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MOULDING CALIXARENES FOR BIOMACROMOLECULE TARGETING

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Abstract. After their successful use as preorganized platform for the preparation of receptors for metal ions and small neutral molecules, in the last 15 years calixarenes are enjoying a renaissance of popularity as scaffolds for ligands able to efficiently and selectively target macromolecules such as proteins/enzymes, nucleic acids and lipids. This Feature Article summarizes the peculiar factors characterizing calixarene structure and properties also outlining the main rules which can be used to turn such macrocycles in efficient and successful ligands for these classes of biomacromolecules. Factors affecting the multivalent properties of calixarenes such as the size, conformation and stereochemical presentation of binding groups or their amphiphilicity and hybrid character will be described in details with the use of few selected examples from the literature. Perspectives and applications of these ligands in Bionanotechnology and Nanomedicine, such as protein sensing and inhibition, gene-delivery, targeted drug-delivery and cell imaging will be also discussed.

1. Introduction

Calix[n]arenes (Figure 1, 1: n ≥ 4 but most commonly 4, 5, 6 or 8), the cyclic oligomers obtained by the condensation of phenol and formaldehyde under basic conditions, occupy a privileged position in Supramolecular Chemistry.

Figure 1: The planar representation (left) and a three-dimensional sketch (right) of the calix[n]arenes 1 (n ≥ 4) with the indications of the two rims.

1 Dedicated to Prof. Andrea Pochini on the occasion of his 76th birthday.
Thanks to well consolidated procedures that allow to synthesise the native macrocycles even in kilo-scale, the chemistry of these compounds undertook a burst in the late 80’s that does not yet seem to have reached a plateau. Their applications as scaffolds to build up receptors for metal and ammonium ions, greatly contribute to this success. The highly lipophilic backbone and the possibility to make the macrocycle fit the guest by choosing the proper cyclic oligomer size resulted in some of the most efficient and selective ligands ever known for different type of cations. Quite soon, it also appeared evident that the possibility to shape the calix[4]arenes in desired structures (cone, partial cone, 1,3-alternate, 1,2-alternate), via functionalization of the lower rim and/or intramolecular bridges, would have permitted to implement the supramolecular concept of preorganization also for calixarene-based ionophores.

![Figure 2: The four limiting conformations for calix[4]arenes 1a (cone, partial cone, 1,3-alternate and 1,2-alternate). For R = Me or Et conformational interconversion takes place even at room temperature, while, when R groups are larger than ethyl, the structure is fixed by the conditions used during lower rim (phenolic oxygen) functionalization.](image)

Each of these calixarene structures, possessing different polarity, stereochemical orientation of binding units and shape demonstrated to be able to discriminate even among spherical metal ions of very similar characteristics. The cone structure usually results to be, indeed, selective for the smaller and harder sodium ion while the 1,3-alternate for larger and softer caesium and rubidium ions. However, the lipophilicity of the scaffold that was first considered an advantage, also turned out to impair the use of such macrocycles in water to target molecules of biological interest. A first example of water
soluble derivative, the tetracarboxylato calix[4]arene 2, was reported in the literature in 1984, but, being its solubility in water quite low, only with the nearly concurrent report on the synthesis of p-sufonatocalixarenes (3) it appeared evident that calixarenes could have a future also as supramolecular hosts in aqueous media. It rapidly came clear that water soluble receptors could be obtained by adorning the macrocycle with positively or negatively charged groups or even neutral but highly hydrophilic moieties. Moreover, these particular molecular architectures allowed to disclose the possibility to flank, often in a synergistic way, hydrogen bonds/electrostatic interactions of the polar and water-solubilizing groups with hydrophobic effects/CH-π/π-π interactions given by the preorganized aromatic cavity. Interestingly, first observations indicated that such amphiphilic host can also complex small organic guests such as anilinium ions and native amino acids or mimic the conditions met in the hydrophobic pockets of proteins where water molecules can be bound via OH-π hydrogen bonds.

Besides the complexation of such small biologically relevant molecules, already in 1996 a first example of a calix[4]arene was reported to possess antimicrobial activity with selectivity towards Gram-positive bacteria and efficiency close to that of the natural antibiotic vancomycin. Remarkably, this study was the first example where the biological activity of a calixarene could be connected with its ability to recognize a biomacromolecule (vide infra). In the following 20 years a number of studies appeared in the literature with investigations on calixarene ligands able to interact with proteins/enzymes, oligonucleotides and nucleic acids or lipopolysaccharides and related potential applications ranging from antiadhesion therapy against viruses, bacteria and bacterial toxins, to those of antitumour agents, stimulators of the immune system and gene- or drug-delivery systems.

This feature article does not aim to comprehensively cover all the plentiful literature reports in this field, already reviewed in different recent essays, but it tries to identify the main
characteristics and structural elements that make calixarenes unique and special scaffolds for the construction of ligands able to target biomacromolecules.

2. Calixarene peculiar characteristics affecting biomacromolecule binding

2.1 Multivalency: size, shape and stereochemical presentation of binding groups

One of the characteristics most exploited in the design of calixarene-based ligands for biomacromolecules is certainly multivalency since it allows to make binding tight and specific. In fact, while a single binding event between a ligating group of a small ligand and a macromolecule is usually endowed with a low affinity (dissociation constants often in the millimolar range), the multiple presentation of several ligating groups properly exposed from a central scaffold gives rise to multivalent ligands endowed with much higher affinity (lower $K_D$). If properly designed in terms of number of and distances between ligating groups (epitopes), the overall binding energy might result much more favourable than the arithmetic sum of the energies of the single binding events. This effect, mostly attributed to entropic gains (high statistical binding probability and high local or effective concentration of the ligating groups in proximity of the receptor sites) is called multivalent effect and is often used also by Nature to make recognition phenomena more efficient and selective. The oligomeric nature of calixarenes, *i.e.* the presence of repeating phenolic units in a cyclic array, makes these macrocycles ideal scaffolds for the construction of multivalent ligands (Figure 3).

![Figure 3](image)

**Figure 3**: Schematic representation of calixarene-based multivalent ligands. Tetravalent calix[4]arenes (a) and (b) having different stereochemical presentation of ligating units and (c) a larger calix[8]arene bearing 16 ligating units.
Thanks to the well-known and developed synthetic procedures for the lower (phenolic OH groups) and/or upper (aromatic para position to the phenolic oxygen) rim functionalization, calixarenes might be easily adorned with a variable number (2, 3, 4, 5, 6, 8 and their multiples) of simple (sulfonate, phosphate, carboxylate, ammonium, guanidinium etc...) or more elaborated (amino acids, small peptides, mono- or oligosaccharides, nucleotides etc...) groups. The features of the resulting multivalent ligand, are strictly dependent upon the valency, size and conformation of the macrocycle (Figure 3). The distance between the ligating units on the macrocycle is often quite important and needs to be correctly designed prior to synthesis to allow the simultaneous formations of all the possible supramolecular contacts. It is determined by (i) the length of the linkers between the binding units and the calixarene scaffold and (ii) the size of the calixarene, being a calix[4]arene considerably smaller than a calix[8]arene (Figure 3). The size of the macrocycle also influences the conformational mobility of central scaffold and consequently the directionality of the peripheral binding units. Calix[4]– (1a) and –[5]arenes (1b) functionalized at the lower rim with small R groups are usually conformationally mobile, as most of the larger calix[6]– (1c) and –[8]arene (1d) derivatives. Multivalent ligands resulting from these scaffolds are therefore usually mobile and might occasionally exploit a beneficial induced fit effect with the target, i.e. can adapt their conformation to optimize the binding process in terms of number of binding events. On the other hand, while calix[6]- (1c) and [8]arenes (1d) can be conformationally locked only by bridging two or more phenolic units or by special functionalizations,25 the insertion of groups larger than ethyl at the lower rim of calix[4]arenes (1a) allows to fix the macrocycle in one of the four possible structures, namely cone, partial cone, 1,3-alternate and 1,2-alternate (Figure 2).26 Interestingly, these structures clearly display a different stereochemical presentation of the ligating units around the central scaffold. Most of the tetrafunctionalized cone derivatives are, however, still characterized by a fast conformational interconversion (sometimes referred as “breathing of the calix”) between two pinched (or flattened) cone structures (Figure 4, top). Bridging of two adjacent phenolic oxygen atoms with short diethylene glycol chains (Figure 4, bottom) ensures a high level of rigidification of the scaffold which often results in higher selectivity and efficiency in including guests in the apolar cavity27 or even in binding to large biomacromolecules.28
Figure 4: Equilibrium of conformational interconversion between the two pinched cone structures of a tetraalkoxy calix[4]arene (top). A calix[4]arene-biscrown-3 derivative (bottom) which exemplifies the bridging strategy to rigidify the calixarene scaffold.

The most studied cone and 1,3-alternate isomers (Figure 3a,b), not only present different shape, orientation and distance between binding units, but also their polarity and amphiphilicity (vide infra) are remarkably different and this often deeply influences their self-assembly properties and affinity for the target biomacromolecules.

Figure 5. Possible modes of protein binding by calixarene ligands: (a) large surface (“hot spot”) recognition; (b) “single point” recognition of a selected aminoacid residue in a peptide sequence; (c) stoppering of lipophilic pockets assisted by electrostatic interactions.

The cone derivatives exhibit a facial disposition of binding units (epitopes) and are more suitable to match large surfaces (Figure 5a) or species having binding domains relatively close each to the others (Figure 6a) and facing the same region of the space. On the contrary, the 1,3-alternate derivatives with a two-faced presentation of the epitopes on opposite regions seem more adapt to bridge different receptor units, often giving rise to large cross-linked multimeric aggregates (Figure 6b).
2.2 Hybrid character

The presence of highly polar groups adorning one of the rims of the macrocycle offers the interesting possibility to present in the same ligand groups able to establish electrostatic and/or hydrogen bonds and lipophilic aromatic nuclei able to give rise to CH-π, π-π interactions or to exploit hydrophobic effects. The simultaneous presence of groups having such different polarity in the same structure, sometimes referred as “hybrid character”, somehow resembles the situation encountered in the interior of protein and enzyme binding pockets where the aromatic nuclei of phenylalanine and tryptophane residues are in close contacts with the hydrogen bond acceptor and donor NH-CO peptide groups of the protein backbone or with the side chains of polar amino acids. The proximity of these quite different ligating units, reinforces the binding within supramolecular partners by exploiting synergistic and solvation-desolvation effects. A text-book example of such hybrid character is given by Vancomycin. Vancomycin is, in fact, a natural antibiotic whose activity is linked to its ability to selectively recognize the terminal L-Lys-D-Ala-D-Ala sequence of peptidoglycan, one of the main cell wall constituents in Gram-positive bacteria. It could be proved that, thanks to this binding ability, Vancomycin can block the cross-linking of this glycopeptide thus causing the cell wall lysis and inhibiting the growth of bacteria. Vancomycin is constituted by a peptide backbone sunk, together with its N-terminal ammonium ion, into a highly lipophilic pocket and therefore shielded from water molecules. This peculiar environment enhances the binding ability of Vancomycin which can strongly trap the terminal part of peptidoglycan through a cooperative combination of H-bonds and electrostatic and hydrophobic interactions. Trying to mimic this binding motif, we reported on a series of calix[4]arenes fixed in the cone conformation and bridged at the upper rim with a short peptide. The most active compound against Gram-positive bacteria resulted to be the macrobicyclic ligand 4 having two N-linked L-alanines at the
upper rim, connected with 1,3,5-diethylenetriamine (DETA). Compound 4 was proved to efficiently bind to N-acetyl-D-Ala-D-Ala,\textsuperscript{29} used as model of the peptoglycan terminal part, thanks to a salt bridge with the central ammonium group of DETA and H-bonds with the calixarene amino acids (Figure 7, left). Quite interestingly, it was also suggested that one of the methyl groups of the guest Ala residue might be included in the lipophilic cavity and bound through CH-π interactions. Besides showing the potentiality of the synergistic combination of the presence of polar groups close to the lipophilic cavity, this class of macrobicyclic calixarenes with antimicrobial activity quite close to that of Vancomycin, probably represents the first example reported in the literature of calixarene based ligands whose biological target could be clearly identified and a mechanism of action proposed.

Figure 7. Possible structure of the complex between the macrobicyclic ligand 4 and Lys-D-Ala-D-Ala (left); sketch of the x-ray crystal structure of the complex between the p-sulfonatocalix[4]arene 3a and Lys (right).

A previous example of a biological active calixarene, in fact, dated back to 1955 is that of a calix[8]arene functionalized at the lower rim with long oligoethylene glycol chains showing antitubercolicotic activity,\textsuperscript{30} but its mechanism of action was only quite recently proposed and, however, a definite target has not yet been ascertained.\textsuperscript{31} The binding mode shown by the Vancomycin mimic calixarene ligands, able to address a single amino acid (or a very limited number of amino acids) in a large biopolymer could be systematically classified as “single point” recognition (Figure 5b), a way of binding very recently elegantly exploited by Crowley and Hof to recognize a protein mutated on single positions upon methylation of the Lysine residues (\textit{vide infra}).\textsuperscript{32} The potential of these hybrid receptors in the binding to specific single points of proteins is certainly attracting the interest of many chemists and biologists also because several are the examples of simple calixarenes able to bind single amino acids \textit{via} inclusion of their lateral side chains in the cavity thanks to the combination of different interactions. Leu, Ile, Phe and Trp were shown
to be bound in water solution by peptidocalixarenes, while several evidences were collected in solution or at the solid state of binding to lysine (Figure 7, right) and arginine side chains by p-sulfonatocalixarenes. In the latter cases it could be clearly identified that the binding is due both to electrostatic interactions of the negatively charged sulfonate groups with the ammonium ions and to CH-π interactions between the calixarene cavity and the CH₂ groups of the basic amino acid lateral chains in σ and ε positions.

Interestingly, the aromatic backbone of the calixarene can also take part to the interaction with the biomacromolecules not only by presenting a lipophilic cavity but also offering a highly lipophilic external surface which might behave as a lipophilic wedge. This is the case of de Mendoza’s tetraguanidinium cone calix[4]arenes (vide infra) able to restore the tetrameric structure of p53 also thanks to the formation of hydrophobic interactions between the external calixarene backbone and the hydrophobic region present on the four assembling p53 units (Figure 5c).

2.3 Self-assembly: facial vs bolaamphiphiles

As a consequence of the simultaneous presence of polar head groups and a lipophilic backbone, water soluble calixarenes have an amphiphilic character. The cone calix[4]arene derivatives adopt a more classical facial amphiphilic structure, being the polar groups well confined at one side of the macrocycle (Figure 8a,b). Such structures have therefore a great tendency to aggregate generally in micelles or Solid Lipid Nanoparticles (SLNs) whose dimensions and nature depend on the type and length of the lipophilic chains and hydrophilicity of the polar head groups. Lipophilic, long and linear chains, either at the upper or the lower rim, increase the self-assembling ability in water and the aggregation number tends to increase by increasing the number of carbon atom per chain. Thus, for alkyl chains longer that 6-10 carbon atoms micelles or SLNs might form. Shorter alkyl groups (up to propyl) or more hydrophilic polyether chains usually enhance the water solubility. Much more common are facial amphiphiles having polar groups at the upper rim and aliphatic chains at the lower rim (Figure 8a), but cone calixarenes with an opposite phobicity, i.e. polar groups at the lower rim and aliphatic chains at the upper (Figure 8b), also behave as facial amphiphiles. It was also noticed that even in absence of alkyl groups at the upper rim, these type of amphiphiles have an
important tendency to self-aggregate, thanks to interactions among the lipophilic backbones of the macrocycles.\textsuperscript{39}

Contrary to cone derivatives, the 1,3-alternate calix[4]arenes show a nearly cylindrical structure and, if the polar groups are all linked to the same positions of the macrocycles (all at the phenolic oxygen atoms or all at the position para to the oxygen atoms), possess a bolaamphiphile structure (two polar region separated by a lipophilic core, Figure 8c). Although this topology has been much less exploited, it appears particularly interesting because such structures, can potentially be included in liposomes (\textit{vide infra}) or, upon self-aggregation, originate vesicles.\textsuperscript{40} Conformationally mobile calix[4]arene amphiphiles in water usually tend to adopt a 1,3-alternate structure which minimizes the lipophilic surfaces exposed to the solvent. Bolaamphiphiles most commonly show higher water solubility and lower aggregation ability compared to the cone analogues.

![Figure 8](image)

\textbf{Figure 8.} Possible geometries of the calix[4]arene amphiphiles showing the different stereochemical orientation of the hydrophilic ligating chains: (a) facial amphiphiles with polar groups at the upper rim; (b) facial amphiphiles with polar groups at the lower rim; (c) bolaamphiphiles.

Rather surprisingly, also calix[6]- or -[8]arenes functionalized at the upper rim with polar groups, although conformationally mobile and more soluble of the lower tetrameric homologues blocked in cone or 1,3-alternate structures, might aggregate as well although they present only short methyl chains at the lower rim.\textsuperscript{41,42}

Thus, many factors such as conformation, length and nature of the alkyl chains, polarity of the head groups and of the linkers to the macrocycle can be finely tuned to obtain aggregates or discrete unimolecular units also as a function of the concentration used in water. Self-aggregation properties, therefore, deeply influence the ability of the calixarene ligands to interact with biomacromolecules or might be usefully employed to trigger special
functions such as nanoparticles/monolayer functionalization or gene-/drug-delivery processes.

The possibility to vary the valency, distance and presentation of the binding groups, the opportunity to exploit the lipophilic cavity/backbone in binding and the option to induce the self-assembly of these macrocyclic amphiphiles, are all characteristics that cooperate in making multivalent calixarenes efficient ligands for biomacromolecules. In the examples herein reported, the targeting properties of these ligands were often compared with their monovalent models, always showing very high multivalent effects. On the other hand, a direct comparison of multivalent ligands based on calixarenes with analogues based on acyclic or cyclic platforms was seldom reported in the literature. However, in these few cases, calixarenes demonstrated to be superior.

3. Targeting protein and enzymes

The cone isomer of the calix[4]arene has the successful geometry for the interaction with protein surfaces and for the inclusion in the apolar cavity either of positively charged groups or, especially in aqueous environment, lipophilic residues. In the first context, particularly significant and interesting are the derivatives reported in the years by Hamilton and colleagues who, taking inspiration from the antibody shape and functionality, proposed a series of calix[4]arenes able to interact with the surface of some proteins by covering specific areas of several hundreds of Å² (Figure 5a). The simultaneous presence of a lipophilic region surrounded by polar groups having the same polarity/charge was identified by Hamilton as a key element for protein targeting by calix[4]arene ligands in cone geometry and adorned at the upper rim with ligating groups bearing charges complementary to those present on the protein. Accordingly, in the case of cytochrome c, a lipophilic region with a positively charged belt surrounds the heme edge responsible for the enzymatic activity. Calixarene 5a with four cyclopeptides at the upper rim was designed to approach and interact with this area and cover the active site working as a cap (see Figure 5a). The Asp units of the GlyAspGlyAsp sequence provide the negative charges for the interaction with the side chain ammonium groups of the Lys residues of the belt positioned at the border of the enzyme lipophilic area. The benzene units of the 3-aminomethylbenzoyl based dipeptide analogue included in the loops extend the aromatic character of the calixarene backbone to increase the possible contact surface with the lipophilic patch of the protein. The successful interaction of this calixarene with the selected area of the enzyme was ascertained by observing that the cyt-c Fe²⁺ reduction by
Ascorbate is inhibited by 5a. This calixarene, forming a 1:1 complex with the enzyme \( K_{\text{ass}} \approx 10^8 \text{ M}^{-1} \), prevents the approach of the reducing agent to the heme edge. Moreover, covering this area for an extension of 450-500 Å\(^2\), 5a is also able to prevent the interaction between cyt-c and cytochrome-c peroxidase.\(^{47}\) For the same structural reasons, 5a binds at nanomolar level to the \( \alpha \)-chymotrypsin surface, disrupting the complex between this enzyme and some proteinaceous inhibitors such as soybean trypsin.\(^{48}\) This remarkable potential in the disruption of protein-protein interactions was later exploited to inhibit the binding between some growth factors and their receptors involved in angiogenesis and tumour progression. This was the case of the platelet derived growth factor (PDGF) that, as cytochrome c and \( \alpha \)-chymotrypsin, presents on its exterior surface an hydrophobic area with a positively charged belt used for the recognition of the corresponding membrane bound receptor PDGFR.\(^{45,49}\) The complex formation between PDGF and PDGFR is critically involved in oncogenesis as key step for the maintenance of blood vessels. Calixarene 5b, characterized by a functionalized surface of approximately 400 Å\(^2\) and a mix of negative charges and hydrophobic residues, binds to PDGF. In this way it prevents the growth factor adhesion to the complementary surface of PDGFR inhibiting its activity with an \( IC_{50} = 250 \text{ nM} \).

As a consequence, Hamilton and colleagues could observe that the growth of different human tumours implanted in nude mice resulted strongly slowed down by the treatment with this molecule. Analogously, compound 6, a simplified evolution of this class of molecules where the combination of negative charges and aromatic moieties was made more essential replacing the pentapeptide loops with acyclic monooacid monoester isophthalic acid units, evidenced inhibition properties towards both PDGF and vascular endothelial growth factor (VEGF).\(^{50}\) This double activity is very interesting because the tumour development depends on angiogenesis that in turn needs both growth factors for initiation and maintenance of blood vessels, respectively. Remarkably, suppression of
human tumour growth and angiogenesis was observed after the in vivo treatment of mice with this calixarene derivative.

The aromatic cavity, that is indeed well defined only in the cone calix[4]arene, confers to this macrocycle the capability of trapping lipophilic and positively charged side chains of selected amino acids, thus providing these ligands also with the potential ability to recognize “single points” in the peptide sequence (Figure 5b). If the upper rim of the cavity is adorned with further functional groups, these latter ones can also assist in the binding. The tetrasulfonato calix[4]arene 3a, for instance, whose cone geometry is ensured by the hydrogen bonding array present at the lower rim and involving the four hydroxyl groups, one of which is in the anionic form at neutral pH, \(^{51}\) includes the ε-ammonium group of lysines (Figure 7, right) present in cytochrome c. Crowley and coll. could prove this ability through NMR studies in solution and resolution of the X-ray crystal structure of the calixarene-protein complex. \(^ {52}\) The asymmetric unit is composed of two proteins and three sulfonato calixarenes each complexing the side chain of a Lys. All the three contact points show very similar features with the amino acid side chain included in the apolar cavity of the macrocycle in a hook-like shape that projects the ammonium group towards the anionic sulfonato units. Charge-charge and hydrophobic interactions then cooperate in the stabilization of the complex. The surface area that becomes inaccessible upon the interaction between cytochrome c and one of the calixarene molecules is 200-300 Å\(^2\) depending on the position of the Lys included. The same sulfonato calix[4]arene 3a showed similar complexation features towards lysozyme but preferring in this case the inclusion of the side chain of one of the 11 arginines present in the protein, namely the C-terminal Arg128, as evidenced by solving, also in this case, the crystal structure of the complex. \(^ {53}\) Again, CH-π interactions and charge-charge salt bridges are simultaneously involved. The accessibility for this unit respect to other ten arginines present in the protein seems to be the reason of the selectivity. Arg side chain results almost entirely submerged in the calixarene that buries ~230 Å\(^2\) of the protein surface. Interestingly, when the nitrogen atom in the side chain of the lysines present in lysozyme undergoes bis-methylation to originate the positively charged [-NH(Me)]\(^2\) head group, the selectivity of 3a shifts from Arg to N,N-dimethyl lysine. \(^ {32}\) The sulfonated, cone shaped structure in fact well mimics the aromatic cage accompanied by carboxylate groups that several proteins, such as chromodomains, uses to recognize and complex di- and trimethylated lysines. Interestingly, 3a selects only one, namely Lys116, among the six possible dimethyllysine sites present in the protein. As ascertained by NMR in solution and X-Ray crystal studies,
its cationic dimethylamino substituent is encapsulated in the calixarene cavity. Differently from the complex between 3a and Lys-NH$_3^+$ in cytochrome c, the methylated ammonium of Lys side chain penetrates into the cavity positioning the charged head group at the core of the macrocycle cavity establishing cation-π interactions, while the methylene units are substantially excluded. The sulfonato calixarene 3a adopts a regular cone conformation, as observed in the complexation of simple tetramethylammonium cations, while in the binding to cytochrome c the macrocycle is in a pinched cone conformation that can entirely accommodate the methylene units of the unmodified amino acid side chain. Methylation of Lys ε-nitrogen is an important post-translational modification in proteins and specifically in hystones it is responsible for relevant effects on gene regulation and signalling pathways. Upon methylation of their Lys side chains, histones trigger protein-protein interfacial interactions and induce changes on the chromatin structure and activity. To this end, Hof and coll. systematically studied the complexation ability of 3a towards the methylated Lys in the case of small peptide sequences representing the unmodified and progressively methylated histone tails. The p-sulfonato calixarene 3a evidences increasing affinity moving from the unmethylated to the trimethylated Lys. The role played by the structured cavity resulted clear when an analogue of 3a, alkylated at the lower rim and then lacking the rigidity typically conferred by the hydrogen bonds among the OH groups, showed poor efficiency in the complexation of the same residues.

A calix[4]arene blocked in cone conformation also resembles a stopper or a wedge. With this idea in mind de Mendoza and coll. designed a series of derivatives (7), shaped in this geometry and bearing guanidinium at the upper rim, to block the activity of voltage-dependent potassium channels. These proteins are important pharmacological targets because many diseases are related to their dysfunctions. The calixarene conical shape suggested the idea of a possible accommodation of the macrocycle, from the side of the lower rim, into the channel vestibule thus allowing the cationic substituents at the upper rim to bind to the negatively charged residues indeed positioned at the external surface surrounding the channel entry (Figure 5c).
Compounds 7a and 7b with OH groups at the lower rim show at 50 μM an interesting reversible inhibition activity towards the Shaker potassium channel that results even more pronounced with the analogue 7c blocked in a rigid cone structure thanks to the short crown ethers bridges. The treatment of oocytes expressing the channel with the two alkylated derivatives 7d and 7e, on the contrary, determined an important but irreversible decrease of the ionic current, probably because of an aspecific, destructive detergent action of the macrocycles against the cell membrane. Compared to 7a-c, compound 7d,e are markedly more amphiphilic because of the presence of lipophilic chains at the lower rim. The importance of the simultaneous presence of the calixarene conical shape and of the guanidinium units is evident because the same calixarene scaffold functionalized at the upper rim with anionic groups such as carboxylates or even with primary ammonium functions did not give appreciable activity. Moreover, a phenol functionalized as 7a and used as monovalent and monomeric model of the tetravalent macrocycle, substantially did not show any activity and this supports the rational that the multivalent, preorganized exposition of the binding units is a winning strategy. Molecular dynamic studies exploiting the crystal structure coordinates of the potassium channel and minimized calixarenes, indicated that the macrocycles with free OH groups at the lower rim (7a and 7b) can deeply penetrate the outer vestibule of the protein placing the cationic groups at standard hydrogen bonding distances with the negatively charged Asp side chains around the vestibule. One year before the same research group proposed, using an analogous design, another cone tetraguanidinium calix[4]arene (8a) to restore the physiological activity of the homotetrameric p53 transcription factor when altered by an inherited mutation. The mutated form of p53 is characterized by the replacement of Arg337 with Hys in each of the four peptide chains of the wild-type protein and, as a consequence,
shows defective association properties. This mutation weakens the tetramerization domain, typical of wild-type p53 and strictly related to the protein activity against tumour cells. The action of p53 as "genome guardian", which induces cell arrest when DNA is damaged and triggers the expression of DNA repair machinery or apoptosis when the damage is irreversible, is thus strongly impaired.

The tetramer destabilization is ascribable to the Hys side chain since the imidazole group is not always protonated at physiological pH as, on the contrary, it is the Arg guanidine in the wild-type form and therefore it cannot ensure a sufficiently strong interaction with a complementary Asp unit of an opposite protein unit. The presence of glutamates Glu336 and Glu339 onto each of the two opposite monomers, just above the hydrophobic pocket generated upon the tetramerization process, defines an almost squared disposition of negative charges at both the surfaces located at the bases of the tetramer. This suggested to de Mendoza and coll. that a cone shaped molecule equipped with four cationic groups could be perfectly complementary to the shape of the squares defined by the four Glu units and, then, stabilize the tetramer through salt bridges and hydrophobic interactions. Differential scanning calorimetry (DSC) and circular dichroism showed indeed that calixarene 8a at 400 \( \mu \)M stabilizes the tetrameric form of the mutated protein, while no significant effects were observed with the wild-type form. The gained robustness allowed to detect by electrospray mass spectrometry the tetramer non covalently bound to two molecules of the synthetic ligand. On the basis of the data collected also through molecular modelling studies, two calixarenes 8a, one for each side of the protein assembly, fit into the hydrophobic regions thanks to hydrophobic interactions that the exterior of the calixarene cavity, rigidified by the short ethylene bridges at the lower rim, establishes with the lipophilic side-chains of the protein (Figure 5c). Cooperatively, as a multivalent four hook harpoon, the cationic heads at the upper rim interact with the carboxylate anions exposed by the Glu residues keeping strongly together the four monomers of the mutated transcription factor. More recently the same authors investigated the effect on the p53 tetramerization of the ligand 8b, a more flexible analogue of 8a with propyl chains at the lower rim instead of the short bridges.\(^{38}\) DSC experiments with 8b at
only 25 µM evidenced, compared to 8a, an increase of the thermal stability of the
tetrameric domain both for the wild-type p53 and, even more substantially, for the mutated
form. Although flexibility in the ligand is often considered a drawback because of the
expected loss of entropy upon complexation, in this case the higher rigidity and degree of
preorganization of 8a appears detrimental. Likely, the flexibility of 8b allows a more
profitable induced fit binding of the guanidinium groups with the complementary
carboxylates, maximizing the charge-charge interactions. Moreover, the lipophilic propyl
chains can, penetrating in the pocket, establish additional hydrophobic interactions with
the protein. Taken together, these two aspects seem to overbalance the entropy decrease
arising from calixarene conformational freezing upon protein binding.

4. Targeting lectins - Interfering with carbohydrate-protein interactions

Other examples of protein recognition where specific shapes of the calixarene skeleton
have shown to have remarkably influence on binding, are those involving carbohydrate-
protein interactions for lectin recognition. Carbohydrate binding proteins, named lectins,
have no enzymatic or immunological activity but are involved in many recognition
processes of physiological and pathological nature. Calixarenes functionalized with
saccharide units, the glycocalixarenes,\textsuperscript{20} demonstrated in these years their relevance as
multivalent ligands for lectins. In the cases here described, the collected data point out
how selectivity and efficiency of the binding to the protein is strongly dependent on the
stereochemical presentation (geometry) of the exposed saccharide units and, then, on the
conformational features of the macrocyclic scaffold.

A series of calix[4]arenes functionalized at the lower rim with β-galactoside units (e.g.
9a,b), for instance, were prepared and evaluated by Cecioni et al. as inhibitors of the
tetrameric galactose-binding lectin PA-IL from the opportunistic bacterium \textit{Pseudomonas}
aeruginosa.\textsuperscript{55} This bacterium is responsible for chronic respiratory diseases and is often
associated to cystic fibrosis. These ligands have inhibitory activity dependent on the
different number of linked sugar units (valency) and/or their presentation geometry. ITC
and SPR experiments, by consent, evidenced the higher binding efficiency of the
tetravalent compound blocked in 1,3-alternate conformation (9a). The stoichiometry found
for the complex between the glycocalixarene 9a and the monomer of PA-IL was 1:4.
Significantly, respect to the $K_D = 176$ nM determined for this compound, the tetravalent cone isomer $9b$ is characterised by a $K_D = 420$ nM. The stoichiometry of the complex involving the cone isomer is 1:3, suggesting that no more than three monomers of PA-IL can approach the four galactose units clustered at the same face of the macrocycle, despite the relatively long spacers between the aromatic units and the sugar epitopes present in this family of derivatives. The lectin shape and the position of its four binding sites, located two by two at the minor faces of the parallelepipedon defined by the tetrameric lectin, can explain the preference for the 1,3-alternate derivative. This in fact can bridge two lectin tetramers by chelating two monomers on each lectin (Figure 9). This binding arrangements was initially hypothesized and supported through docking calculations. Later, AFM measurements on samples containing PA-IL and $9a$ allowed to visualize linear species consistent with the proposed chelation model, although sometimes interrupted by bifurcations.

**Figure 9**: Schematic representation of the chelation model used by 1,3-alternate galactocalix[4]arene $9a$ to bind to PA-IL.

Very recently, the same 1,3-alternate calixarene scaffold with the same spacers was functionalized with L-fucose units to obtain an inhibitor ($9c$) of PA-IIIL, a second lectin expressed by *Pseudomonas aeruginosa* and, together with PA-IL, an important bacterial virulence factor. With experiments also *in vivo* on infected mice, the authors could point
out the ability of both multivalent galactosylated (9a) and fucosylated (9c) compounds to block the infection and the bacterial biofilm proliferation through the inhibition of the two lectins. For the interaction between the fucosylated cluster (9c) and PA-IL, the chelate mode of binding indicates for 9a and PA-IL is not conceivable. PA-IL in fact, although tetrameric and similar to PA-IL, has the binding sites too far to allow their simultaneous complexation with two fucose units of the same calixarene. So, a wide and less ordered cross-linked multivalent aggregate (Figure 6b) is more reasonably the mode of interaction which takes place.

Another small library of glycocalix[n]arenes (10-12), functionalized at the upper rim with galactose and lactose units and connected to the macrocyclic scaffolds through thiourea moieties, was designed and synthesised by us to obtain inhibitors of the galectin mediated adhesion processes. These lectins selectively bind galactose epitopes on cell surfaces. Human galectins are strategic targets because they are involved in the progression and development of tumours and metastases. Several subfamilies of human galectins are known, with different activities, roles and impact on the correlated pathologies. Endogenous human gal-1, gal-3 and gal-4, used in the reported studies, can be considered the model of these sub-families and show different structures. In particular, gal-1 and gal-4 are characterized by the presence of two carbohydrate binding sites, covalently linked in the latter and noncovalently associated in the former, thus facing opposite regions of the space. Gal-3, on the contrary, is a monomeric lectin that tends to self-assemble in a pentameric ensemble. Limiting the attention to the lactosylated derivatives, since they resulted in general significantly more efficient than the galactosylated ones, the inhibition of the galectin adhesion to surface immobilized asialofetuin indicated a marked multivalent effect, being the monovalent D-lactose always a worse inhibitor than lactocalixarenes. A peculiar binding selectivity was also observed, since different lactocalixarenes show rather different IC$_{50}$ values respect to the same galectin and, vice versa, each multivalent ligand has different IC$_{50}$ values towards the three different galectins. Much more remarkably, the occurrence of selectivity in the recognition between these glycocalixarenes and these subfamilies of lectins was confirmed in experiments with cells, even with amplified differences.$^{57}$ Through fluorescence assisted cell sorting tests, it was in fact observed that the larger and conformationally mobile lactocalix[6]- (11d) and -[8]arenes (11f) are particularly efficient in the inhibition of the binding of gal-4 to human pancreatic carcinoma cells. Together with them, also the 1,3-alternate calix[4]arene (12b) potently interferes with this adhesion process. In addition,
cluster 12b, together with the calix[8]arene analogue (11f), resulted to be the best inhibitor also of gal-1 adhesion to the same tumour cell line. 

The similarity in the disposition geometry of the carbohydrate binding sites in the two galectins could be one of the reasons of the major activity of these two multivalent systems towards these two subfamilies. The availability of ligands able to simultaneously block the activity of gal-1 and gal-4 is indeed of relevant biomedical interest since the two lectins are both involved in the development of the colon tumour. However, from a structural point of view, the most impressive aspect in term of shape dependent selectivity is that the cone isomer 10b, with the same valency of the 1,3-alternate 12b but with a facial display of the saccharide units, is very poorly active towards gal-1. *Vice versa*, it is the most potent inhibitor of the galectin-3 adhesion to the human colon adenocarcinoma cells while the 1,3-alternate (12b) is the worst one. In a more recent work, two derivatives of the previous series, the calix[4]arene in cone geometry and the calix[6]arene, were modified by insertion of the LacNAc moieties bearing a benzyl group at the 3’ position (13a and 13b) in place of the simpler lactose units. This modification was suggested by the analysis of previously reported X-ray crystal structures of the complexes between lectin and model substrates that evidenced possible additional interactions of the protein with the aromatic and amide moieties of the sugar. The selectivity in the cell assays of the calix[4]arene 13a for gal-3 over gal-1 appeared even more amplified, since its antiadhesion efficiency against gal-3 increased respect to the lactosylated analogue (10b), and a complete
inactivity towards gal-1 was observed. The cluster based on the calix[6]arene scaffold (13b) confirmed the preference of this larger and conformationally mobile structure for the inhibition of gal-4, with an efficiency higher compared to the corresponding lactosylated derivative 11d. The inter-group selectivity showed by the cone derivative has a not negligible medical relevance. In fact, gal-1 exerts, under some conditions, a positive role, with a potent pro-anoiéisis activity against some tumour cell lines, while gal-3 hampers its beneficial function. The possibility thus to block only gal-3 could help gal-1 to exert its positive effect of induced tumour cell programmed death.

Differently from the case of galectins where the best shape of the calixarene based ligand for the best binding and inhibition activity could not reasonably be predicted, the particular structure of Cholera Toxin (CT) suggested us the choice of the calix[5]arene as scaffold to build a multivalent inhibitor of this lectin. CT in fact is a well-known and widely studied AB₅ protein where each of the five B units presents a binding site for the pentasaccharide of the GM1 ganglioside (GM1os). These binding sites are all located on the same face of the toroid-like B₅ assembly (Figure 6a), allowing CT to adhere on the cell membrane and form a very stable complex which kicks off the infection process by insertion of the toxic A unit in the cell. Because of this particular geometry, we considered the possibility of achieving a ligand potentially able to form with CT a 1:1 multivalent complex (Figure 6a), provided that it is pentavalent and equipped with a spacer between core and saccharide units long enough to allow the simultaneous interaction of the five epitopes with the five CT recognition sites. The calixarene family can indeed provide calix[5]arene as platform with the right valency and we prepared and studied, in collaboration with Zuilhof and Wennekes, the derivative 14. Five GM1os units enzymatically prepared were connected to the calixarene upper rim through a 31 atom chain obtained via click chemistry. Although so far we could not obtain evidence of the actual formation of a 1:1 complex, ligand 14 has towards CT a IC₅₀ = 450 pM as determined by ELISA inhibition tests, which is the lowest value found so far for a pentavalent CT ligand and showing a gain in the activity per single GM1os installed on the macrocyclic core of 20,000 folds.⁵⁹
5. Targeting NA and polyphosphates

Polyphosphates and in particular nucleic acids (NAs), having a highly dense presentation of negative charges in the phosphodiester backbone and of hydrogen bond acceptor groups on the nucleobases, can be predicted to be proper targets of multivalent ligands possessing positively charged and/or hydrogen bond donor head groups. Pioneering studies during the 80’s, showed that positively charged azamacrocycles or cyclophanes could effectively interact with oligonucleotides and nucleic acids in water with rather high association constants.\textsuperscript{60-62} However, a series of systematic studies on the role of multivalency, conformation of the macrocycle and type of positively charged groups on calixarene-based DNA-binders started to appear only at the beginning of the new millennium.\textsuperscript{63,64} Quaternary ammonium,\textsuperscript{39,61} N-methylimidazolium,\textsuperscript{65} primary ammonium, and guanidinium groups\textsuperscript{43,66,67} have been since then studied in details. Guanidinium ion, in particular, thanks to its ability to effectively interact with tetrahedral anions such as phosphate groups via electrostatic interactions and H-bonding and to its low acidity which allows to be always protonated at physiological pH even when present on a macrocyclic structure at short distance with other positively charged head groups, reveals to be the best groups for DNA binding. We first studied the interaction of a small library of multivalent p-guanidinocalixarenes 15 which differ for their valency, conformational properties, length and lipophilicity of the alkyl groups present at the lower rim.\textsuperscript{63,66}
Electrophoresis Mobility Shift Assay (EMSA), melting curves and ethidium bromide displacement assays nicely showed a rather high affinity of all these compounds for DNA, independently of its circular (plasmid) or linear structure. Schrader and coll. also studied in details and compared the mode of binding of a series of primary ammonium and guanidinium calix[4]arenes fixed in the cone structure.\(^{64,67}\) Combining the information from a series of complementary experimental techniques and molecular modelling calculations, these authors suggest that these ligands bind in the major groove of B-DNA. Ligands bearing primary ammonium ions mainly interact with the nucleobases, while in the guanidino derivatives the charged head groups are also binding to the phosphate anions of the backbone. Quite different is the binding mode to shorter and destructured oligonucleotides such as triadenosine found in solution, where H-bonds between the calixarene NH protons and the negatively charged phosphate groups were evidenced while no interactions with the nucleobases could be pointed out.\(^{68}\) By studying the supramolecular properties of the small library of p-guanidinocalixarenes 15, our attention was especially drawn by the unprecedented ability shown by some of these calixarenes to transfer DNA into the cells and thus behaving as gene-delivery systems.\(^{66}\) Although cationic lipids and amphiphiles have been widely studied and used as gene vectors, to the best of our knowledge, no examples of multivalent cationic macrocycles had been reported to be able to transfect cells before 2006 when we published our first results in this field. Nearly in this same period, however, few examples of cationic cyclodextrin-based gene-delivery systems started to independently appear in the literature,\(^{69}\) thus eliciting an extensive number of studies on the use of macrocyclic preorganized cationic amphiphiles.
for cell transfection as potential alternative to widely studied and used nonviral vectors based on lipids and polymers. In general, the process aimed at introducing genes into a cell by the use of gene-delivery systems is a quite complicated phenomenon and results from the sequence of a series of positive events: i) a strong interaction between the carrier and the NA filament; ii) neutralization of NA charges; iii) condensation of the NA structure in small aggregate able to cross the phospholipid bilayer of cells; iv) activation of the uptake processes; v) release of the genetic cargo from the endosomes into the cytoplasm; vi) internalization of the NA into the cell nucleus. The final efficiency of transfection should therefore be a successful combination of so many and different steps that is usually quite difficult to predict the effects of even small changes in the structure of the carrier on the overall process. However, the ability to neutralize the NA charges and to compact the its structure to small aggregates (40-100 nm) is certainly a prerequisite for transfection even though this does not necessary ensure an efficient expression of the protein encoded by the genetic material. Quite interestingly, we realized that AFM can be profitably used to preliminary check the ability of the different calixarene-based carriers to bind and compact DNA thus allowing to predict how the structural modifications of the carriers can at least affect the first three steps of the gene-delivery process.\textsuperscript{66} The comparison of AFM images obtained by depositing on mica DNA alone or previously incubated with proper amounts of calixarene-based surfactants, allows to visualize the effect of the ligands on DNA folding (Figure 10).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure10.png}
\caption{Different proposed compactation modes of plasmid DNA by p-guanidinocalixarene: (a) tetraguanidinocalix[4]arene fixed in the cone structure; (b) tetraguanidinocalix[4]arene in 1,3-alternate; (c) a conformationally mobile.}
\end{figure}
octaguanidinocalix[8]arene. The AFM images of the starting relaxed plasmid (left) and the three calixplexes (right) are also reported.

These data, combined with transfection experiments on different cell lines, allowed us to point out a structure activity relationship extremely beneficial for the design of other families of calixarene-based gene-delivery systems with improved transfecting abilities. Although first defined for p-guanidinocalixarenes these rules are found to be valid, in general, also for other calixarene-based DNA binders. These amphiphilic calixarenes can therefore be grouped in three different categories of macrocycles: i) conformationally mobile, ii) blocked in the 1,3-alternate structure, and iii) fixed in the cone structure. The high number of anion binding groups (high valency) present in calix[6]- (15f) and calix[8]arene (15g) and the complete flexibility usually give rise to rather efficient DNA binders, able to link several different DNA molecules and thus originating extremely large gorgone-like aggregates (Figure 10c) not suitable for transfection even in the presence of helper lipids such as DOPE (dioleoyl-sn-phosphatidylethanolamine). These helper lipids are generally phospholipids not able alone to compact and transfect DNA, but effective in increasing the lipophilicity of the complexes between DNA and carriers, thus improving the transfection potency. With bolaamphiphilic calix[4]arenes fixed in the 1,3-alternate structure (15d) or conformationally mobile derivatives (15e) adopting a 1,3-alternate structure in aqueous solution, condensation of single DNA molecules takes place and originates aggregates of proper dimensions for transfection (Figure 10b). However, condensation is mainly driven only by charge-charge interactions and thus results to be not very efficient. For these reasons, it usually requires higher calixarene concentrations. The addition of DOPE, improving the overall lipophilicity of the calixplexes (lipoplexes made of calixarenes) triggers the cell transfection mediated by these bolaamphiphiles. Finally, in the last category might be grouped all those facial amphiphiles possessing a clear separation between the region bearing hydrophilic DNA binding groups and the lipophilic region (calixarene skeleton plus hydrocarbon chains). Usually such compounds are in a fixed cone structure (e.g. 15a-c), and present the polar head groups more or less fixed on a single face of the macrocyclic structure and therefore directed towards a defined region of the space. Quite important to preserve a correct amphiphilic character is a correct balance between hydrophilic and lipophilic character of the amphiphile, that can be properly programmed by tuning the length of the linear alkyl chains linked to the macrocycle. The facial amphiphiles fixed in the cone structure strongly bind to the DNA
filaments through multivalent interactions, neutralizing the DNA charges. These electrostatic and H-bond interactions thus coagulate calixarenes on the phosphoribose backbone increasing the local concentration of amphiphiles to a critical level which triggers hydrophobic interactions among the lipophilic aromatic and alkyl tails. The combined action of these hydrophobic and polar interactions generates strong and compact condensates containing a single double-stranded DNA and of the proper dimensions to allow cell transfection (Figure 10a). Rather efficient condensation takes place even when short alkyl chains (prooxy groups in 15a) are present on the calixarene structure although also in this case transfection is observed only upon incubation of the carrier and DNA with the helper lipid DOPE. AFM resulted therefore a rather valuable tool to observe DNA condensation and predict if the dimensions of the aggregates are suitable for transfection, even though the formation of these aggregates does not necessary implies that transfection can take place, since a certain degree of lipophilicity is also needed. Hexyl or octyl chains are usually enough to create a compact aggregate of proper lipophilicity, while the use of longer linear alkyl groups might even be undesirable since it increases the tendency to self-aggregation of the carrier with formation of SLNs or might result in highly cytotoxic vectors.

Thanks to these guidelines, more efficient and less cytotoxic systems could be obtained by inverting the polarity of the facial amphiphile that is by introducing guanidinium groups at the lower rim (16) instead than at the upper rim. Even if linear alkyl chains are absent, this compound is able to condense DNA, form tight aggregates through the interaction of unsubstituted aromatic rings and transfect cells.\textsuperscript{39,70} Quite interestingly, however, by far the most efficient compounds for cell transfection are the upper rim functionalized lysino- 17b and especially the arginino-calix[4]arene 17a.\textsuperscript{43} Not only they represent the best calixarene-based gene-delivery systems reported so far, but it was demonstrated that they are even much better that some of the most efficient commercially available transfecting agents such as Jet-PEI and LTX. Although polyarginino based amphiphiles already found
important applications as gene delivery systems, the facial calixarene amphiphile 17a presents several unprecedented characteristics. It is characterized by a rather high concentration of arginine moieties clustered at the upper rim that not only ensure a tight binding to DNA but, by interacting with negatively charged glycosaminoglycans of the extracellular matrix, might trigger the signal leading to endocytosis mechanism. Moreover, in compound 17a a fairly low (1:1) arginines to lipophilic tails ratio is necessary to ensure high activity, while in classical polyarginines up to 4-8 amino acids per lipophilic tail are needed. Contrary to the head-to-tail disposition of arginines in classical polyarginine carriers, here we have a parallel disposition of Arg units that, from one side ensures a more conformationally fixed and convergent array of positive groups and, from the other side, leaves all the α-amino groups free to interact with the target nucleic acid or, thanks to the so-called proton sponge effect, to favour the protection of the vector–DNA complex from the lysosomal degradation and to facilitate the release of DNA from the endosomes into the cytosol.

Figure 11: Different proposed mechanism of DNA compactation for calixarenes 17a and 18. (a) DNA binding followed by aggregation of the facial calixarene amphiphile 17a; on the right, the snake-like ultra-thin structure evidenced by a TEM image is reported. (b) The self-aggregation of the amphiphile 18 followed by DNA binding (hierarchical self-assembly).

Finally, two different mechanisms of DNA compactation by calixarene amphiphiles seem to be operative. In the case of compound 18, it could be demonstrated that a hierarchical self-aggregation mechanism takes place (Figure 11b). The carriers first self-assemble into small micelles and then these aggregates bind to DNA. However, with other derivatives, endowed with a reduced tendency to self-aggregate, free amphiphiles or smaller oligomers in equilibrium with the larger aggregates, progressively clot onto the DNA filaments (Figure 11a). When a critical local concentration is reached, the lipophilic
groups of the carriers start to attract each to the other bringing to the compaction of the DNA structure. This type of mechanism is also supported by a TEM micrograph image (Figure 11a) corresponding to the nanocomplexes formulated with compound 17a. A snake-like ultra-thin structure is evidenced where darker regions corresponding to the DNA filaments are alternated with lighter regions occupied by tail-to-tail double layers of calixarenes directing the arginine groups towards the phosphoribose backbone.

Besides nucleic acids, other phosphate containing biomacromolecules were identified as important targets of calixarene ligands. With the idea to mimic the amphiphilic structure of bactericidal peptides such as βpep peptides and dodecapeptide SC4, Mayo and coll. studied a series of cone amphiphilic calixarenes (similar to those above reported for DNA binding) in the binding to lipopolysaccharide (LPS) endotoxins produced by Gram-negative bacteria. These toxins are slowly released from bacterial membranes and induce an inflammatory response involving different proteins such as TLR4, CD14 and MD2. These processes promote a cascade of events associated with bacterial infections that might even bring to sepsis and septic shock. LPSs are rather variable in structure depending from the different bacteria, but possess a conserved region known as the Lipid A, a disaccharide structure bringing two negatively charged phosphate groups and lipophilic tails formed by modified fatty acid residues. On the other side, most of the bactericidal peptides, have an amphiphilic β-sheet or helix-forming structure that seems to be responsible for their activity. Their highly hydrophilic cationic face, characterised by the presence of Lys and Arg residues, is able to interact with the anionic region of Lipid A, while the highly lipophilic face exposing the Trp, Phe, Leu and Ile side chains generates hydrophobic interactions with the fatty acid chains of Lipid A. Twenty-three topomimetic calix[4]arenes were tested in vitro for their ability to neutralize LPS from different Gram-negative bacteria. The most active compounds (19-20), showing IC_{50} values in the nanomolar range, are all present in the cone structure. Some of them were also tested in vivo in mice administered with a lethal dose of LPS, and demonstrated a good protection efficacy having a 25-100% survival (20c being the best), depending on the microbial origin of LPS. Binding to Lipid A, with a possible 1:1 stoichiometry, was also suggested by NMR binding studies which evidenced strong electrostatic interactions between the ammonium and phosphate groups.
All these studies therefore suggest that these amphiphilic calix[4]arene ligands, exposing positively charged groups (preferably guanidinium or primary ammonium) at the lower rim and alkyl chains or the calixarene aromatic nuclei (upper rim), might mimic a small portion of peptide α-helix or β-sheet of the antibacterial peptides. Switching the phobicity, as in calixarenes with hydrophilic groups at the upper rim and lipophilic alkyl chains at the lower, causes a substantial inactivity in LPS neutralization. However, although it could be clearly evidenced the ability of these calixarenes to bind to LPS, it still remains to be clarified their protection mechanism in vivo where interferences with the cell receptors might also be possible.

In addition to the previously described recognition of phosphate containing substrates (NAs and LPS), another very important supramolecular function widely explored in this context is the cleavage of the phosphodiester bonds in relevant biomolecules (di-, oligo-nucleotides and ATP). Since several years cone calix[4]arene has been, in fact, widely used as platform also for the preparation of supramolecular catalysts able to cleave
carboxylic or phosphoric ester bonds.\textsuperscript{76,77} The general approach used consists in the functionalization with two or more catalytically active moieties. In particular, the presence of metal ions bound to the upper rim of the macrocycle through the positioning of the chelating moieties in well-defined geometries and at a more or less fixed distances imposed by the cone geometry, often ensures a high degree of cooperativity that highly increases the rate of ester bond cleavage compared with the background (uncatalyzed) reaction. Several metal ion and binding units were indeed used in the past\textsuperscript{76} but most of the generated catalysts only works in alcoholic or hydroalcoholic solutions because of the higher catalyst solubility and, especially, stability of the metal complexes in these media. More recently, however, the introduction of 1,5,9-triazacyclododecane ([12]aneN\textsubscript{3}) units at the upper rim of tetraethoxyethoxy calix[4]arenes made possible the preparation of Cu(II) dinuclear (\textit{21-Cu}\textsubscript{2} and \textit{22-Cu}\textsubscript{2}) or trinuclear (\textit{23-Cu}\textsubscript{3}) complexes with high solubility and stability to allow their studies as artificial metallonucleases in water.\textsuperscript{78,79}

![Chemical structures](image)

The polar ethoxyethyl groups at the lower rim not only fix the calixarene in the cone structure and ensure solubility in water, but also avoid self-aggregation phenomena so that such complexes can be reasonably well considered present as monomers in aqueous solution. The remarkable stability of the [12]aneN\textsubscript{3} Cu(II) complex (Log $K_{ass}$ = 12.6, at 25°C)\textsuperscript{80} also allows to study the catalytic activity of complexes \textit{21-Cu}\textsubscript{2}, \textit{22-Cu}\textsubscript{2} and \textit{23-Cu}\textsubscript{3} even at less than $10^{-3}$ M concentration. Quite interestingly, it has been observed that, in
the cleavage of the model substrate HPNP (2-hydroxypropyl p-nitrophenyl phosphate) in water at pH = 7 (T = 25 °C), the catalytic efficiency of these Cu(II) complexes (0.2 mM in buffer) is strictly dependent from the relative position of the metal centres at the upper rim. When the two copper ions are bound in 1,2-vicinal position (catalyst 22-Cu$_2$) a rate enhancement of 1090-fold higher than that of the reaction carried out in the presence of the buffer alone (background), is observed. This indicates a high cooperation between the metal ions that is not present in the distal 1,3-difunctionalized regioisomeric catalyst (21-Cu$_2$) which shows only a 40-fold rate enhancement compared to the background. Kinetic studies could also point out the presence of a moderately stable complex between 22-Cu$_2$ and HPNP ($K_{ass} = 500 \text{ M}^{-1}$) so that the model of substrate activation reported in Figure 12a could be proposed. The substrate electrophilicity is activated via two Lewis acid interactions with the copper ions present in 1,2-positions at the upper rim while an hydroxy group, coordinated to one of the metal ion (general base), deprotonates the β-OH group of HPNP favouring the intramolecular transesterification.\textsuperscript{78}

![Figure 12: Proposed mode of a) HPNP transesterification, catalyzed by 22-Cu$_2$; b) RNA-dinucleotide transesterification, catalyzed by 25-Cu.](image)

Compared with 22-Cu$_2$, the presence of an additional Cu(II) metal ion coordinated to the calixarene like in 23-Cu$_3$ does not cause any further acceleration in the cleavage of HPNP, so that a dimetallic catalysis is suggested to be operative also in this case, with the third metal ion acting as a more or less innocent spectator. Remarkably, the same catalysts result to be active also in the cleavage of diribonucleoside 3',5'-monophosphates with rate enhancements up to $10^5$ (at T = 50 °C) and a similar activity of 22-Cu$_2$ and 23-Cu$_3$. Substrate selectivity in the cleavage of different dinucleosides is, in some cases, interesting with a preference for UpU and UpG (up to 39 folds) over GpU, GpG, ApG, CpC, CpG, GpA and being CpA cleaved only at a very low rate. A rather different frame
emerged from the studies on the cleavage of oligomeric ribonucleotides possibly also due to the presence of an additional phosphate group in the terminal 5' position. With the hexa- or epta-ribonucleotides tested, no clear indication of a preferred geometrical disposition of the catalytic groups appears although extended degrees of cooperation between two metal ions and accelerations (up to $5 \times 10^5$ at $T = 50 \, ^\circ\text{C}$) were observed. With some oligonucleotides the 1,2-vicinal $22\text{-Cu}_2$ is better than the 1,3-distal $21\text{-Cu}_2$ complex, but in other cases the opposite is true. In many cases, however, the trimetallic $23\text{-Cu}_3$ complex behaves similarly to the dimetallic catalysts, once again excluding the involvement of the third metal ion in the catalytic mechanism. In sharp contrast with the diribonucleoside cleavage where a selectivity for UpU and UpG bonds was observed, in the tested oligonucleotides the phosphodiester bond best cleaved is the CpA. A fair decrease of the observed catalytic rate constants with the distance of the cleaved bond from the doubly charged phosphate group in terminal 5' position finally also suggests a possible role of this dianionic phosphate as a primary anchoring site for the catalysts.

More recently, the catalytic activity of p-guanidinocalix[4]arenes 24 was tested in the cleavage of HPNP. In slightly basic conditions (DMSO/H$_2$O = 80:20) where a guanidine-guanidinium pair is present, the neutral guanidine is acting as general base while the positively charged guanidinium as electrophilic activator. In this case, the 1,3-distal guanidinocalixarene 24a is slightly more efficient than its 1,2-vicinal isomer 24b. The tetrafunctionalized catalyst 24c, when monodeprotonated at pH 9.8, cleaves ATP into ADP and phosphate, at least three orders of magnitude faster than the background hydrolysis. These observations prompted us to combine a guanidinium group and a copper ion held in place by a triazamacrocycle (1,4,7-triazacyclononane, [9]aneN$_3$) in 1,3-positions of the calix[4]arene scaffold (25-Cu). Compared to the background hydrolysis, the reactions at 1 mM concentration of 25-Cu are $6 \times 10^5$-fold and $10^7$-fold faster for HPNP and for the most
favorable RNA dinucleotides, respectively. Kinetic measurements suggest the operation of a general-base/general-acid mechanism (Figure 12b) and the binding of the pentavalent phosphorane dianion transition state by the guanidinium group.\textsuperscript{83}

6. Exploiting the amphiphilic character for biomacromolecule sensing and targeted drug-delivery

The peculiar macrocyclic structures of calixarenes amphiphiles\textsuperscript{17} combine the possibility to organize cavities for drug-delivery\textsuperscript{84} with that to properly arrange, even through a noncovalent approach, ligating groups for recognition of biomacromolecules. This attractive opportunity, prompted several scientists to set up new materials and devices to be potentially used in bio-nanotechnologies and nanomedicine.\textsuperscript{16,85} In particular, the calixarene amphiphilic character allows to easily embed these powerful ligands in different types of monolayers (Langmuir films, self-assembled monolayers on metal surfaces) or to organise them in different kinds of self-aggregates such as micelles, SLNs or vesicles and liposomes. Depending on the hydrophilicity of the polar groups implanted on the calixarene backbone, usually at the upper rim, the lipophilicity should be properly balanced by the insertion of linear alkyl groups of proper length (C3-C8) at the lower rim, to deserve the proper stability for the self-assembly. Longer alkyl groups usually end-up in extremely low solubility of the amphiphiles and in the aggregation in SLNs.\textsuperscript{86} Most commonly, thanks to their wedge structures, cone derivatives are used, but recently it has been shown that also 1,3-alternate bolaamphiphiles can give rise to rather stable aggregates spanning a phospholipid bi-layer.\textsuperscript{40} We already extensively reported in a previous section on the use of calixarene amphiphiles for DNA binding and gene-delivery which clearly represents a very successful application where the self-assembly of calixarene ligands might be exploited for nanomedicine applications. However, other important examples were reported where calixarene amphiphiles were used to devise biomacromolecule sensors or targeted drug-delivery systems.

Langmuir films embedded with proper calixarene amphiphiles were, for example, used to set up efficient devices able to sense proteins and nucleic acids in the underneath aqueous solution. Schrader and coll.\textsuperscript{87,88} by observing changes in the pressure/area diagrams of the monolayers of stearic acid containing the charged cone calix[4]arenes 26, 27 and 28a turned out to be able to reveal proteins with a surface charge complementary to that of the macrocycles, even at $10^{-5}$ M concentration. Monolayers containing the
negatively charged tetraphosphonate calix[4]arene 26, giving pronounced shifts towards larger surface area values, were used to sense the arginine rich cyt c, lysine-rich histone 1b and chymotrypsin which have an isoelectric point (pI) higher than physiological pH. The same system was also able to recognize proteins with pI close to 7 but having well separated anionic and cationic domains. Although these recognition phenomena might take place also in solution upon leaching of the ligand in the aqueous phase, the neutralization of the proteins upon binding prompts the less polar protein-calixarenes complex to be reincorporated in the lipophilic layer resulting in significant increases in the surface areas that allow the protein detection. Also in these cases, the role of the multivalent calixarene ligand appears crucial, since similar stearic acid monolayers embedding anionic amphiphilic mono-phosphonates showed only rather minor effects.

Similarly, acidic proteins such as the acyl carrier protein (pI = 4) are detected by embedding the tetraammonium calixarene 27 in the layer, while the partially protonated tetraniline 28a allows the identification of both negatively and positively charged proteins. Quite interestingly, monolayers of dimyristoylphosphatidylcholine and the described calixarenes but doped with chromatic polydiacetylene enable the direct detection of the proteins by “naked eye”. Shahgaldian and coll. could prepare stable Langmuir monolayers using similar tetraminocalixarenes 28b but in absence of a cosurfactant. The partially protonated macrocycles in the layer thus interact with DNA in the subphase causing an expansion of the monolayers and a phase transition from a liquid-condensed to a liquid-expanded phase that can also be used to detect the presence of the nucleic acid. Interestingly, similar monolayers but formed of p-guanidinododecyloxy-calix[4]arene 29
showed isotherms with significant differences that depend on the duplex DNA sequence present in the subphases. The interactions with poly(AT) give increases of the surface area that are significantly more pronounced than those for the interactions with poly(GC) or with the random sequence DNA. The solvent displacement method causes the formation of SLNs of 29 that resulted composed of particles of 190 nm in diameter and with positive ζ-potential. Also with these SLNs a slightly higher preference for a DNA consisting in a A_{30}T_{30} oligonucleotide over a G_{30}C_{30} oligonucleotide was observed. According to the authors, while these SLNs bind to the minor groove of a A_{30}T_{30} oligonucleotide, with a G_{30}C_{30} oligonucleotide the binding proceeds via major groove. The possibility to use calixarene aggregates to transport drugs even into cells has also been reported. Lee and coll. found that the amphiphiles 30a,b can aggregate into vesicles able to include calcein, used as fluorescent model of a drug, and to release it upon drop of the pH of the solution to 5. Zhou and coll. showed that 31 is able to form complexes with Chlorin e6, a model of photosensitizers, indicating that this system may potentially be useful in photodynamic therapies. This host-guest complex self-assemblies in micelles (ca. 140 nm in diameter), showing high biocompatibility due also to the presence of the PEG chains and low cytotoxicity. Interestingly, it could also be
demonstrated that the calixarene carriers highly facilitate the photosensitizer uptake by HeLa cells. Weeden et al.\textsuperscript{95} prepared and characterized nanoparticles formed by p-aminocalix[4]tetrachydroxyloxy \textit{28c} and paclitaxel and stabilized by polyvinyl alcohol, showing that the morphology of these particles is dependent even on small pH variations (from 4 to 6) of the solution. Quite interestingly, Liu and coll.\textsuperscript{96} exploited a noncovalent approach to generate supramolecular binary vesicles. Although the p-sulfonatocalix[4]arene \textit{3a} has no tendency to self-aggregate in aqueous solutions, by the inclusion of the N-methylpyridinium residue of \textit{32}, an amphiphilic 1:1 complex was formed that self-assemblies in vesicles and efficiently includes the anticancer Doxorubicin hydrochloride. These vesicles can be disrupted thanks to multiple external stimuli such as temperature, reduction of \textit{32} or sequestration of the viologen by complexation of its alkyl chains in \(\alpha\)-cyclodextrins. Experiments with cancer cells also verified that the controlled release of Doxorubicin does not affect the therapeutic properties of the drug. With the aim to increase the possibility of interactions with the phospholipid head groups of the cell membrane, Fujii et al.\textsuperscript{97} proposed the use of calix[4]arenes \textit{33} in the cone structure and bearing choline phosphate groups (CP) at the upper rim. These micelle-generating amphiphiles were demonstrated to interact quite favourably with the phosphoryl choline (PC) groups of phospholipids, the major component of cell membranes, \textit{via} multiple CP-PC complementary interactions due to the formation of quadrupoles between the quaternary ammonium groups and the phosphate anions.

All these studies, however, although proposing efficient drug-delivery systems represent examples of passive drug-delivery where no programmed and specific interactions with biomacromolecules present on the cell membrane were devised and where selectivity of delivery is simply entrusted to the ability of the assembled carrier and cargo to preferentially accumulate, for example, in cancer tissues than in normal ones through the so-called enhanced permeability and retention effect. On the other hand, other examples were recently published were the possibility to have targeted drug-delivery was proposed. Liu and coll.\textsuperscript{98} found that p-sulfonatocalix[4]arenes \textit{34a,b} functionalized at the lower rim with butyl or hexyl chains, can co-assemble in 5-10\% mixture with DPPC (1,2-dihexadecanoyl-\textit{sn}-glycero-3-phosphocholine) giving liposomes of roughly 100nm in diameter. The presence of lipophilic cavities provided by the cone calixarenes embedded in the phospholipid bilayers (Figure 13) allows the noncovalent functionalization of the liposomes through host-guest interactions. The surface modification was achieved by incubation with a FITC-conjugated pyridinium (FITCPy) or a biotinylated pyridinium (BtPy)
and occurs thanks to the pyridinium inclusion in the macrocyclic cavity ($K_{ass} = 7.4 \times 10^4 \text{ M}^{-1}$). While the former guest is used as fluorescent probe, the latter conjugate is aimed at conferring targeting properties. The MCF7 cancer cells used in \textit{in vitro} experiments, in fact, are cells of human breast adenocarcinoma which overexpress biotin receptors on their surface. Quite interestingly, only those liposomes also decorated with BtPy are able to transfer the fluorescence of FITC into the cells, while experiments driven with nonbiotinylated liposomes or with cells previously saturated with biotin give much less fluorescent cells.

Figure 13: Incorporation of tetraalkoxy-p-sulfonatocalix[4]arenes 34 in a phospholipid double layer of liposomes. Inclusion of the piridinium group into the calixarene cavity allows the noncovalent functionalization of the liposome surface for targeted delivery and fluorescence imaging.

In a parallel study, the same authors found that 34b can self-assemble in small micelles in the absence of other surfactants, while it can give co-assembly into vesicles by using proper cationic amphphilic anticancer drugs such as Mitoxantrone hydrochloride (MTZ-HCl) or Irinotecan hydrochloride (IRC-HCl) at an optimum molar ratio (drug:calix) of 0.5 and 0.8, respectively.99
**Figure 14:** Co-assembly of tetrahexyloxy-p-sulfonatocalix[4]arene 34b and cationic amphiphilic anticancer drugs (Mitoxantrone hydrochloride MTZ-HCl) into a double layer of micelles. Noncovalent surface modification with a pyridinium functionalized biotin allows targeted drug-delivery.

This feature ensures to this “drug chaperone” (Figure 14) a high drug loading with a minimum nondrug component and a remarkable protection of the drug from premature degradation. Moreover, also in this case a targeted delivery to the MCF-7 cell line could be demonstrated by decoration of the exterior chaperone surface with BtPy or hyaluronic acid functionalized with pyridinium ion (HAPy). The targeting activity of the BtPy or HAPy functionalized vesicles was attributed to a strong biotin or hyaluronic acid binding to their corresponding cell receptors evolving in an efficient internalization *via* receptor-mediated endocytosis.

Our group has proposed to exploit carbohydrate-protein interactions and the multivalent effect (or glycoside cluster effect) to provide the calixarene aggregates with targeting abilities. As previously outlined, in fact, cells and especially tumour cells overexpress lectins on their surface. It appears therefore rather appealing the possibility to functionalize self-assembled nanoparticles with specific carbohydrates able to selectively interact with lectins to obtain targeted drug-delivery. In a first study, we used calix[4]arene-β-cyclodextrin heterodimers 35a and 35b (Figure 15) to form nanospheres (22-25 nm diameter) made up of 17-25 molecules. The calixarenes, blocked in the cone structure and functionalized with hexyl or dodecyl chains at the lower rim, tightly self-assemble projecting the hydrophilic β-cyclodextrin units towards the exterior bulk water solution. The release profiles of docetaxel from the loaded nanospheres suggest that part of the drug molecules are hosted in the cyclodextrins cavity and are quickly released, while a slow release kinetics is observed for those drug molecules hosted in the lipophilic calixarene core.
Figure 15: Self-assembly of calix[4]arene-β-CD heterodimers 35 showing possibility of loading with a lipophilic antimitotic drug (DXT) and noncovalent functionalization with mannosyl dendrons for targeted drug-delivery.

These nanospheres can be, moreover, noncovalently functionalized with a trivalent mannosyl dendron taking advantage of the particularly high binding of adamantane for the cyclodextrin cavity (Figure 15). A strong affinity of the mannose decorated nanospheres for human macrophage mannose receptor (h-MMR) could also be achieved already at 30% cyclodextrin functionalization thanks to specific multivalent mannose-protein interactions.

Glycocalixarenes, thanks to their remarkable ability to recognize lectins, clearly represent an obvious option to be employed to specifically target tumour cells or tissues. We have interestingly found that glycocalixarenes fixed in the cone structure (Figure 16) can be conveniently exploited to noncovalently functionalize dodecanthiol-stabilized gold nanoparticles (AuNPs).
Figure 16: Enhanced targeting efficiency of the mannose receptors by AuNPs functionalized by mannosylated calixarene 36 (clustered multivalent interactions, right) compared to AuNPs covered by monovalent mannosyl ligand 37 (simple multivalent interaction, left)

By mixing a chloroform and an aqueous layer containing the AuNPs and the mannosylthioureidocalix[4]arene 36, the AuNPs adsorb glycocluster 36, become water dispersible and are transferred to the aqueous layer. This protocol therefore represents a facile, straightforward and robust method to functionalize AuNPs which exploits stable hydrophobic interactions between the dodecanthiol layer and mainly the calixarene backbone since only short propyl chains are present at the lower rim. These mannosyl-coated nanoparticles exhibit remarkable "stealth" properties. When dipped in culture media, they show a rather low tendency to be coated by a protein corona that often affects the colloidal stability and uptake by cells. Experiments with HeLa cells, a mannose receptor expressing cell line, indicate that the NP uptake is mediated by the monosaccharide and that the mannosylcalixarene functionalized AuNPs are much better uptaken than AuNPs covered by an equimolar amount in sugar of monovalent mannosyl ligand 37 (Figure 16). This also demonstrates that the AuNP uptake is significantly dependent by the topology of presentation of the mannosyl units on the NP and that the presence of the calixarene units ensures a high local concentration of sugar units (clustered multivalency) rather beneficial in the interaction with the mannose receptors on the cell membrane. As showed with all these examples, cone-shaped calixarenes represent well preorganized prototypes of facial amphiphiles which display a clear attitude to form micelles and to be included in lipid mono- and bilayers. Other calixarene structures however, may result very useful to be incorporated in nanoobjects. The alternate structures of calixarenes, for instance, when properly adorned at the periphery with
hydrophilic groups display a bolaamphiphile character.\textsuperscript{40} The functionalization of the 1,3-alternate calix[4]arene with long spacers terminating with glucose units, thus afforded the bolaamphiphile 38 which can be included in 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) double layer of liposomes (Figure 17).

![Figure 17](image)

**Figure 17**: Mixed DOPC/bolaamphiphile 38 liposomes able to efficiently interact with a lectin (ConA) and to be loaded with lipophilic or hydrophilic model drug molecules.

A 15 atom length spacer on these alternate structure is indeed enough to allow the bolaamphiphile to span the lipid bilayer and to project the sugar units into the aqueous phases. Therefore, not only the lipid-calixarene co-formulation (90/10) ensures a high rigidity and stability to the liposome, but represents a facile and robust methodology to thickly functionalize with sugar units the external liposome surface. It could be demonstrated that glucose functionalized 38/DOPC liposomes interact with Concanavalin A (ConA) through efficient and specific multivalent interactions. These results therefore pave the way to develop glycocalixarenes analogue to 38 and decorated with more complex saccharides for the preparation of stabilized liposomes loaded with proper cargo and able to target cells exposing specific carbohydrate receptors for targeted drug-delivery.

7. Conclusions and outlook

The knowledge on calixarene chemistry developed to such an extent in the latest 30 years that nowadays it is possible to clearly control the reactivity, conformation and structure of these macrocycle. It became therefore feasible to functionalize calixarenes with rather simple water solubilising groups (ammonium, sulfonate, phosphate etc...) or with much more sophisticated moieties such as amino acids and small peptides, mono- and oligosaccharides. The ligands thus obtained are often characterised by rather different
properties which are strictly dependent on the size, geometry of presentation of the polar groups and by an amphiphilic character that is function of a subtle balance between polarity of the head groups and lipophilicity of the tails/backbone. Chemists working in this field are starting to understand also the rules, which allow to design and synthesize ligands to target biomacromolecule. The state of the art, herein reported, often discloses a fundamental role played by the multivalency of such compounds and by the stereochemical presentation of the ligating groups exposed on the macrocyclic scaffold. The hybrid character, ensured by the presence of the hydrophobic cavity and of the lipophilic external backbone surface, often plays key roles especially in the recognition of amino acid residues in peptides or in controlling interactions with proteins. Quite interestingly, the self-assembly properties of facial- or bola-amphiphiles can be exploited to control gene condensation and internalization into cells or to build up large assemblies such as micelles and liposomes which demonstrated to be useful for drug-delivery and cell imaging. The covalent or noncovalent functionalization of the calixarenes embedded in the double layers of such assemblies, allows to target specific receptors present on the membrane of given cell-lines. All these exciting data seem therefore to indicate great potentiality of calixarene ligands in bioorganic chemistry with possible successful application in Bionanotechnology and Nanomedicine.

8. Acknowledgements

We thank the Italian Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR, PRIN project 2010JMAZML) and to EU-COST Actions CM1102 “MultiGlycoNano” and CM1005 “Supramolecular Chemistry in water”.

9. Notes and references


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