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Protein Surface Recognition by Calixarenes

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1. Introduction

Protein surface recognition by synthetic binders is a topic of great interest both from a Supramol Chem. and analytical application point of view.[1] Despite the pivotal role of protein–surface interactions in many fundamental processes of living cells, a directed targeting of protein surfaces by external agents remains a challenge because the programmed synthesis of molecules with a predetermined complementary pattern of solvent-exposed functional groups is still in its infancy.[2]

At the interface between biochemistry and Supramol Chem., multivalent interactions that are based on individually weak, noncovalent bonds and are thus generally reversible, play a decisive role in recognition, adhesion, and signal processes (Figure 1). In contrast to weak monovalent binding, multivalent interactions increase binding affinity and specificity on a molecular scale by just increasing the number of simultaneously and synergistically operating ligands and receptors. [3] The concept of multivalency has been utilized in recent years by supramolecular chemists to create well-defined structures and elicit a biological response, giving rise to new nanomaterials and molecular devices.[4]

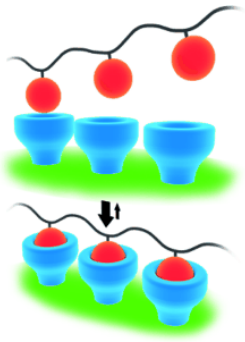


Figure 1. Multivalent complex formation.[3a]

Calixarenes, the cyclic oligomers obtained by condensation of phenols or resorcinols with aldehydes, are ideal scaffolds for the synthesis of multivalent ligands with unique properties.[5]

The calixarene core structure provides a very versatile template which allows building numerous structures with preorganized shape and programmed multivalent pattern of binding sites. It is easily accessible by conventional synthetic transformations, and can be functionalized at will on its upper and/or lower rim. Thus, functional variability and conformational stability conferred by the calixarene scaffolding have inspired chemists to design promising candidates for the study of noncovalent interactions involved in the molecular recognition of protein surfaces.[6]

The aim of this review is to summarize recently developed calixarene derivatives for protein surface recognition which are able to identify, inhibit, and separate specific proteins.

2. Protein Recognition

Protein-protein interactions (PPIs) play key roles in many cellular processes, including DNA replication, transcription and translation, signal transduction, and metabolic pathways.[7] Therefore, the development of artificial protein surface binders, which may deliberately interfere with these processes in a predictable way, is one of the most important and challenging topics for Supramol Chem. today.[8] The general principles in the design of a protein surface binding agent are usefully illustrated by the case of cytochrome-c (cyt-c) recognition, an ideal model protein with essential roles in electron-transfer and apoptosis pathways.[9]

Cyt-c is a hemoprotein consisting of a single polypeptide chain of 104 amino acid residues and covalently attached to an iron protoporphyrin IX prosthetic group (heme group) with a notably asymmetric distribution of surface charge.[10] Positively charged residues on the cyt-c (19 lysines and 2 arginines) are distributed over most of the protein surface including the heme edge region, in contrast with the backside of cyt-c to the heme group involving 12 glutamate and aspartate residues, which act to neutralize the backside cation charges (Figure 2).[11]

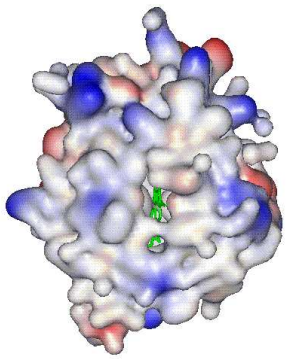


Figure 2. The charge distribution of cyt-c. Blue represents positively charged lysine and arginine. Red represents the negatively charged glutamate and aspartate. The heme is represented in green.[12]

Thus, the excess positive charge around the heme edge facilitates a strong interaction with negatively charged functionalities on an electrode surface.[13] In addition to electrostatic interactions, hydrophobic interactions operating over significantly shorter distances between nonpolar residues, rearrange both components for a more productive ET complex (Figure 3).[14]

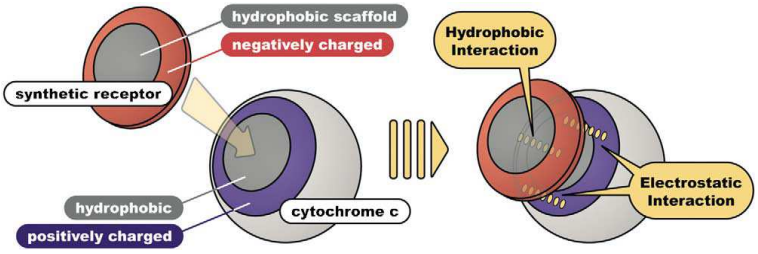
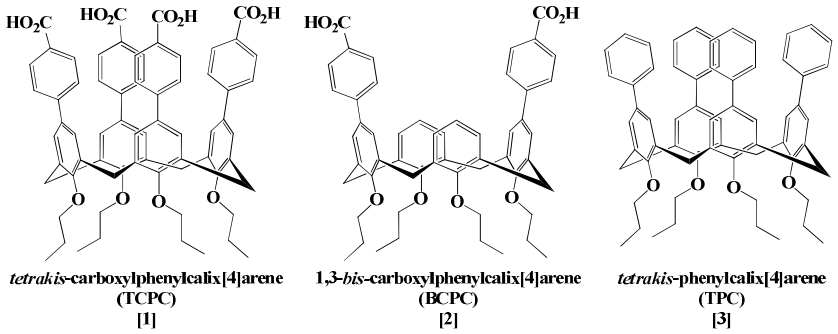


Figure 3. Schematic representation of cyt-c binding by synthetic receptors.[15]

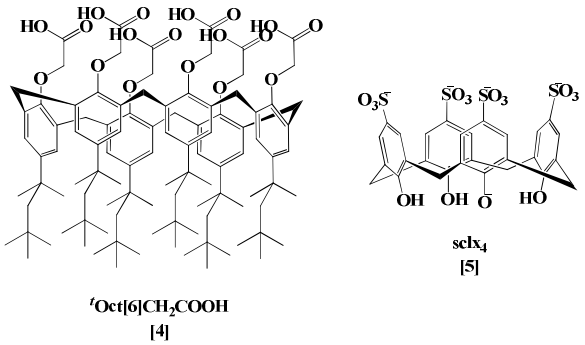
Various techniques and instruments such as circular dichroism spectroscopy, dynamic light scattering and Langmuir film balance have been used to study these electrostatic and hydrophobic interactions of proteins with artificial protein binding agents.[15] However, despite the excellent ability of the unpaired electrons in the heme group of cyt-c to quench fluorescence upon binding to a fluorogenic receptor, fluorescence-based methods have only recently been employed for cyt c recognition processes.[16] One of the most recent reports on this concept is based on a thorough investigation of the binding properties of carboxylphenyl-substituted calix[4]arene derivatives with cyt-c by fluorescence spectroscopy (Scheme 1).[17]



Scheme 1. The molecular structures of carboxylphenyl-substituted calix[4]arene-based fluorescent receptors.

The donor-acceptor biphenyl platform endows calix[4]arene derivatives with a strong fluorescence property,[18] and exhibits an efficient Förster resonance energy transfer based on the quenching of fluorescence upon binding to proteins, which makes it possible to utilize sensitive fluorescence methods to examine interactions of the calix[4]arenes with cyt-c. The remarkably high binding affinity of these calix[4]arene derivatives towards bovine heart cyt-c in dimethylformamide (DMF) in the strict order of TCPC > BCPC >> TPC reflects the prominent role of multiple carboxylate groups in providing anionic ligating sites for the cationic surface of cyt-c and suggests that the electrostatic force may be the predominant factor which drives these complexing process.

An elegant way to study the mechanisms by which proteins interact with the cell surface involves the use of lipid vesicles and supported bilayer lipid membranes (sBLM) that serve as convenient models of biomembranes.[19] In this context, the binding properties of cyt-c towards large unilamellar vesicles (LUV) composed of dimyristoylphosphatidylcholine (DMPC) or sBLMs, both modified with a highly sensitive calix[6]arene carboxylic acid derivative (^{t}Oct[6]CH$_2$COOH, Scheme 2) were explored and compared with both membrane models in the absence of this receptor.[20] Electrochemical impedance spectroscopy analyses of the binding process demonstrated that the main driving force in the interaction of cyt-c with the investigated membrane structures is of electrostatic nature and results from the negative charge at the membrane surface.



Scheme 2. The molecular structures of the highly sensitive calix[6]arene carboxylic acid derivative (^{t}Oct[6]CH$_2$COOH) and the water-soluble *p*-sulfonatocalix[4]arene (sclx$_4$).

In spite of the growing interest in protein–calixarene interactions, only limited structural information has been available to date. A crystal structure of the complex formed between the anionic water-soluble *p*-sulfonatocalix[4]arene (sclx$_4$, Scheme 2) and cyt-c is the most

prominent example in this regard.[21] The complex structure contains an asymmetric unit composed of two molecules of cyt-c referred to as chains A and B and three molecules of $sclx_4$. NMR titrations and X-ray crystallography both support the postulated three $sclx_4$ binding sites (A.Lys89, B.Lys4 and B.Lys22, Figure 4). All three binding sites involve a Lys side chain trapped inside the calixarene, with the sulfonate-bearing rim making at least one non-covalent bond with other polar groups on the protein. Taken together, these data indicate a dynamic complexation event in which $sclx_4$ explores a contiguous surface area on cyt-c, and adopts varying transient complex geometries on neighbouring exposed lysine residues.

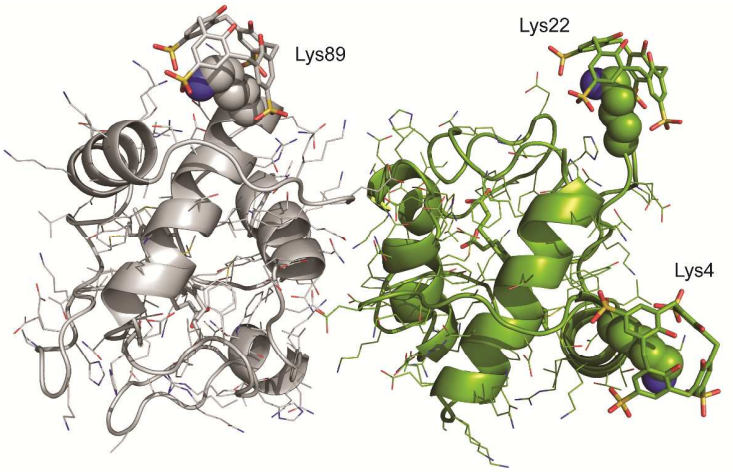
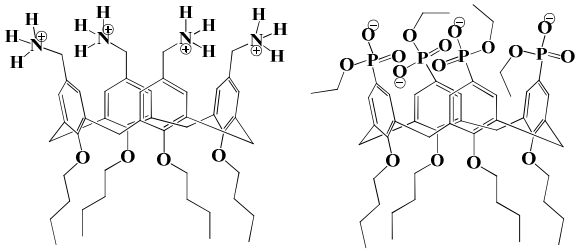


Figure 4. Crystal structure of the cyt c–$sclx_4$ complex. The asymmetric unit comprises two molecules of cytochrome c (chain A in grey and chain B in green) and three molecules of $sclx_4$. The $sclx_4$-bound Lys side chains are shown as CPK and labelled. Blue, N; red, O; yellow, S. Carbon atoms are shown in grey and green for the ligands interacting with chains A and B, respectively. Heme groups are shown as sticks.[21]

The above results confirm the profound importance of the distribution and organization of charges on a protein surface in determining protein function as well as interaction with other proteins and biomolecules, with consequences for complex stability and conformational changes.[22] A remarkable supramolecular characterization of protein surface charges has been achieved through the treatment of proteins with chromatic lipid/polydiacetylene (PDA) films with embedded charged calixarene-based ligands (Scheme 3).[23]



Scheme 3. Multiply charged calixarene-based ligands used to determine surface charges on proteins.

The glass-supported films comprising only lipids and PDA (negative net charge) undergo strong visible and quantifiable blue–red transformations induced by PDA interaction with positively charged proteins. In contrast, complexes formed between charged-proteins and oppositely charged calixarene amphiphiles are immobilized in the neutral lipid region of in the mixed films, and attenuate or totally counteract color changes on the film surface (Figure 5).

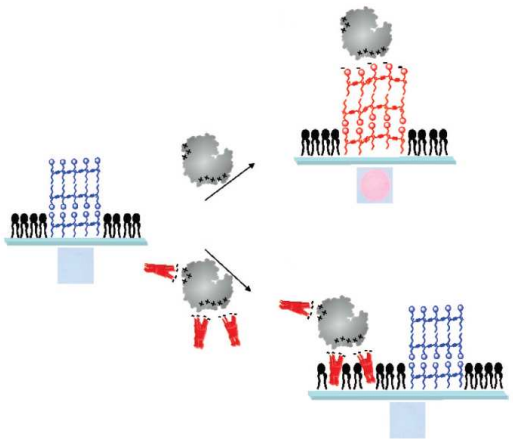
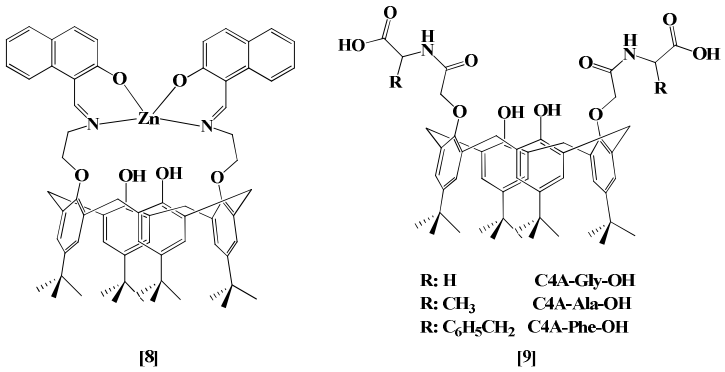


Figure 5. Supramolecular concept for protein surface-charge analysis with a colorimetric assay based on PDA/lipid vesicles and amphiphilic calixarenes.[23]

A further important goal in protein surface recognition processes is the molecular recognition of amino acids present in the proteins.[24] In this respect, intensive research has been focused on the design and synthesis of calix[n]arene derivatives, especially derivatives of calix[4]arenes with specific properties and functions revealing their affinity and selectivity towards biologically relevant molecules. A lower rim naphthylidene conjugate of calix[4]arene [ZnL] represents an interesting attempt to recognize Asp, Cys, His, and Glu

residues in α-helical proteins (bovine serum albumin and human serum albumin) as well as β-sheet proteins (jacalin and peanut agglutinin) by fluorescence quenching from among the naturally occurring amino acids owing to the protonation and chelating ability of the amino acid and the π-π interaction ability of the side chain of the amino acid with [ZnL] (Scheme 4).[25]



Scheme 4. Zn(II) complex of lower rim naphthylidene conjugate of calix[4]arene [ZnL] and the lower rim 1,3-diamido-calix[4]arene conjugates of amino acids.

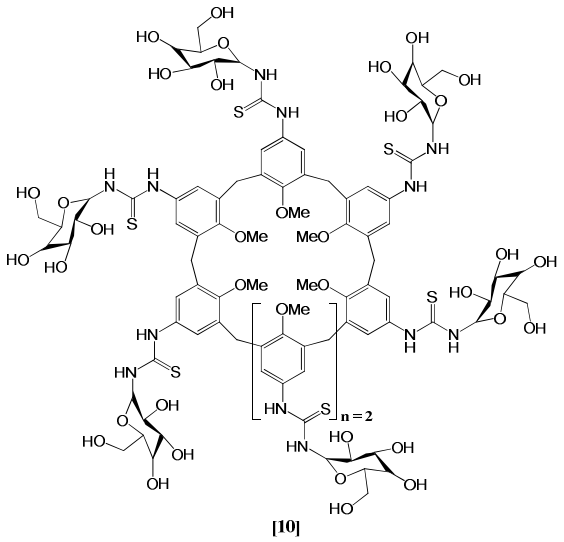
Proteins containing amino acids possessing side chain –COOH functionality are prime interest in biological systems.[26] The lower rim 1,3-diamido conjugates of calix[4]arene have been shown to exhibit recognition toward Asp/Glu residues present in α-helical proteins, viz., bovine serum albumin (BSA), human serum albumin (HAS), and α-lactalbumin by switch-on fluorescence in aqueous acetonitrile and methanol solutions when compared to the control molecules via forming a 1:1 complex (Scheme 4).[27] The calix[4]arene platform, amido arm, and terminal –COOH moiety are required for selective recognition of amino acids and peptides possessing side chain –COOH moiety.

Another important supramolecular challenge is the specific recognition of aromatic amino acids, peptides and proteins by synthetic organic molecules.[28] Recently, the host-guest interaction of $sclx_4$ with tryptophan (TRP) residues of BSA and ovalbumin has been studied using UV–Vis absorption, fluorescence, and theoretical methods.[29] Ovalbumin exhibits stronger binding with $sclx_4$ than BSA, due to the projection of three TRP moieties outside the protein.

Apart from PPIs, protein-carbohydrate interactions (PCIs) between oligosaccharides and lectins are also involved in many biologically important processes. Among others, clearance

of glycoproteins from circulatory system, adhesion of infectious agents to host cells, leukocyte activity in inflammatory sites, cell interactions with the immune system, malignancy and metastasis, control of enzymatic activity, endocytosis, cell migration, routing, adhesion and communication, sperm-egg communication and targeting all involve specific carbohydrate-protein recognition.[30] Similar interactions are believed to be also involved in various protein misfolding diseases such as type 2 diabetes mellitus, Alzheimer's disease (AD), Creutzfeldt-Jacob disease (CJD) and prion diseases (PD).[31] This small collection of typical examples demonstrates the enormous potential of external control over CPIs. Recently, synthetic glycocalixarenes have been synthesized, i. e., macrocycles belonging to the calixarene family which bear at least one carbohydrate unit covalently appended to their calixarene core.[32]

In this respect, a glucosylthioureidocalix[8]arene derivative was designed for multivalent recognition of the jack bean lectin Concanavalin A (ConA, Scheme 5).[33]



[10]

Scheme 5. Glucosylthioureidocalix[8]arene derivative for multivalent ConA recognition.

The ligands first aggregate in small nanoparticles (3–10 nm large); these multivalent species then interact with ConA leading to powerful agglutination due to the formation of large supramolecular aggregates which progressively evolve towards precipitation because of the extensive lectin cross-linking (Figure 6).[33]

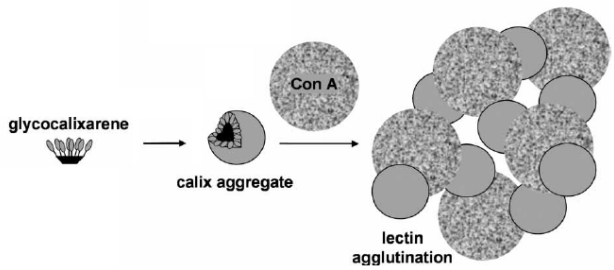
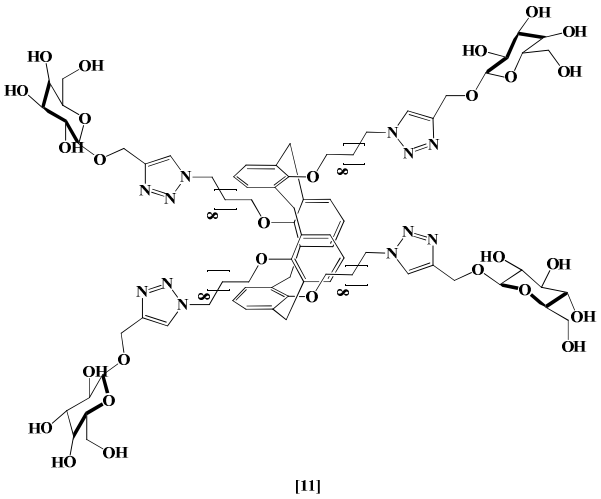


Figure 6. The aggregation process involving glucosylthioureidocalix[8]arene derivative and ConA.[33]

Another fascinating example of multivalent ConA recognition is the synthesis and incorporation of the multivalent 1,3-alternate glucocalixarene **11** in a bolaamphiphilic (BA) shape into liposome formulations of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Scheme 6).[34]



[11]

Scheme 6. Tetravalent glucocalix[4]arene **11**

The presence of the new membrane-spanning glucocalixarene BA **11** in the DOPC lipid bilayer rigidifies the membrane, and thus reduces the permeability of the liposomes and hence the orientational freedom of an embedded fluorescent probe. The multivalent interaction of the novel glucocalixarene BA **11** with the tetrameric glucose binding protein ConA was investigated by fluorescence techniques, exploiting fluoresceine-labelled ConA (FITC-ConA), and turbidimetry experiments. As a potential application, the present study provides a new strategy to functionalize liposomes with saccharides, exploiting multivalent glycosylated ligands able to target specific lectins.

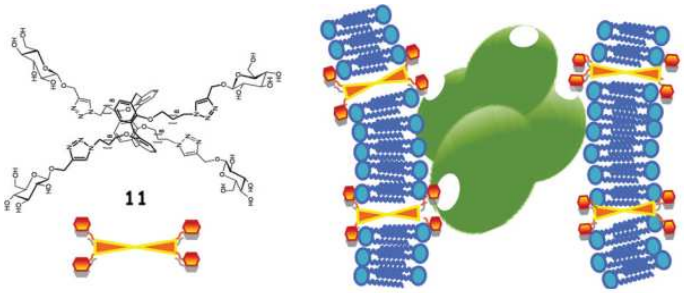
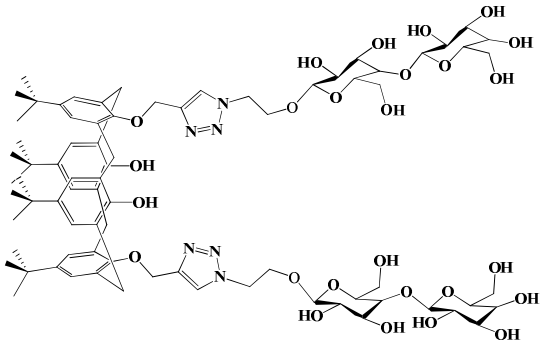


Figure 7. Interaction of the glucosyl units of bolaamphiphile **11**, embedded in a DOPC bilayer, with an external teravalent lectin.[34]

Another example in this series is a lactosyl-appended calix[4]arene endowed with triazole functionality at the lower rim. It was synthesized by 1,3 dipolar cycloaddition of lactosyl azide and calix[4]arene alkyne (**12**), and behaved as moderately potent jacalin receptor.[35] The interaction of these conjugates with jacalin is largely attributed to the interactions of the glycoconjugates with the side chain of Trp moieties present on the protein surface (Scheme 7). Considerable conformational changes occurred during the interaction of **12** with Jacalin, which were inferred from large molar ellipticity changes detected by circular dichroism spectroscopy (CD).



[12]

Scheme 7. Calix[4]arene conjugate with triazole-linked lower rim lactosyl appendices.[35]

In spite of the promising properties of calixarenes to modulate PPIs, little precedents refer to molecules that could induce oligomerization or allow the stabilization or recovery of proteins that have lost their ability to oligomerize.[36] In an attempt to enhance the thermal stability of p53-R337H mutant, two ligand molecules based on a conical calix[4]arene with four cationic guanidiniomethyl groups at the wider edge (upper rim) and hydrophobic loops at the narrower edge (lower rim) has been introduced, fitting nicely and cooperatively into the

hydrophobic clefts between two of the monomers at each side of the protein and keep the tetrameric structure, like molecular templates, by both ion-pair and hydrophobic interactions.[37]

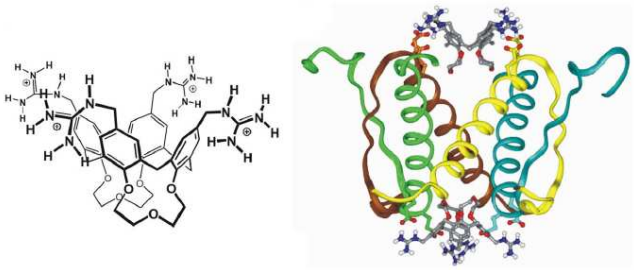
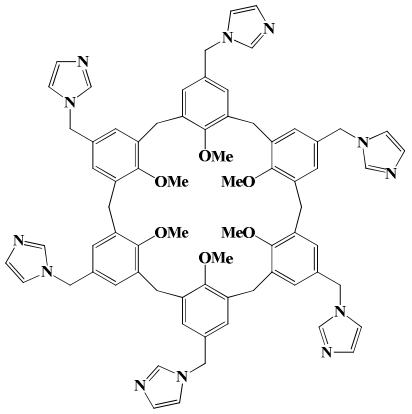


Figure 8. The tetraguanidiniomethylcalix[4]arene and the model its complex with p53-R337.[37]

Along the same line of reasoning, calix[6]arene decorated with imidazole groups on the upper rim (**14**) leads to enhancement of the in vivo transcriptional activity of the most common Li–Fraumeni p53 mutant (p53-R337H) through stabilization of the oligomer formation (Scheme 8).[38] The imidazole groups from the calix[6]arene can interact with the glutamic acid residues and stabilize the mutant tetrameric structure.



[14]

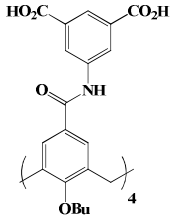
Scheme 8. The molecular structures of the imidazole-calix[6]arene **14**.[38]

3. Protein Inhibition

The last few years have seen an explosion of interest in PPIs study since recognition of their pivotal role in disease development.[39] In order to understand and selectively inhibit cellular signaling pathways, there is a pressing need for small molecules that target PPIs.[40] However,

inhibition of PPIs still remains a significant challenge because it is unclear how they can be effectively and selectively targeted using small molecules.[41] A rational approach to inhibitor design based on the calixarenes is the focus of much research, and different classes of designed ligands have emerged, some of which effectively and selectively disrupt targeted PPIs.

Coinfection of the human immunodeficiency virus 1 (HIV) and hepatitis C virus (HCV) is an important public health concern.[42] The opportunity to investigate the effect of a series of calix[4]arene derivatives on the replication of HIV has been provided by the discovery of a series of calix[4]arene derivatives that block vascular endothelial growth factor (VEGF) and platelet derived growth factor receptor (PDGF) with their respective receptors.[43] In this respect, a potent compound (**15**) based on a tetrabutoxy-calix[4]arene scaffold that possesses dual inhibition for both HIV and HCV was described (Scheme 9).[44] Structural activity relationship studies demonstrated that lower-rim alkylation, resulting in maintaining cone conformation, is important for antiviral activity. Additionally, aromatic isophthalate spacers at the upper rim and the diacid groups turned out to be essential for the observed anti-HIV activities and anti-HCV effects, respectively.

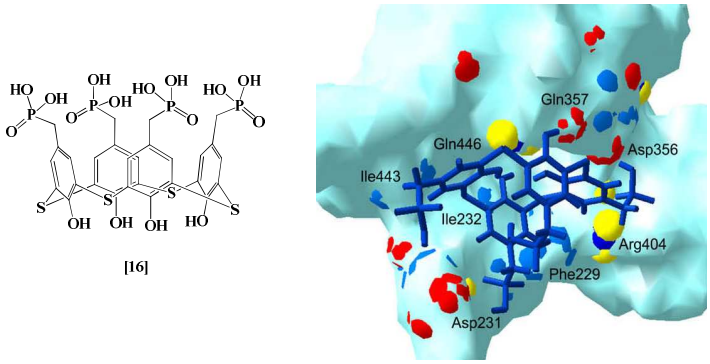


[15]

Scheme 9. The molecular structures of the synthetic dual inhibitor of HIV and HCV infection based on a tetrabutoxy-calix[4]arene scaffold.[44]

The first step of the HIV viral entry into the host cell involves binding of the gp120 protein on the viral envelope to CD4 cell receptor.[45] It has been shown that compound **15** can bind to the HIV-1 envelope protein gp120 and modulate viral entry processes, mode of inhibition that is advantageous since the inhibitor remained active against several pseudotype viruses derived from primary isolates and the resistant strains isolated from existing drug candidates with equal potency. [46]

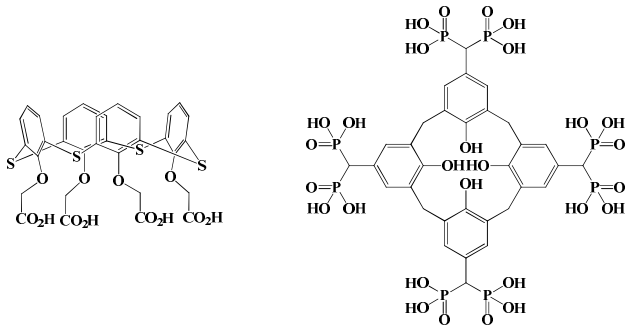
Protein tyrosine phosphatase (PTP) termed Yersinia outer protein H (YopH), secreted by pathogenic bacteria Yersinia, can dephosphorylate multiple focal adhesion proteins such as focal adhesion kinase and focal adhesion protein p130Cas to disrupt the signaling pathways and to escape the immune responses.[47] Based on the literature indicating that inhibitors with two or more anionic groups show enhanced binding to PTPs, thiacalix[4]arene tetrakis(methylphosphonic) acid was confirmed as a potent competitive inhibitor of Yersinia PTP (Figure 9).[48] As observed in the computational model of compound **16** bound with the Yersinia PTP, one of the phosphonate groups was bound to Asp356 of WPD loop and Arg404 of P-loop at the entry of the catalytic pocket. The favourable interactions were also found between phenol oxygens of this macrocycle and residues of Gln357 and Gln446. The macrocyclic platform of compound **16** is involved in hydrophobic interaction with Ile443, Phe229 and Ile232.[48b]



[16]

Figure 9. The thiacalix[4]arene tetrakis(methylphosphonic) acid and its possible binding modes in the active site of Yersinia PTP.[48b]

Another derivative of tetrasubstituted thiacalix[4]arenes (**17**), proved to be a specific antagonist of the leukocyte surface receptor CD69, and that provide complete protection against CD69 dependent apoptosis induced both by multivalent carbohydrate ligand and antibody cross-linking (Scheme 10).[49]

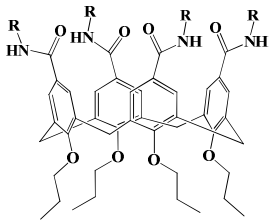


[17] [18]

Scheme 10. The molecular structures of compound **17** and **18**

Calix[4]arene bearing four methylenebisphosphonic acid groups (**18**) at the macrocyclic upper rim has been introduced as a powerful and specific inhibitor of the final step of blood coagulation, fibrin polymerization, and can be used as the basis for the design of new class of antithrombotic agents.[50] This inhibition is a result of the blocking of fibrin polymerization site "A" (Aα17-19, GlyProArg) by the calixarene in a 'knob-hole' manner.

Transglutaminases (TGs) are ubiquitous enzymes which catalyse the cross-linking of glutaminyl residues of a protein/peptide substrate to lysyl residues of a protein/peptide co-substrate.[51] Recent findings suggest that TGs activity may involve in the pathogenetic mechanisms responsible for several human diseases, including celiac sprue and neurodegenerative diseases, and cancer, therefore, TG inhibitors have therapeutic potential in such situations.[52] In this regard, the peptidocalix[4]arene diversomers with sequences Gly-Phe-Gly-Tyr (**19**) and Gly-Phe-Gly-Phe (**20**), bearing at least an apolar aromatic moiety have been demonstrated to effectively inhibit TGs by means of a tissue TG/ peptidocalix[4]arene complex formation promoted by a specific surface recognition on a region noncomprising the enzyme active site (hot spot) leading to a conformational rearrangement of the active form (Scheme 11).[53]



19 : R = Gly-Phe-Gly-Tyr(*t*Bu)-OMe (Fully protected)
20 : R = Gly-Phe-Gly-Phe–OH (Fully unprotected)

Scheme 11. The molecular structures of compound **19** and **20**

Moreover, calix[n]arenes have emerged as promising anti-tumoral agents.[43a,43c,54] Compound **21** is a calixarene-based histone deacetylase inhibitor (HDACi), which performs its biological task by fitting the linker chain into the 11 A° binding channel on the HDLP (histone deacetylase like protein), binding the carboxylate moiety to the ZnII ion, at the bottom of the channel, in a bidentate fashion establishing hydrogen bonds with Hε2 of His132, and hydrogen bonding ability of the amide group with Nδ1 of HIS170 (Figure 10).[55]

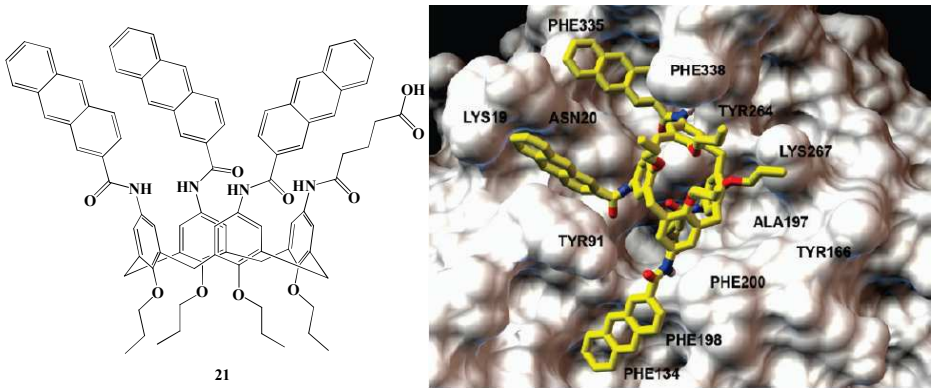


Figure 10. The molecular structures of compound **21** and its putative binding mode in the binding sites of HDLP.[55]

Moreover, aromatic arms of compound **21**, establishing van der Waals interactions with the enzyme counterpart, a π-stacking interaction with TYR91 and TYR264, and a cation-π interaction with LYS19, thus confirming arylamidocalix[4]arenes bearing large aromatic arms constitute moderately active HDACi. Therefore, appropriately substituted arylamidocalix[4]arenes are promising anti-tumor agents as they may affect the cell cycle, inhibit proliferation, stimulate differentiation and induce apoptotic cell death.[56]

In a more recent study, compound **22** has been shown to selectively disrupt binding of the CHD4 PHD2 finger to H3K9me3 (histone H3 trimethylated at Lys9) while not affecting the interaction of this protein with H3K9me0 (unmodified histone H3), Improving understanding the role of CHD4/NuRD in regulation of chromatin structure and gene expression patterns.[57] Notably, the ability of this compound to disturb the association of chromodomain of HP1γ (heterochromatin protein 1γ) with H3K9me3, points to a general mechanism of methyl-lysine caging by calixarenes (Fig 11).

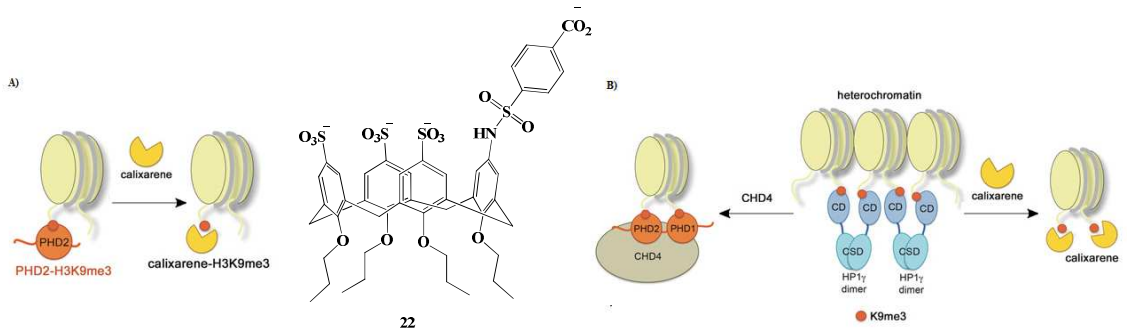
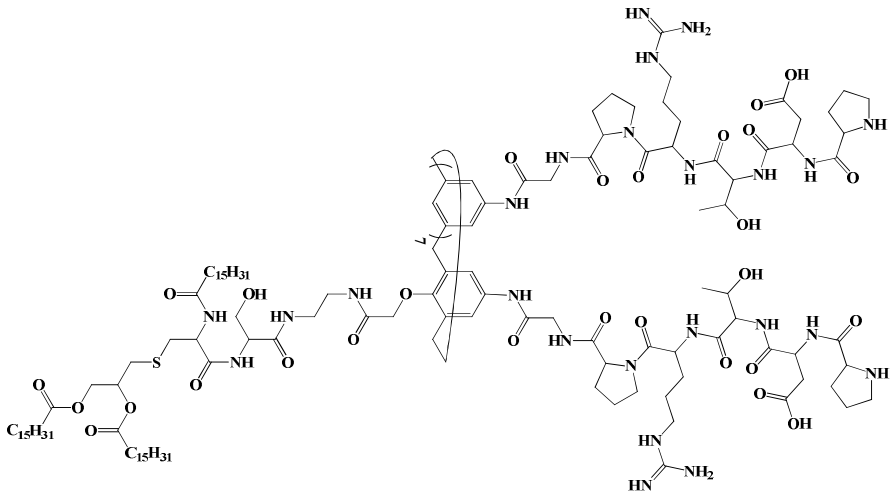


Figure 11. A) Compound **22** abrogates interaction of the CHD4 PHD2 finger with H3K9me3. B) Compound 22 disrupt pericentric heterochromatin.[57]

The three negatively charged sulfonate groups can make contact with the positively charged residues of H3K9me3, including Arg8 and Lys4, and suggests a high potential for these compounds in biochemical applications.

MUC1 protein, the large transmembrane glycoprotein present at the apical surface of the normal glandular epithelial cell, overexpressed in human epithelial carcinoma is another important target for anticancer drug development.[58] In this context, the flexible calix[8]arene platform containing TLR2 ligand (Toll-like receptor 2), conjugated to eight units of immunodominant B-cell epitope PDTRP MUC1 core sequence introduced as the first self-adjuvant multicomponent potential vaccine candidate (Scheme 12).[59]

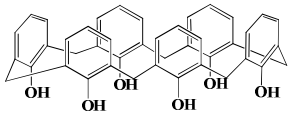


Scheme 12. The molecular structures of Octa-PDTRP-Gly-C8-P_3CS (**23**)

In the synthesized construct, the presence of the P_3CS lipopeptide moiety determines the magnitude of the vaccine candidate immune response while the multivalency and conformational flexibility of the calix[8]arene scaffold provide a better spatial arrangement of

the PDTRP antigens, which ensures effective molecular recognition interaction with the MHC antigen processing machinery.

In the same year, calix[6]arene has been shown to overcome the aggressiveness of a human pancreatic cancer cell line (Panc-1) (Scheme 13).[60] Calix[6]arene dowregulated key protein kinases localized in different cellular compartments resulting in cell cycle arrest, downregulation of pro-survival mediators, endoplasmatic reticulum stress and cell death by autophagy.



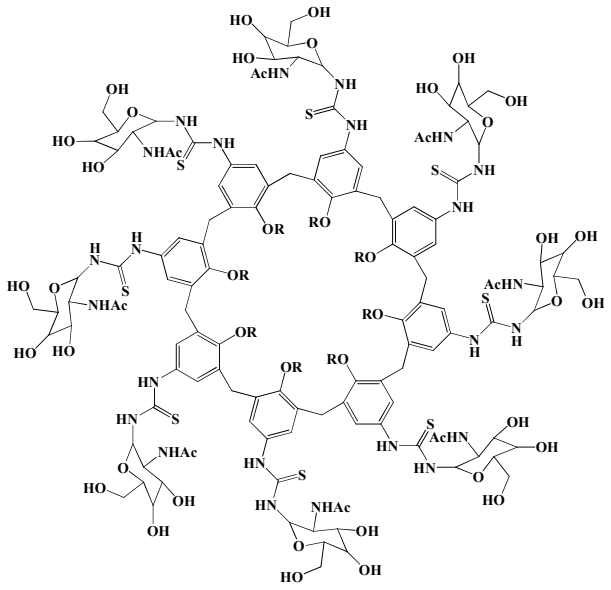
[24]

Scheme 13. The molecular structure of calix[6]arene

Most recently, the water-soluble *p*-sulfonatocalix[4]arene has been shown to inhibit the human papillomavirus 16 L1 (HPV16 L1) pentamer formation via selective binding to Arg and Lys residues at the monomer interface.[61] This study lays the groundwork for the development of assembly inhibitors as a new class of prophylactic and/or therapeutic agents for the treatment of HPV infections causing cervical cancer and other anogenital and oropharyngeal cancers.

In addition to PPIs, the involvement of PCIs in disease processes such as growth regulation, tumor cell adhesion, cell migration or host-pathogen recognition has raised interest towards the synthesis of carbohydrate-based synthetic binders.[62]

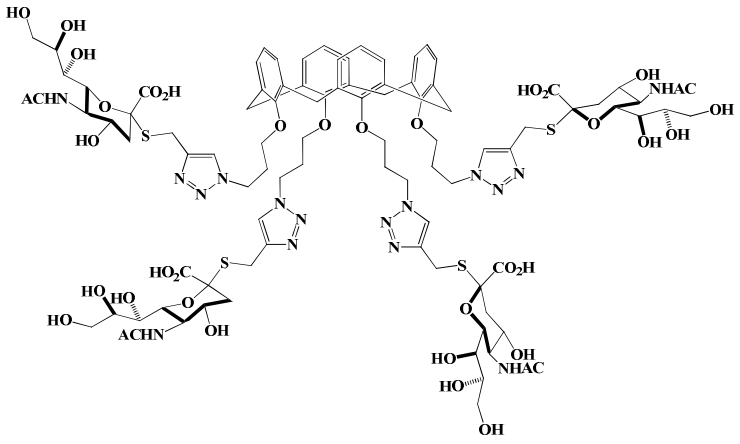
One of the most prominent examples of such carbohydrate-based synthetic binders is a glycocalix[8]arene exposing N-acetylglucosamine (GlcNAc) residues, which exerted inhibitory effects on rat C6 cell migration through the inhibition of focal adhesion kinase phosphorylation (β1,4-Galactosyltransferases (β1,4-GalTase)).[63] β1,4-GalTase upon binding with terminal GlcNAc residues, form highly branched N-linked oligosaccharides, which are markers of glioma and are involved in migration. Thus, artificial GlcNAc decorated structures able to interfere with this sugar-protein recognition process, represent an alternative approach to control glioma tumor migration and invasiveness.



[25]

Scheme 14. The molecular structure of the glycocalix[8]arene exposing N-acetylglucosamine

In another study in the same year, the calix[4]arene functionalized with Neu5Ac, the most widespread form of sialic acid, at the lower rim via multiple azide-alkyne cycloaddition (**26**), was found to inhibit hemagglutination and the viral infectivity mediated both by BK and influenza A viruses.[64] These effects were achieved through interaction of the Neu5Ac-based ligand with the corresponding lectins.

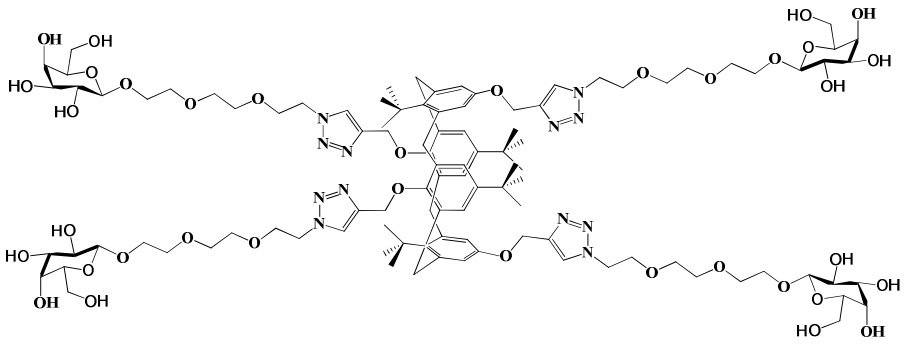


[26]

Scheme 15. The molecular structure of the calix[4]arene functionalized with Neu5Ac.

On the same line of reasoning, to fight PA-IL bacteria lectin from *Pseudomonas aeruginosa*, a major causative agent of lung infections in cystic fibrosis patients, galactose units were

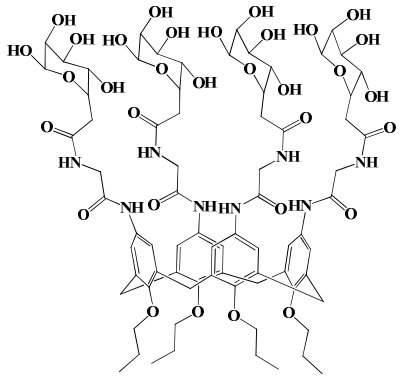
introduced to the lower rim of the calix[4]arene.[65] The results of the study indicated that galactose-based multivalent calix[4]arene ligands interacts with higher affinity to PA-IL bacteria lectin than the monomeric reference compound, the best one being the tetravalent derivative in the 1,3-alternate conformation (Scheme 16).



[27]

Scheme 16. The molecular structure of the tetravalent calix[4]arene glycoconjugate in the 1,3-alternate conformation

In a more recent study, due to the affinity of PA-IIL for α-fucose produced by *P. aeruginosa*, the tetravalent calixarene-based C-fucosyl derivative has been described as a new potential *Pseudomonas aeruginosa* biofilm inhibitor.[66] This is important because antimicrobial resistance of bacteria in the biofilm mode is recognized as causing treatment failure of serious infections from *P. aeruginosa*.[67]



[28]

Scheme 17. The molecular structure of the tetravalent calixarene-based C-fucosyl derivative

4. Protein Extraction

The design of ligands that are able to extract proteins efficiently and without degradation is an important goal for structural and functional studies of proteins, and also in this field calixarenes have prominent roles.

In this direction, the calix[6]arene carboxylic acid derivative (^{t}Oct[6]CH$_2$COOH (**4**)) exhibited high affinity for cationic proteins such as cytochrome c (Cyt c) by promoting its extraction in organic media.[68] Indeed, the ^{t}Oct[6]CH$_2$COOH strongly interact with NH_3^+ groups of the side chain of large number of lysine residues making it hydrophobic enough to be transferred into an organic solution. In addition, the extracted Cyt-c recovered into fresh aqueous solutions at acidic pH containing alcohol as a stripping agent (Figure 12).

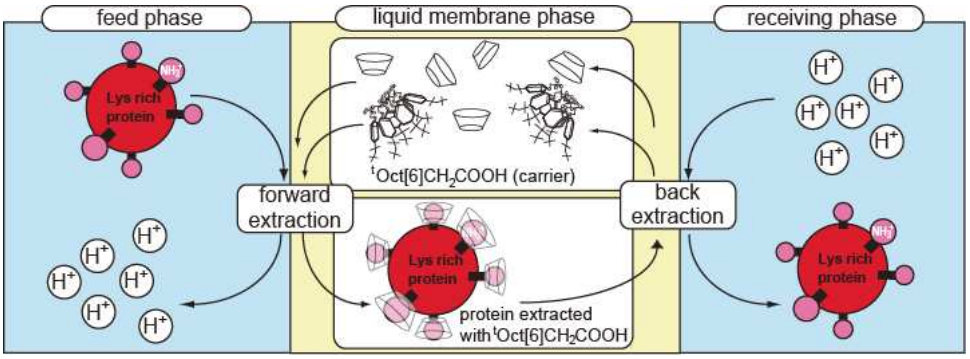
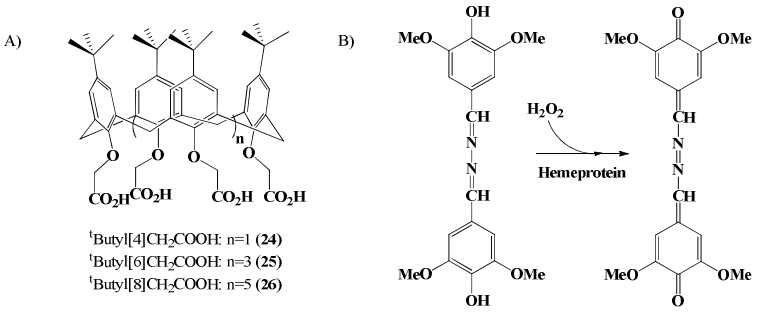


Figure 12. Schematic illustration of liquid membrane transport of a protein, using ^{t}Oct[6]CH$_2$COOH as a carrier.[68]

Due to the presence of multiple amino functions on the surface of hemoglobin, the possibilities of the selective extraction of hemoglobin by *p-tert*-butylcalix[4,6,8]arene carboxylic acid derivatives (^{t}Butyl[4,6,8]CH$_2$COOH) was also investigated.[69] The calix[6]arene resulted in the highest value of the degree of extraction, calix[4]arene gave the next highest yield, while the yield with calix[8]arene was lower (^{t}Butyl[6]CH$_2$COOH> ^{t}Butyl[4]CH$_2$COOH> ^{t}Butyl[8]CH$_2$COOH). Quite interesting the hemoglobin–^{t}Butyl[6]CH$_2$COOH complex exhibited pseudoperoxidase activity which catalysed the oxidation of syringaldazine in the presence of hydrogen peroxide in organic medium containing chloroform (Scheme 18). Hemoglobin–calixarene complex successfully recovered with an aqueous alkaline solution with a recovery of pseudoperoxidase activity of over 100%. An important finding of this study consisted in developing an efficient strategy for protein extraction and solubilisation in organic media for biocatalysis by the use of calixarene derivatives.



Scheme 18. (A) The molecular structure of *p-tert*-butylcalix[n]arene acid derivatives. (B) Reaction catalysed by Hb A–calixarene complex with syringaldazine and H_2O_2 as the substrates in organic media.

More recently, anionic calix[4]arene based detergents (C4Cn, n = 1–12) were designed to structure the membrane domains through hydrophobic interactions and a network of salt bridges with the basic residues found at the cytosol-membrane interface of membrane proteins (Figure 13).[70] Membrane proteins account for up to two thirds of known drugable targets, emphasizing their critical pharmaceutical importance.[71] One important factor in purifying membrane proteins is to extract proteins without losing their activity. Detergents frequently used to purify membrane proteins, do not stabilize membrane domains as efficiently as natural lipids in membranes resulting in partial or total loss of activity.[72] The current compounds were designed to fill this gap. They extract membrane proteins from different origins behaving as mild detergents, and retain protein functionality, as shown for BmrA (*Bacillus* multidrug resistance ATP protein), much more efficiently than SDS (sodium dodecyl sulphate), FC12 (Foscholine 12) or DDM.

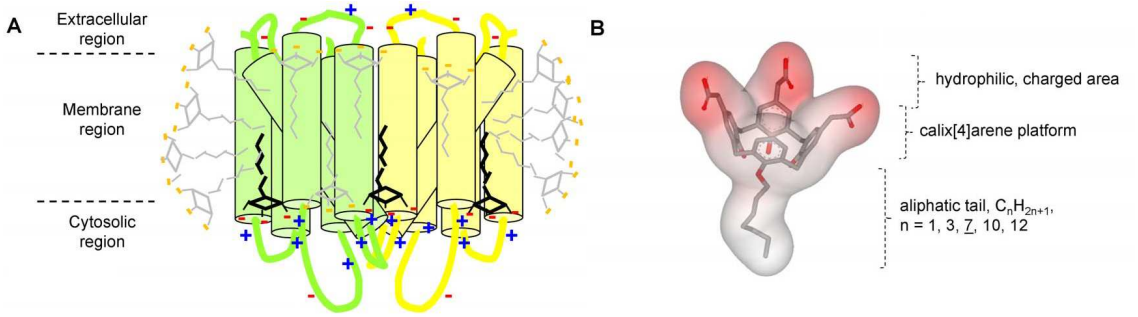


Figure 13. (A) Scheme of a hypothetical dimeric membrane protein typically displaying basic residues at the cytosol-membrane interface, as established by von Heijne[73]. In the absence of lipids, the membrane domain remains in a native conformation due to compounds displaying detergent properties for keeping the membrane protein in solution (grey molecules), but also mild-anionic groups (black molecules), for generating a network of salt bridges close to the membrane domain with basic amino acids carried by the intracellular loops (or domains)

of the membrane proteins. (B) Chemical structure of the designed molecules, C4Cn. Three aromatic rings are substituted by a methylene carboxyl group, -CH_2COOH, at the para position. An aliphatic chain R, $O(CH_2)_{0-11}CH_3$, is grafted onto the fourth phenolic group.[70]

5. Conclusions

The selected examples presented in this review demonstrate that calixarenes fulfil indeed some of the requirements to serve as platforms for the design and synthesis of protein-specific ligands, which are able to identify, inhibit, and/or separate specific proteins. A deeper understanding of how these compounds exert their biological activity as well as more structural information about their protein complexes is urgently needed and will provide the necessary predictive index for their future development and therapeutic application. Calixarenes are attractive for a number of reasons: the possibility of controlling their conformational properties and three dimensional shape, their multivalent nature when considered as scaffolds, multi-functionalization being possible both at the lower and upper rim, and their apparent lack of toxicity. Especially the synthetic versatility of these macrocycles has inspired many chemists to design calixarene conjugates as multifunctional materials for protein recognition. Although considerable research effort has been devoted to synthesize various hybrid compounds, most of them are highly symmetrical, and protein specificity remained limited. New preparative protocols for unsymmetrical calixarenes are needed, and the ability of higher calix[n]arenes to adopt their conformation to the shape of the protein surface area must be fully exploited. Further challenges are bioavailability, metabolism and toxicity. However, the development of protein surface binders has just begun, and calixarenes are expected to play an important role in this endeavor.

Acknowledgments

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Protein Surface Recognition by Calixarenes

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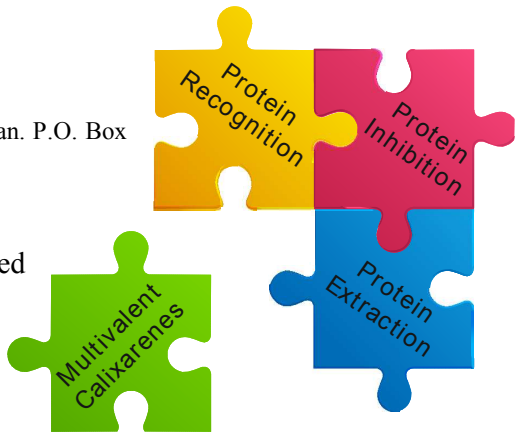

The present review summarize recently developed calixarene derivatives for protein surface recognition which are able to identify, inhibit, and separate specific proteins.

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