of the ligands in determining the selectivity. The conformationally mobile calix[6]- and –[8]arenes **17b** and **17c** resulted particularly efficient in the inhibition of Galectin-4, while the isomeric tetralactosylcalix[4]arene derivatives **18** and **19** showed opposite selectivity: the cone derivative **18** is an excellent inhibitor of Galectin-3 but has low affinity for Galectin-1 while an opposite selectivity is observed for the 1,3-alternate compound **19**.



17a: n = 4, R^1 = H, R^2 = O-β-D-Gal
17b: n = 6, R^1 = H, R^2 = O-β-D-Gal
17c: n = 8, R^1 = H, R^2 = O-β-D-Gal



18: R^1 = H, R^2 = O-β-D-Gal

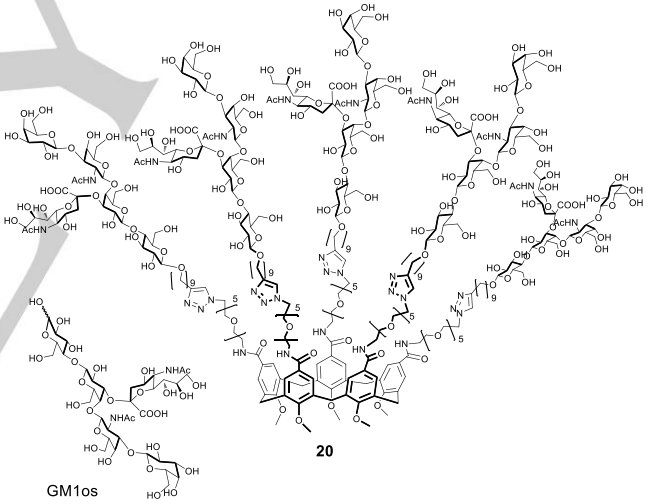


19: R^1 = H, R^2 =O-β-D-Gal

The role of the cluster topology assumes relevance also for the galactosylcalixarenes designed by Vidal and coll.[60] as ligands for the tetrameric lectin PA-IL (or Lec A) produced by *Pseudomonas aeruginosa*. This harmful bacterium, associated to severe respiratory diseases such as cystic fibrosis, uses PA-IL for adhesion processes and biofilm growth. Within a small library of compounds exposing the sugar epitopes with different orientation due to the different scaffold geometries, the 1,3-alternate derivative evidenced the lowest K_d in titration experiments towards the lectin. This ligand exposes its binding sites two by two on opposite faces of a parallelepipedon with the possibility of assembling a chain of alternating calixarene and lectin units, as visualized by AFM images.[61] The disposition of the four carbohydrate recognition domains explains the particular efficacy of the 1,3-alternate macrocycle and the peculiar assembly formed.

The same calixarene scaffold functionalised with L-fucose units instead of galactose was shown to bind PA-IIL (or LecB) from the same bacterium, even if the gain with respect to a monovalent reference ligand (multivalent effect) was in this case rather modest.[62] On the other hand, this cluster evidenced inhibitory activity against a wide spectrum of *Pseudomonas aeurginosa* strains, differently from what observed for the galactosyl calixarene whose efficiency is indeed strongly dependent on the sequence mutations present in the different strains.

In a joint work with Fieschi and coll.,[63] we recently prepared calixarene derivatives bearing α-mannoside units with the aim of finding out a ligand efficient in the interaction with DC-SIGN receptor but, in the meanwhile, unable to inhibit Langerin. In fact, although both these two lectins complex mannoside residues of viral gp120, DC-SIGN is used by HIV as Trojan horse to infect T-cells, while Langerin, upon recognition of the same substrate, triggers a defence mechanism for the organism. However, experiments with Langerin were not performed because, unfortunately, only a weak binding to DC-SIGN was observed without any particular difference among the mannosylated calixarenes. Very recently, Fieschi et al.[64] explored the possibility to block the infection by Ebola virus targeting DC-SIGN receptor with a calix[4]arene bearing four units of L-fucose. Interestingly, this cluster exhibits an IC_{50} of 218 nM, resulting a stronger inhibitor than dendrimers with higher densities of mannose units.



20

GM1os

Impressive is the behaviour of a pentavalent cluster (**20**) we proposed as ligand for cholera toxin.[65] Five units of the **GM1os** were linked to a conformationally mobile calix[5]arene through long spacers designed to allow the simultaneous interaction of each saccharide unit with the five toxin carbohydrate binding sites. The IC_{50} evaluated by ELISA tests reached the value of 450 pM, with an amazing multivalent effect: each of the five GM1 units installed on the pentavalent calixarene ligand binds the lectin 20,000-fold stronger than a monovalent reference compound.

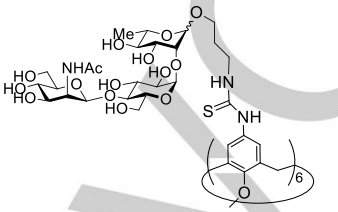
Other researchers have resorted over the years to calixarenes for the preparation of polyglycosylated compounds with the aim of targeting various microorganisms. Marra and colleagues, for instance, prepared two sialylated derivatives that evidenced hemagglutination activity characterized by a significant multivalency against influenza A virus.[66] This pathogen indeed exploits for the adhesion to cells multiple copies of a surface trimeric lectin. Consoli et al. produced and studied a tetra-L-fucosylcalix[4]arene in cone geometry able to interact with the

previously mentioned PA-IIL.[67] Very recently, *P. aeruginosa* was selected by Benazza et al. as target for calix[4]arenes functionalized at the lower rim with L-fucose or D-mannose units linked to the macrocycle through a spacer incorporating an hydroxamic acid moiety.[68] Beyond lectins, in fact, bacteria also exploit Fe^{3+} cations to invade host cells and create the biofilm. The presence of hydroxamic acids, strong Fe^{3+} chelators, can therefore hinder the metal uptake. Indeed, since an analogue calixarene exposing glucose residues and used as negative control evidenced equivalent efficiency in the inhibition, the authors concluded that the hydroxamic acid groups play a more relevant role in the observed process than the sugar units.

In the inhibition of the interaction between tumour cells and GM3 ganglioside binding proteins, a calixarene based multivalent ligand[69] proved to be superior in comparison to other multivalent clusters built on different scaffolds and displaying the same or an even higher valency. In these cases, the multivalent inhibitors are functionalised with multiple copies of a mimic of the GM3 saccharide epitope that melanoma cells exploit to grow and spread through adhesion processes mediated by complexation between the overexpressed ganglioside and specific tissue lectins.

Polyglycosylated calixarenes are usually characterized by amphiphilic character due to the presence in their structures, together with the polar sugar units, of a lipophilic region. This feature allowed us to insert glycocalixarenes with proper geometry and sugar epitopes in liposome double layers[70] or on the surface of gold nanoparticles.[71] In the first case, a 1,3-alternate calix[4]arene spanning the thickness of the phospholipid bilayer and exposing glucoside units provided the liposome with the ability of interacting with glucose binding Concanavalin A used a model of cell membrane receptor. In a second case, we noncovalently functionalised the surface of AuNPs coated with dodecanthiol chains with cone calixarenes bearing α-mannoside units at the upper rim and propyl chains at the lower rim. The presence of the macrocycles determined an enhanced uptake of the NPs by cells presenting mannose binding proteins on the outer surface. The key-point of this work consists in proving that NPs functionalised with calixarene glycoclusters are significantly more efficient than others NPs simply bearing a comparable number of copies of a monovalent ligand. This means that the cluster of sugars present on the macrocyclic structure, together with the epitope proximity and disposition offer an added value - "clustered multivalent effect" - to the interaction with the target multivalent lectin.

Saccharide structures are recognized also by certain antibodies. This phenomenon can, therefore, be exploited to generate immunogenic molecules and, in a subsequent step, sugar-based vaccines for which the epitope multiple exposition could again be an important resource. With this perspective, Geraci et al.[72] investigated the immunogenicity of a cone calix[4]arene bearing at the upper rim four units of the galactoside tumour associated antigen S-Tn and at the lower one an unit of the lipid immunoadjuvant tripalmitoyl-S-glycerylcysteinylserine (P3CS). The treatment of mice with the calixarene determined an increased antibody production with respect to a monomeric model. In the same context, we recently explored the potentiality of calixarenes as platform for the efficient functional presentation of *Streptococcus Pneumoniae* (SP) saccharides.[73] In particular, a calix[6]arene functionalised with six copies of the trisaccharide repeating unit (β-DManpNAc-(1→4)-α-D-Glcp-(1→2)-α-L-Rha) constituting the capsular polysaccharide of SP19F serotype (**21**) resulted interesting and promising. In ELISA assays it was indeed able to significantly inhibit the binding between SP19F native polysaccharide and anti-19F antibodies reaching the 75% of the inhibition observed with the SP19F polysaccharide used as reference ligand. In the same tests, with the trisaccharide alone only the 52% of inhibition as maximum was achieved. In this case the concept of multivalent effect acquires a different meaning with respect to that established for the inhibition of lectins. That obtained with **21** is a relevant result since it suggests how this glycocluster, although projecting in the space only six trisaccharide units and thus relatively simpler than the natural high-weight capsular polysaccharide, can efficiently mimic a variety of arrangements and geometries of the immunogenic epitopes that interact with the polyclonal antibodies.



21 **22** R = C_nH_{2n+1}

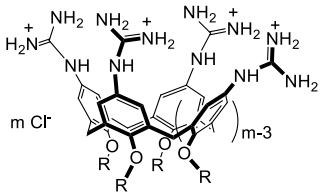
2.5. Calixarene-based Detergents for Membrane Proteins

In 2011, Coleman et al. pointed out the possibility to use calixarene-based detergents (**22**)[74] to extract and stabilise the membrane protein Bacillus multidrug resistance ATP protein (BmrA). This class of detergents, not only allowed the 3D-crystallisation of the protein, but also preserves the ATPase activity much better than other common detergents. It was proposed and partially proved that these amphiphiles freeze the monomeric structure of the protein, preventing its aggregation and denaturation, through hydrophobic interactions with the protein membrane region and through a network of salt bridges with the basic residues at the cytosol-membrane interface. With the aim of overcoming what has been indicated as a bottleneck in the membrane protein structural biology,[75] in the latest years other studies appeared where the stabilisation, isolation and characterisation of membrane proteins such as Human Multidrug Resistance Protein 4[76] and G protein-coupled receptors,[77] using similar calixarene detergents, were reported. A glycocalixarene having glucose units at the upper and an heptyl chain at the lower rim also showed to preserve and stabilise, even over long periods of time, the function of three native membrane proteins.[78] Very recently, the use of **22** ($R=C_3H_7$) was proposed as an alternative detergent for influenza virus inactivated split vaccine preparation. The use of such calixarene allows to obtain Hemagglutinin antigens fully functional and stable for at least 6 months,[79] offering an important improvement in the vaccine manufacturing process and preventing the use of prohibited detergents such as Triton X-100.

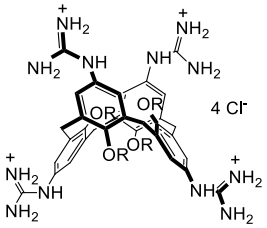
3. Ligands for Nucleic Acids and Biologically Relevant Anions

Another important area where we[18] and others[80] gave a relevant contribution to the applications of calixarenes in bionanotechnology is that of the interaction with and delivery of

nucleic acids. After a pioneering paper in 1999 by Schneider reporting on the results of complexation between single nucleotides or ds-DNA and ammoniumcalix[4]arenes,[81] no other publications on this topic involving this family of macrocycles followed for some years. In 2004[82] and then in 2006[83] we published on the interaction of calixarenes functionalised with guanidinium cationic head groups with linear and plasmid ds-DNA. Already at the time of those first studies, it was clear for us that the final goal was to use calixarene amphiphiles as nonviral vectors for gene delivery, the transport of nucleic acids into cells. We synthesized a small library of calix[n]arenes (**23a-g**, **24**) bearing guanidinium groups directly linked at the upper rim, while aliphatic chains of different length were tethered at the lower rim.

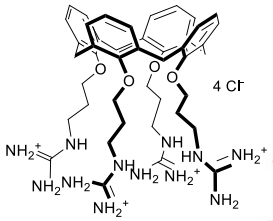


23a: m = 4, R = propyl
23b: m = 4, R = hexyl
23c: m = 4, R = octyl
23e: m = 4, R = methyl
23f: m = 6, R = methyl
23g: m = 8, R = methyl
23h: m = 5, R = dodecyl
23i: m = 5, R = 4-methylpentyl
23l: m = 5, R = methyl
23m: m = 4, R = butyl



24: R = propyl

Calixarenes characterized by different conformational properties and size were included in the series, some being blocked in the cone or 1,3-alternate geometry and others showing a mobile conformation. For the first time it could be evidenced the ability of some calixarene derivatives in transfecting cells, behaving as nonviral vectors. By using the plasmid ds-DNA encoding for green fluorescent protein (pEGFP-C1), the expression of the protein in cells, as a consequence of the delivery of the corresponding gene into their nucleus, was monitored and revealed. Derivatives with a marked amphiphilicity, where polar and apolar regions are well and permanently distinguished, are able to transport the DNA across the cell membranes. On the contrary, derivatives lacking this structural feature, namely the 1,3-alternate calix[4]arene **24** and the conformationally mobile methoxycalix[4]- **23e**, -[6]- **23f** and –[8]arene **23g**, do not transfect cells. AFM experiments nicely evidenced that, although the latter compounds are able to bind DNA and to strongly change the folding of its relaxed form, they do not induce in the plasmid the compactation process necessary, although not sufficient, for transfection. Calix[4]arenes fixed in the cone **22a-c**, instead, form 50-100 nm aggregates, the “calixplexes” (in analogy with the term lipoplexes used for lipids-DNA aggregates and polyplexes for polymers-DNA aggregates), where DNA is protected from the action of nucleases and its negative charges are masked allowing its integrity and delivery across the cell membrane. When used in formulation with helper lipid DOPE, the cone derivatives showed an interesting transfection activity although significantly lower compared to that of LTX lipofectamine used as reference.[83] Following this rational, we later designed calix[4]arenes functionalised with guanidinium groups at the lower rim and blocked in the cone geometry, having different modifications at the upper rim.[84,85]



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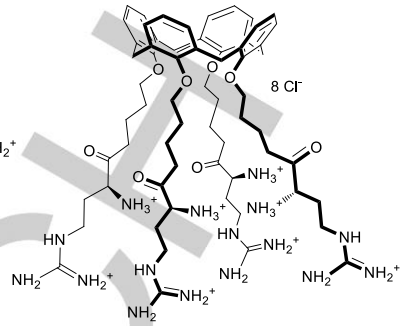

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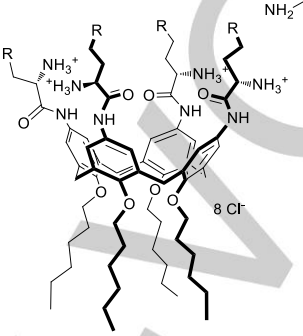


27a: R = $NHC(NH_2)NH_2^+$
27b: R = $CH_2NH_3^+$

In the case of macrocycle **25**, lacking substituents at the upper rim and therefore having as lipophilic region simply the calixarene aromatic scaffold, an important improvement of the transfection efficiency was observed with respect not only to other p-substituted analogues but also to the previously reported upper rim guanidinocalix[4]arenes **23a-c**. The percentage of transfected cells, using rhabdomyosarcoma (RD-4) cells, reached, in presence of DOPE, almost the 50%, overtaking LTX (30%). The concomitant synthesis of non-macrocyclic gemini models and the evaluation of their properties evidenced that also the macrocyclic nature of the calixarenes plays a crucial role in determining their ability as vectors, being the non-macrocyclic analogues completely unable to give transfection.[85]

In 2007 Matthews and coll.[86] reported on the use of multicalixarenes (first generation dendrimers having calixarene units both as core and branching units) bearing aromatic or aliphatic primary amines at the upper rims. Although all these compounds were shown to be able to bind plasmid DNA in gel-electrophoresis, only calixarenes bearing aliphatic amines transfect cells albeit with low efficiency (10%), suggesting that an extensive protonation of amino groups is essential.

An important breakthrough of the research was the replacement of the simple guanidinium head groups with arginine units.[87] The effect of this modification was impressive. Although compound **26**, with four arginine units at the lower rim, is a very poor vector also in the presence of DOPE as adjuvant, compound **27a** with the four arginine units linked to the upper rim through carboxy groups resulted the most efficient delivery system among all the calixarene derivatives synthesized so far.[87] The use of this vector, even in absence of adjuvant, determines the transfection of 80% of the RD-4 cells and reaches high percentages in general with other tested cell lines. The efficiency is always at least similar but frequently higher or even largely higher than that of common transfecting agents like PEI and LTX. Noteworthy, the toxicity associated to this compound is negligible. Also in this case, a non-macrocyclic

analogue of **27a** completely failed in transfection experiments, due to its inability in compacting DNA. Calixarene derivative **27b** bearing four lysines instead of arginines is much less active than **27a** and substantially transfects cells only in presence of DOPE, demonstrating the superiority of guanidinium over ammonium for these purposes. Interestingly, the studies on the mechanism of DNA compactation indicate that, when long aliphatic chains are present on the vector, self-assembled micelles form even in absence of the cargo and then these assemblies bind to the DNA without rearranging significantly their structure (hierarchical self-assembly).[88] On the other side, in the presence of shorter aliphatic chains, first the multivalent calixarenes singularly bind to DNA and subsequently compactation takes place thanks to intermolecular hydrophobic interaction of the neighbouring lipophilic tails (template induced self-assembly).[83]

In parallel with these studies, other research groups reported examples of calixarene-based derivatives able to bind DNA often not directly connected to the delivery of DNA into cells but significant for giving interesting contribution to the comprehension of the recognition mechanism. It is the case of a series of calixarene dimers bearing NH_2/NH_3^+ or guanidinium groups, proposed by Schrader and Zadmard, that bind to the major groove of ds-B-DNA and ds-RNA.[89,90] In these cases, the authors suggest that the interaction is not merely due to salt bridges between ammonium and phosphate units but also to the "insertion of the extended apolar calixarene dimer backbone into the major groove accompanied by hydrogen bonding to the nucleic bases".[91] Not purely electrostatic interactions are also those that determine the binding between DNA and solid lipid nanoparticles (SLNs) constituted of a tetraguanidinium-tetradodecyloxycalix[4]arene.[92] It could be evidenced that the SLNs tend to bind the major or the minor groove depending on the nucleobase sequence. In particular, ITC experiments showed that with AT sequence (dA_{30}–dT_{30}) the recognition proceeds via minor groove binding while with GC (dG_{30}–dC_{30}) via major groove with a concomitant conformational change of the nucleic acid double strand. More recently, Zadmard described a calix[4]arene bearing two arginine units at the lower rim that binds DNA thanks to major groove interactions in the early stages of the recognition process, while it is able to recognize a mismatched DNA by extensive interactions with its minor groove.[93]

The evidence collected that these cationic vectors may also directly interact with nucleobases prompted us to investigate the possibility to use polycationic calixarene, in particular **27a**, in binding to microRNAs (miRNAs) and peptide nucleic acids (PNAs), lacking both a groove and, for PNAs, even the negative charges of the phosphate group. In the case of miRNAs, we could verify that argininocalix[4]arene **27a** is able to afford an efficient delivery of premiRNA and antimiRNA molecules, with percentages of transfection higher than those of reference gold standards available. Remarkably, the biological activity of the transported premiRNAs and antimiRNAs is maintained. This was demonstrated by evaluation of the expected biological activity of the cargos and in particular by i) the induction of apoptosis by antimiR-221 in glioma U251 cells; ii) the induction of apoptosis by premiR-124 in U251 cells; iii) the inhibition of pro-inflammatory IL-8 and IL-6 genes in cystic fibrosis IB3-1 cells.[94] Moreover, the lead vector **27a** resulted rather surprisingly able to deliver into cells also PNAs.[95] Such nucleic acid mimics have a big potential for therapeutic applications, but this is currently strongly limited by poor cellular uptake. The availability of vectors that, through a noncovalent process, can facilitate PNA delivery to cells is fundamental for boosting their use. Calixarene **27a** is an excellent candidate to act as "universal" delivery system for PNAs, avoiding tedious, time consuming and expensive covalent insertion of an oligoarginine tail at one end of the peptide scaffold that represents the current most effective alternative.[96] The mode of binding between the cationic calixarenes and PNAs is still under investigation and needs to be rationalized. The absence of phosphate groups in PNA makes the hydrogen bonds of the calixarene ammonium and amide groups with the PNA nucleobases and peptide skeleton the most important and likely interactions for binding. However, due to the aqueous environment where the binding and the consequent transfection occurs, hydrophobic effects cannot be ruled out.

In addition to their use as gene-delivery systems, guanidinocalixarenes were also proposed in a series of applications for the detection of biologically relevant phosphates and of biomacromolecules bearing multiple negative charges. Guo and coll. proposed the use of a complex between a photosensitizer (PS) and p-guanidinocalix[5]arene (**23h,i**) as a targeted phototheranostic system *in vivo*. The PS, aluminum phthalocyanine or 4-(sulfonatophenyl)porphyrin, are completely deactivated (OFF state) when complexed in the calixarene-based nanocarrier. However, when exposed to the rather high concentration of the biomarker ATP present in tumour cells (>100 μM), ATP is included and the PS released, giving rise to the complete recovery of fluorescence and photoactivity (ON state). The low ATP concentration ([ATP] ≈ 1-10nM) present in normal tissues is not sufficient to trigger the PS release and this avoids undesired off-target leak during its delivery.[97] The p-guanidinocalix[5]arene (**23i**) was also used, in combination with fluorescein (Fl), to generate an Indicator Displacement Assay (IDA). Fluorescence is quenched when Fl is included in the host, but it immediately turns "on" in the presence of lysophosphatidic acid (LPA), a biomarker for early diagnosis of ovarian cancers.[98] The biomarker is threaded through the calixarene cavity held in place thanks to electrostatic ($PO^{-}\cdots Gua^{+}$), CH-π and CH-O interactions, and hydrophobic effects. Recently, a fluorescence displacement sensing array based on four p-guanidinocalixarenes (**23h-m**) and a fluorescent dye (Eosin Y) was devised for the discrimination of six glycosaminoglycans (GAGs).[99] GAGs are a wide family of polysaccharides having structures quite different in type and density of the sulfate or carboxylate groups and their relative abundances significantly change in different tumour tissues making their discrimination a prognostic index for a series of carcinomas. Again, upon complexation of Eosin Y by the calixarene, the fluorescence is quenched, but when the sensing array comes into contact with the analyte, the fluorescence is regenerated, giving rise to different patterns, function of the number and type of charged groups present on the GAG.

4. Supramolecular Catalysts based on Multifunctional Calixarenes

Enzymes are extremely efficient catalysts due to the simultaneous presence in the active site of a recognition pocket for the reactants and of a variable number of active units which cooperatively: i) promote the cleavage of bonds by general/Lewis acid activation; ii) deliver the nucleophile at a proper distance and direction; iii) facilitate the release of the leaving group by coordination to a proton or a Lewis acid. Since

1992,[100] calixarenes, thanks to well consolidated regioselective functionalisation procedures which allow the insertion of multiple metal ligating units and/or basic/nucleophilic moieties in close proximity to the lipophilic cavity, have been often proposed as scaffolds for the development of multifunctional supramolecular catalysts endowed with esterases and phospodiesterases activity. The barium salt of p-tert-butylcalix[4]arene **28-Ba** acts as a catalyst with turnover activity in the methanolysis of activated arylacetates (Figure 6). In a first step, the catalyst is acylated by transfer of the acetyl group from the substrate (ArOAc) to the calixarene phenolate oxygen atom that takes place thanks to the Lewis acid activation by the barium ion on the carbonyl bond and to the proximity of the nucleophile (phenolate). In the second step, the catalyst **28-Ba** is restored thanks to Lewis acid activation of the carbonyl group of the acetylated catalyst (**29-Ba**) as a result of the barium coordination and of the methoxide attack to the carbonyl group.

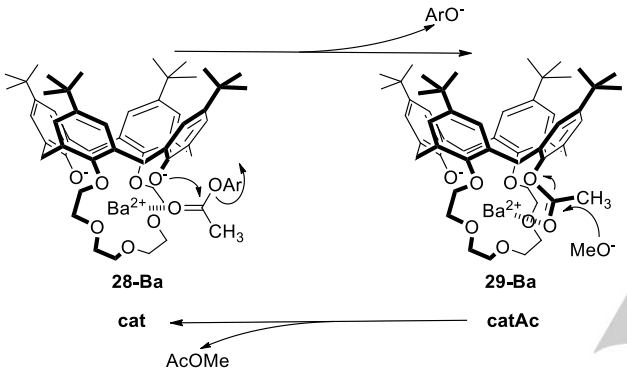
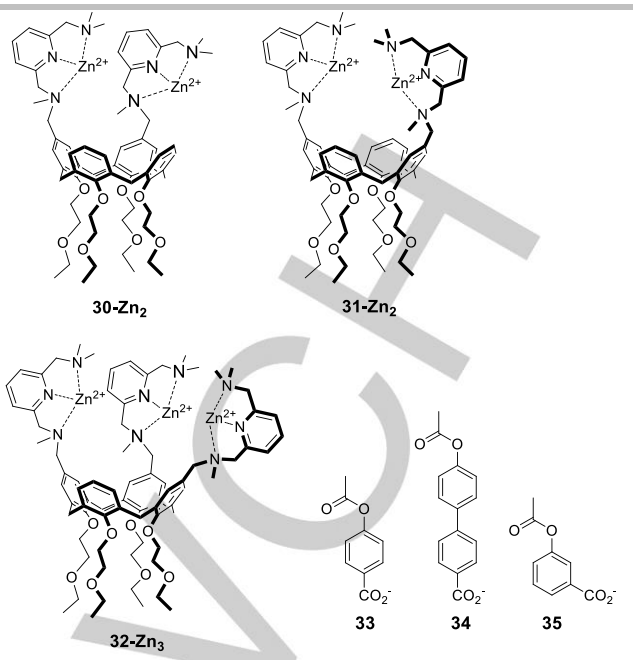
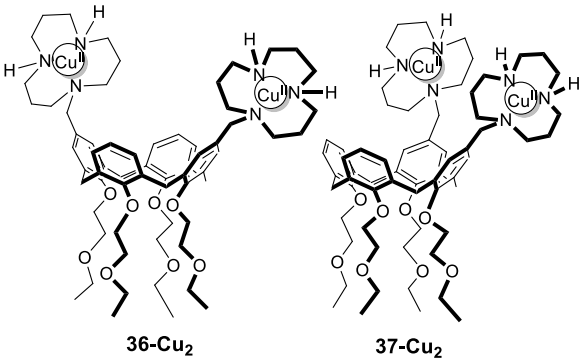


Figure 6. Mechanism for the methanolysis of phenyl acetates catalysed by barium salt of p-tert-butylcalix[4]arene **28-Ba**..

The introduction of BAMP (bis-aminomethyl-pyridine) units at the upper rim of calix[4]arenes fixed in the cone structure (**30-Zn_2**, **31-Zn_2** and **32-Zn_3**) allows to preorganise two or three zinc metal ions thus obtaining artificial catalyst for the methanolysis of phenyl acetate esters (**33**-**35**) bearing carboxylate (-COO^-) anchoring groups on the aromatic unit.[101,102] The dinuclear 1,2-vicinal (**30-Zn_2**) and 1,3-distal (**31-Zn_2**) complexes catalyse the cleavage of esters **33-35** two orders of magnitude faster than a monometallic BAMP-Zn model. The two metal ions cooperate, one of the zinc ion blocking the substrates through an interaction with the negatively charged carboxylate group, while the other Zn ion intramolecularly delivers the MeO^- nucleophile onto the carbonyl group of the complexed substrate. The trinuclear complex **32-Zn_3** exhibits clear evidence of a trimetallic catalysis where three different effects appear to operate: i) substrate selection through carboxylate binding, ii) Lewis-acid activation of the ester C=O bond, and iii) delivery of the activated methoxide anion.

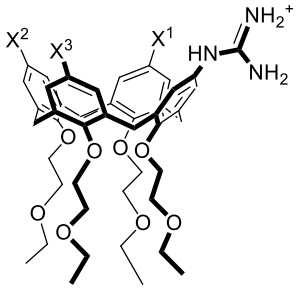


Interestingly, Zn and especially Cu calixarene complexes also demonstrated to be effective catalysts for the cleavage of phosphodiester bonds (artificial phosphodiesterases activity). A first family of these complexes was prepared and studied by Reinhoudt and coll.[103,104] The Zn complexes were able to efficiently catalyse the transesterification of 2-hydroxypropyl p-nitrophenol phosphate (HPNP), a model of RNA. Very efficient rate enhancements were observed for the 1,3-distal bimetallic catalyst **31-Zn_2** compared to the monometallic model. However, the use of such Zn-complexes is limited to 50% acetonitrile-water mixture, since the metal ions dissociate when the water content is increased. Looking for more stable chelating units able to strongly coordinate Cu(II) or Zn(II) even in pure water, we introduced the 1,5,9-triazacyclododecane unit at the upper rim of calix[4]arenes fixed in the cone structure and studied their di- and trimetallic complexes. We especially studied the metallonuclease activity in water of the dinuclear Cu(II) 1,3-distal (**36–Cu_2**) and 1,2-vicinal (**37–Cu_2**) complexes.[105,106] Remarkably, the catalytic activity in the cleavage of the HPNP model compound observed in water at pH = 7 for the 1,2-vicinal derivative (**37–Cu_2**), is more than 25 times higher than that of its 1,3-distal regioisomer (**36–Cu_2**), showing how the distance and relative disposition of the two metal centres at the upper rim might play a crucial role.

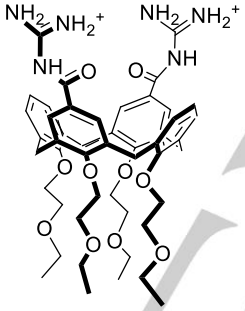


The proposed mechanism of cleavage implies the cooperation between the two metal ions. While a Cu(II) metal ions, carrying a deprotonated water molecule (OH^-), acts as a general base

and deprotonates the β-hydroxyl group of HPNP thus inducing an intramolecular attack of the latter alkoxide ion onto the phosphate group, the second Cu(II) ion binds the substrate and activates, as a Lewis-acid, the P=O bond.[105] Interestingly, both 1,2- and 1,3-dinuclear complexes cleave the diribonucleoside 3',5'-monophosphates at T = 50 °C with rate enhancements 10^5 times higher than the background reaction carried out in the absence of the catalyst. Even a certain degree of selectivity is observed for dinucleotides such as UpU or UpG.[106] An extended degree of cooperation between the two metal ions - but with no significant difference in efficiency between the two regioisomeric **36-Cu₂** and **37-Cu₂** catalysts - was observed also in the cleavage of hexa- and eptaribonucleosides, in H_2O, pH 7.4, 50 °C. Accelerations up to 5 x 10^5 are observed but no marked difference in efficiency for the vicinal or distal disposition of the catalytic groups emerges. In sharp contrast with the selectivity found with diribonucleoside monophosphates, a remarkable preference for cleavage of the CpA phosphodiester bond is always registered. The catalytic rate constants of phosphate bonds cleavage significantly decrease with distance from the doubly charged phosphate group in terminal 5' position, suggesting a possible role of the 5' dianionic phosphate as anchoring site for the catalysts.



38a: $X^1=X^3=H$; $X^2=NHC(NH_2)NH_2^+$
38b: $X^2=X^3=H$; $X^1=NHC(NH_2)NH_2^+$
38c: $X^1=X^2=X^3=NHC(NH_2)NH_2^+$



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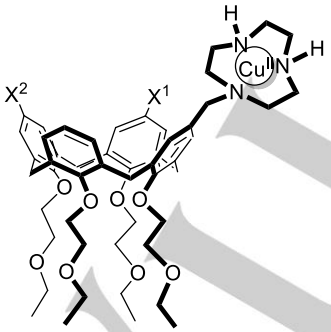

40a-Cu: $X^2=H$; $X^1=CH_2NHC(NH_2)NH_2^+$
40b-Cu: $X^1=H$; $X^2=CH_2NHC(NH_2)NH_2^+$

More recently, we also studied p-guanidinocalix[4]arenes (**38a-c**) for the cleavage of HPNP. At proper pH (DMSO/H_2O = 80:20), the bisguanidylated **38a** and **38b** possess a protonated guanidine (guanidinium ion) and a neutral guanidine groups. The former acts as an electrophilic activator, while the latter as a general base. A high degree of cooperation between the catalytic groups in **38a,b** (up to three orders of magnitude compared with the background hydrolysis) was observed with the 1,3-distal guanidinocalixarene **38a** slightly more effective than the 1,2-vicinal regioisomer **38b**.[107–110] Interestingly, by increasing the number of guanidinium groups, and therefore the positive charges at the upper rim, it is possible to obtain a tetra-guanidinium derivative **38c** that, upon monodeprotonation, is able to efficiently cleave ATP into ADP and phosphate. In this case, the rate enhancement is due to a combination of neutral guanidine (nucleophilic catalysis) and protonated guanidinium units which bind the substrate, activate it and stabilise the leaving group.[111] The insertion of a carbonyl group between the calixarene and the guanidinium ion, to obtain **39**, not only lowers the pH of maximum rate enhancements for the cleavage of both HPNP (RNA model) and BNPP (DNA model) to nearly neutral values (pH=7.9), but also increases the preorganisation of the catalyst that seems to perfectly fit a strainless ring-shaped transition state.[112] Finally, the combination of a (methyl)guanidinium group acting as electrophilic activator and a metal ion, Cu(II) or Zn(II) ligated to the calixarene scaffold, ensuring the presence of a metal-bound OH^- group close to the substrate, gives an effective heterobifunctional catalysis, studied in the cleavage of HPNP, BNPP and dinucleosides. Interestingly, the two regioisomeric Cu(II) complexes turned out to have opposite selectivity, with the 1,2-vicinal derivative (**40a**) being selective for CpA while the 1,3-distal isomer (**40b**) preferentially cleaves GpA.[113,114]

5. Perspectives and Outlooks

Calixarenes, thanks to their three-dimensional shape and possibility to be adorned with preorganised binding groups, demonstrated to be smart scaffolds for the development of efficient and selective endo-receptors for a plethora of small ions and neutral molecules. In the latest 15 years, especially exploiting their ease of multiple functionalisation with ligating units of different nature, they have been used to prepare multivalent ligands for biomacromolecules and multifunctional catalysts for the cleavage of carboxylate and phosphate esters. Compared to other multivalent scaffolds, calixarenes offer the possibility to shape the size, the valency and the stereochemical disposition in the space of the multiple ligating units. Moreover, the introduction of proper aliphatic chains and the presence itself of the lipophilic aromatic external walls of the macrocycle ensure the possibility to tune the amphiphilic character of the multivalent ligand[115] so that they can be noncovalently included in the lipophilic monolayers of nanoparticles[71] and nanocapsules[116] or in the double layers of liposomes[70,117] to address specific biological targets. Playing with this palette of instruments, organic chemists might design proper systems, based on calixarenes, able to interact with proteins, nucleic acids or even biomacromolecules present on the surface of cells/bacteria/viruses. This paves the way to modulate protein activity or to develop site-specific drug/gene delivery systems or sensors for pathological biomarkers. It is therefore expected that in the next decade calixarene-based nanosystems can be further exploited for the development of diagnostic and therapeutic tools for bionanotechology and nanomedicine applications.

Laura Baldini obtained her PhD in Chemistry with Prof. Rocco Ungaro in 2002, with a thesis about the self-assembly and molecular recognition properties of functionalised calixarenes. In 2002 she joined the group of Prof. Andrew D. Hamilton at the Yale University (USA) as a post-doctoral fellow working in the field of protein surface recognition by synthetic receptors. Since 2005 she has been first a researcher and then an associate professor at the University of Parma. Her present research activity is focussed on the development of calixarenes as active systems in the field of biomacromolecule recognition and functional materials.

Alessandro Casnati, after his PhD in Chemistry in 1991 and a research period in Twente (Prof. D. N. Reinhoudt) started to work with Prof. A. Pochini and R. Ungaro on the selective functionalisation of calixarenes for the development of receptors for ions and neutral molecules. Since 2015 he is Full Professor of Organic Chemistry. His research interests focus on the use of calixarenes as multivalent ligands and multifunctional biomimetic catalysts. He has been awarded the ''G. Ciamician'' medal (1997) and the "A. Mangini" gold medal (2019) from the Italian Chemical Society. He is author of more than 230 contributions in books and international journals and of 10 patents.

Francesco Sansone graduated in Chemistry at Parma University. He spent in 1992 a research period at Twente University (NL) by prof. David N. Reinhoudt and in 1995 at Ferrara University by Proff. Dondoni and Marra. In 1998 he received his Ph.D. in Organic Chemistry working under the supervision of Prof. Rocco Ungaro. He is Associate Professor at Parma University, and his scientific interests are in the field of supramolecular and bioorganic chemistry, particularly on the synthesis and study of calixarene-based derivatives for bionanotechnology applications. He is co-author of 110 publications among papers on international journals and book chapters and 6 patents.

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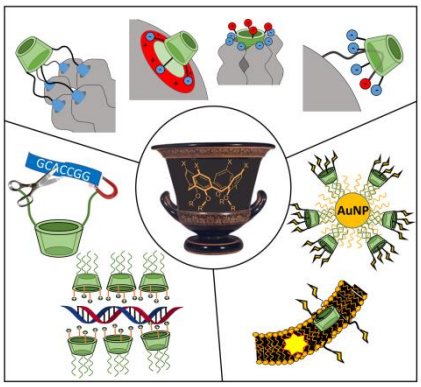

In the 70's, when they started to appear in the literature, no one would have bet even a cent on the future of these strange cyclic oligomers. Fifty years later, a series of appealing features make calixarene a versatile scaffold for the design of ligands with applications ranging from nuclear waste technology to bionanotechnology and nanomedicine.