Supramolecular Medicine of Diverse Calixarene Derivatives

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8.1 Introduction

Supramolecular chemistry is "beyond molecular chemistry," focusing on the construction of complex structures with specific functions through non-covalent interactions. Compared to covalent bonds in traditional chemistry, supramolecular chemistry emphasizes reversible non-covalent and weak interactions between molecules. These intermolecular interaction forces generally include static

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electricity, π-π stacking, hydrogen bonding, hydrophobic interaction, metal coordination, and van der Waals forces. Supramolecular chemistry is rooted in life and medicine. Molecular recognition, the fundamental concept in supramolecular chemistry, is similar to the interaction between enzymes and substrates or more like a model of "lock and key." In medicine, the mechanism of drug action is often the supramolecular interaction between drugs and receptors. Supramolecular medicine was arised combining supramolecular chemistry with modern medicine, which emphasis the supramolecular recognition and assembly in medical applications, promoting the level of modern medicine. Broadly speaking, supramolecular medicine can be defined as the supramolecular agents for the prevention, diagnosis, and treatment of diseases [1]. The unique and beneficial properties of supramolecular materials have led to extensive research of their use in the fields of disease diagnosis, imaging, drug delivery, drug discovery, and precision medicine. Medically, the emergence of drug-receptor complexes and nanostructure-based drug delivery systems provides new ways to optimize the pharmacokinetic profile of drugs, achieving more effective treatments with fewer side effects and inactivating toxic substances to achieve detoxification. This provides new momentum for developing groundbreaking strategies on treatment of cancer and other major diseases [2].

In supramolecular medicine, host-guest interactions are attracting increasing attention arising from their distinctive properties due to the introduction of macrocyclic hosts into supramolecular systems. Calixarenes, as third-generation macrocyclic molecules, usually have hydrophobic cavities in which the guests can be embedded. Calixarenes provide ideal platforms for the fabrication of supramolecular medical agents through host-guest molecular recognition. In fact, it can effectively solve some restrictions that hinder the use of traditional medicine for clinical applications by taking advantage of host-guest chemistry. For example, the host-guest complexes can significantly improve the solubility/stability of certain anticancer drugs under physiological conditions. Supramolecular self-assembly can facilitate high accumulation of anticancer drugs in tumors, significantly enhance the therapeutic effect of the anticancer drugs, and reduce their side effects on normal tissues. Furthermore, functional groups (such as targeting ligands, imaging agents, or even therapeutic agents) can be readily integrated into the calixarene-chemotherapy system, giving these systems multifunctional therapeutic diagnostic properties. Most importantly, the release of the drug/prodrug loaded in the tumor can be controlled, as it can be based on the different environments (e.g., pH, redox, enzyme) presented between the tumor and normal tissue. The dynamic nature of non-covalent interactions makes supramolecular chemotherapy more versatile than traditional chemotherapy and nanomedicines that lack stimuli responsiveness.

The aim of the present chapter is to summarize the latest research results from us and other research groups about calix[n]arenes and their derivatives with respect to their supramolecular medicine applications in biosensing, bioimaging, gene delivery, drug carriers, and treatment agents, as well as advancing some hints on future areas of scientific research related to the above topics. We hope that this review will constitute a useful tool for nonspecialized readers who wish to obtain an overview of current trends related to calixarenes in supramolecular medicine or for experts who want to look for a precise entry in a particular application domain.

8.2 Calixarene Overview

Calixarenes are a class of third-generation supramolecular host molecules, appearing after cyclodextrin and crown ether. These molecules are formed by the ortho-condensation of para-substituted phenols with formaldehyde, generally in the presence of inorganic bases, although more rarely, acid-catalyzed cyclization reactions are used (Fig. 1a) [3]. Calixarene was created by C. David Gutsche, who likened three-dimensional structure of the molecule to "calix" (the ancient Greek Holy Grail) and "arene" to the building block of the aromatic structure. Given the complexity of the IUPAC terminology for calixarenes, the name has remained and is now in general usage. The simplified nomenclature of the calixarenes uses [n] to denote the number of phenolic units in the macrocycle. For example, calix[4]arene contains four units. The nature and position of substituents on the aromatic rings are given by sequential numeration, and the appropriate term for the function is placed before the term calix[n]arene. Hydroxyl substitution follows sequential numeration with the substituent name generally placed after calix[n]arene. The calixarene history (Fig. 1b) begins with the pioneering work of Adolf von Baeyer in 1872, who first studied the phenol-formaldehyde reaction and obtained a resinous material.

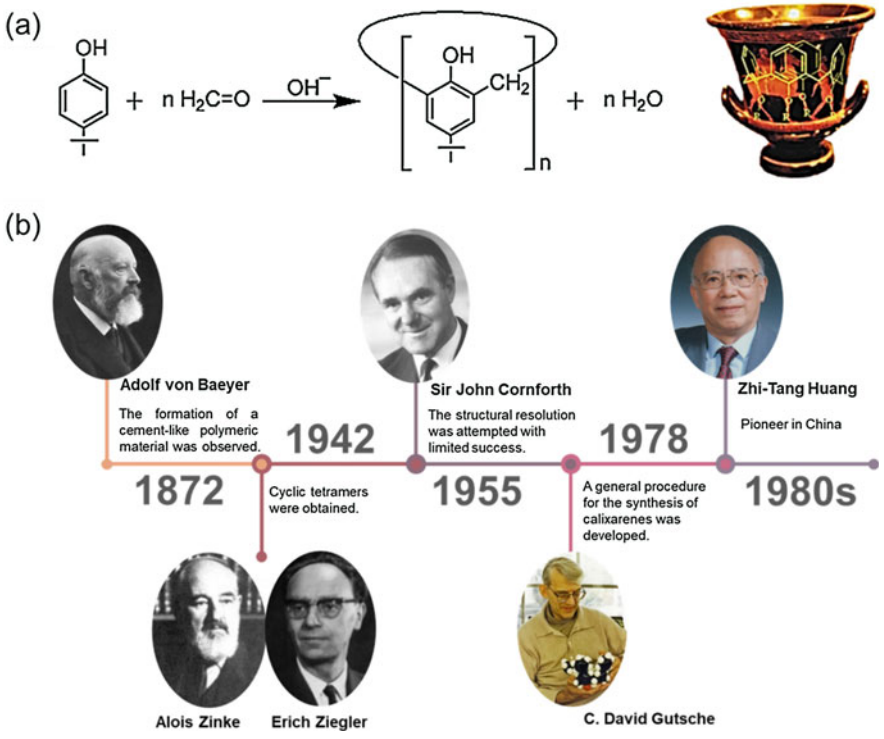

Fig. 1 (**a**) General synthetic method for calixarenes. (**b**) The history of the development of calix[n] arenes

Despite numerous attempts, Adolf von Baeyer was unable to isolate or characterize the products from this reaction, describing them simply as a substance resembling cement. Subsequently, Leo Baekeland (1905–1909) developed a method for synthesizing phenolic plastic from phenol and formaldehyde in an alkaline medium. Due to the growing interest in this material, Zinke and Ziegler (1942) analyzed that the products of the condensation reaction of alkylphenol and formaldehyde may be the tetramers in the presence of NaOH. In 1955, Sir John Cornforth studied these tetramers and found that there were four different conformational isomers. Finally, Gutsche's research indicated that these polymers were cyclic homologs, typically tetramers, hexamers, and octamers, while odd species such as pentamers and heptamers were present in small amounts. Thus, Gutsche and colleagues explored the experimental conditions for the synthesis of common calixarene macrocycles and finally determined various reaction conditions for adjusting the synthesis products, such as the type of base, the source of formaldehyde, solvent, and temperature. It should be mentioned that the research of calixarene chemistry in China began in the 1980s, pioneered by Prof. Zhi-Tang Huang (1928–2016) [4].

8.3 Calixarene Decoration

The general structure of calix[n]arene is shown in Fig. 2, wherein the number of phenolic units is 4–20. However, the most studied members are those constituted by 4–8 aromatic units. Calixarenes have good chemical stability, high melting point, adjustable cavity size, and other unique physical and chemical properties, and they can be functionalized on demand [5]. For example, after modification by ionic groups, the resultant calixarene derivatives can reach high water solubility, allowing their potential for applications in supramolecular medicine to be considered.

The chemical modification of calix[n]arenes has been thoroughly investigated with the main aim of synthesizing hosts with novel supramolecular properties. The easiest and most common transformations regard:

- The para position of the aromatic rings (the upper, wide, or exo rim) by aromatic electrophilic substitution (carboxylates, phosphates, guanidiniums, ammonium groups, sulfonate functionalities, etc.)
- Phenolic hydroxyl groups (the lower, narrow, or endo rim) through alkylation and acylation reactions
- Methylene bridges
- Aromatic walls

The chemistry of the modification of the calix[n]arenes has been widely reviewed (Fig. 3) [6, 7]. Two particularly relevant modification types may be important in medical applications: (a) preparation of water-soluble derivatives used as transport molecules for molecules relevant to supramolecular medicine and (b) synthesis of amphiphilic derivatives used to prepare self-assembling systems such as micelles, liposomes, or solid lipid nanoparticles.

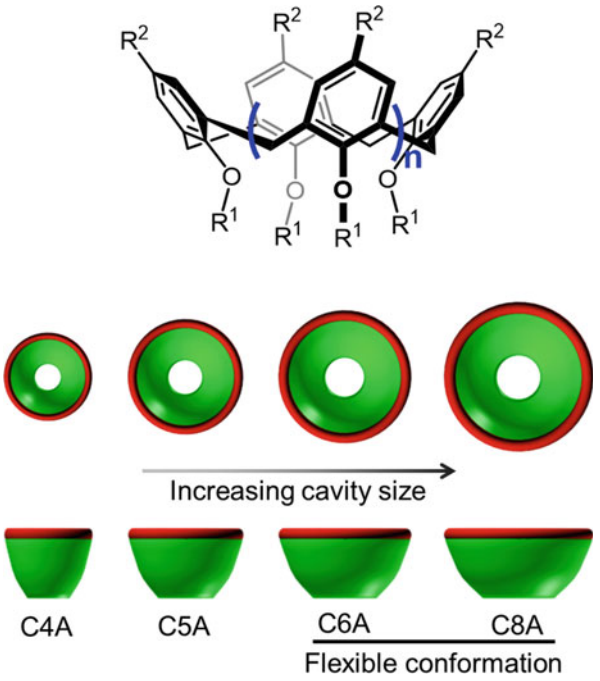

Fig. 2 (Top) The structure of calixarenes. (Bottom) Schematic representation of calix[n]arenes with different numbers of aromatic units

8.4 Calixarene Biocompatibility

For the clinical application of calixarenes in medicine, they must have excellent properties and application prospects. They are not only able to provide chemical/physical benefits but must also have toxicological or biosafety. If calixarenes are used as carriers, it is desirable that calixarenes not only reduce the toxicity and side effects of the drugs but also allow the drug to have a targeted and stimulating response. Therefore, the biological application of calixarene should be evaluated for the inherent toxicity of calixarenes themselves, including cytotoxicity (the ability to inhibit or kill cells) and in vivo toxicity (short-term toxicity, long-term toxicity, side effects).

Biological studies investigating the toxicity of calixarene for clinical use have been reported [8, 9]. Various studies have shown that calixarene, especially water-soluble derivatives, has good compatibility and low cytotoxicity, which are important prerequisites for the applications of calixarenes in supramolecular medicine. Taking sulfonated calixarenes as an example, it was found that *para*-sulfonato-calix[4]arene exhibited extremely low toxicity to mice at doses up to 100 mg/kg [10]. Such property clearly ensures the safety of calixarenes in the clinic to enhance drug dissolution and facilitate delivery. Amino-calix[4]arene-based fluorescent probes were found in cytotoxicity evaluation to be similar in toxicity to phosphate-buffered saline in Chinese hamster ovary cells (CHO) and human

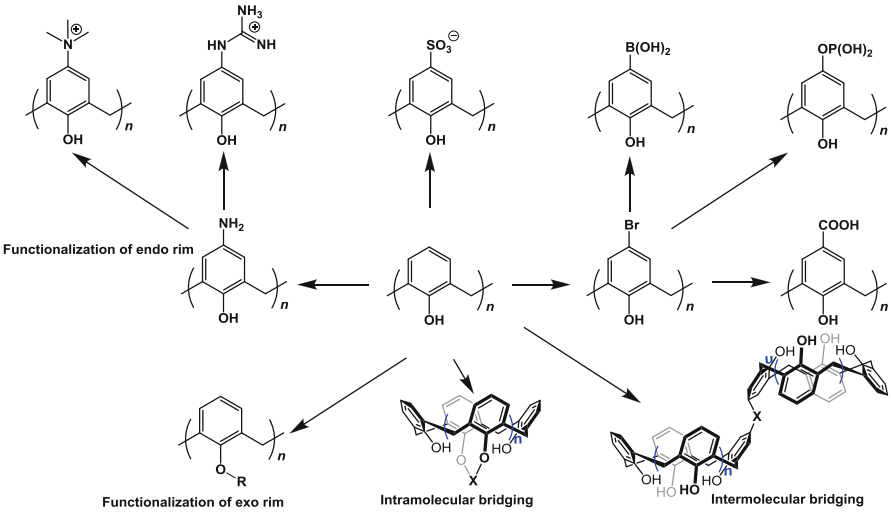

Fig. 3 Examples of calixarene functionalization

promyelocytic leukemia (HL-60) [11]. *Para*-Sulfonato-calix[4]arene was proved to be non-cytotoxic, according to the MTT test results of the human ovarian cancer cell line A2780 and the corresponding cisplatin-resistant subline A2780cis [12]. In the study of calixarene hemolysis, *para*-sulfonato-calix[4]arene and its derivatives did not cause hemolysis even at concentrations up to 200 mM. *Para*-Sulfonato-calix[6]arene and *para*-sulfonato-calix[8]arene relative to *para*-sulfonato-calix[4] arene were enhanced hemolytic effects at the same concentration. But for all sulfonated calixarenes at lower concentrations, this effect was greatly reduced [13]. No hemolytic effects were observed for solid lipid nanoparticles derived from a series of amphiphilic calixarenes [14]. In immunization, three above *para*-sulfonato-calixarenes also did not show activation of neutrophils even at relatively high concentrations, suggesting that these molecules did not induce an immune response [15].

Overall, most of the calixarene derivatives show low or no toxicity in vitro and in vivo, further increasing their attractiveness in the field of supramolecular medicine applications. However, it is important to note that there are still many calixarene derivatives that need to be assessed for their toxicity or immunological responses.

8.5 Calixarenes for Biosensing

Sensing biomolecules are critical for early screening diseases and accurate diagnosis. In general, generalized biomolecules are substances found in blood, urine, stool, or tissues of patients with diseases. These substances are generally metal ions, small molecule compounds, proteins, RNA, etc. Various sensing systems based on calixarene have been applied to the identification of various biomolecules, and

there are many related reviews [16–20]. Therefore, we only selected the following topics to make some comments: sensing metal ions through coordination and sensing biomolecules through indicator displacement assay (IDA) or tandem assay and the surfaces. Many metal ions and inorganic anions play important roles in human growth and development. Additionally, there are some ions that can harm health and induce diseases. Recently, the sensing and recognition of these ions have been a significant goal in the field of chemical sensors. By simple modification, calixarenes can be used to sense and detect various cations (e.g., alkali and alkaline earth metal cations, lead, transition metal cations, rare-earth metal cations, and organic cations) or anions (e.g., some bio-anions) [19]. Recently, Karakurt et al. designed the "switch-on" fluorescence sensor for the determination of Hg^{2+} ion based on the perylene bisimide derivative containing calix[4]arene units (PB-CX[4]) (Fig. 4) [21]. In the mixed solvent of DMF and water, the PB-CX[4] sensor had a very high selectivity and sensitivity to Hg^{2+} ions due to the fluorescent switch-on signal generated by the photoinduced electron transfer (PET) effect. According to the results of the JOB experiment, it was found that PB-CX[4] formed a 1:2 complex with Hg^{2+}. The association constant of PB-CX[4] with Hg^{2+} was determined to be $1.66 \times 10^{9}\ M^{-2}$, and the detection limit was 5.56×10^{-7} M. Finally, PB-CX[4] was applied to imaging Hg^{2+} in human colon cancer cell lines by confocal fluorescence microscopy, which has potential bio-application value. M. Yilmaz and co-workers also completed a similar work and synthesized two kinds of water-soluble fluorescent calixarenes for sensing and imaging Hg^{2+} in living cells [22]. The complexation with Hg^{2+} would result in fluorescence quenching due to the PET process. The resulting LODs were 1.14×10^{-5} and 3.42×10^{-5} M. These nontoxic sensors were then used to sense and image Hg^{2+} in the SW-620 cell line, and excellent results were observed.

A distinctive feature of calixarenes is that they tend to quench fluorescence after complexation with fluorescent dyes. The mechanism is generally considered to be the PET effect [23]. The calixarene skeleton is constructed by base-catalyzed condensation of 4-substituted phenols with formaldehyde. As a rule, the electron-rich hydroxyl- or alkoxyl-substituted aryl rings are prone to act as electron donors toward excited states, which lead to fluorescence quenching upon binding of fluorescent dyes. In fluorescence detection technology, the fluorescence signal from "off" to "on" is a more reliable signal conditioning means. A variety of "switch-on" fluorescence sensing supramolecular systems have been developed based on the quenching fluorescence property and applied to the detection of tumor markers and other diagnostically significant biological analytes. These fluorescence detection systems are based primarily on the principle of IDA (Fig. 5a): When the calixarenes and dyes act as sensing pairs, the fluorescence of the dyes is in a quenched state; after the analytes are added to the sensing system, the analytes replace dyes by binding to the calixarenes, and the dyes recover their own fluorescence. Whether the sensing system is suitable for detecting analytes depends mainly on the sensitivity and selectivity of the system. To improve sensitivity, the dye should have a high fluorescence quantum yield and maximum quenching fluorescence after being encapsulated by calixarene; to minimize interference from nontarget species, the

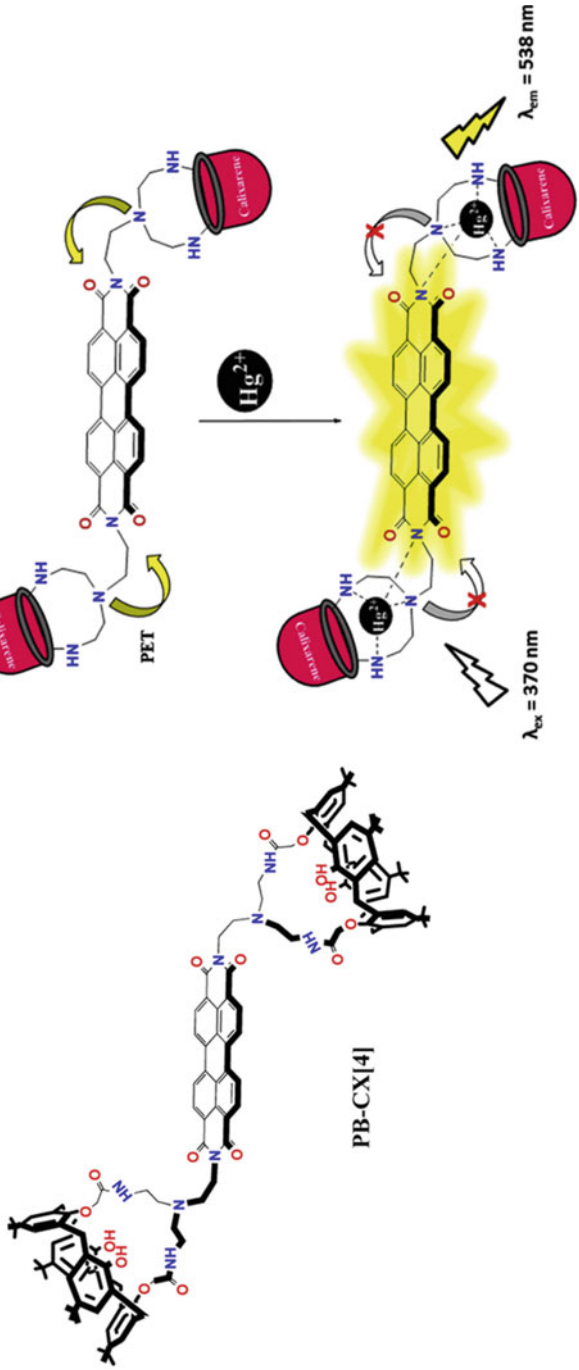

Fig. 4 The chemical structure of PB-CX[4] and sensing mechanism of PB-CX[4] with Hg^{2+} and the PET process [21]. (Reproduced from Ref. [21] with permission from Elsevier)

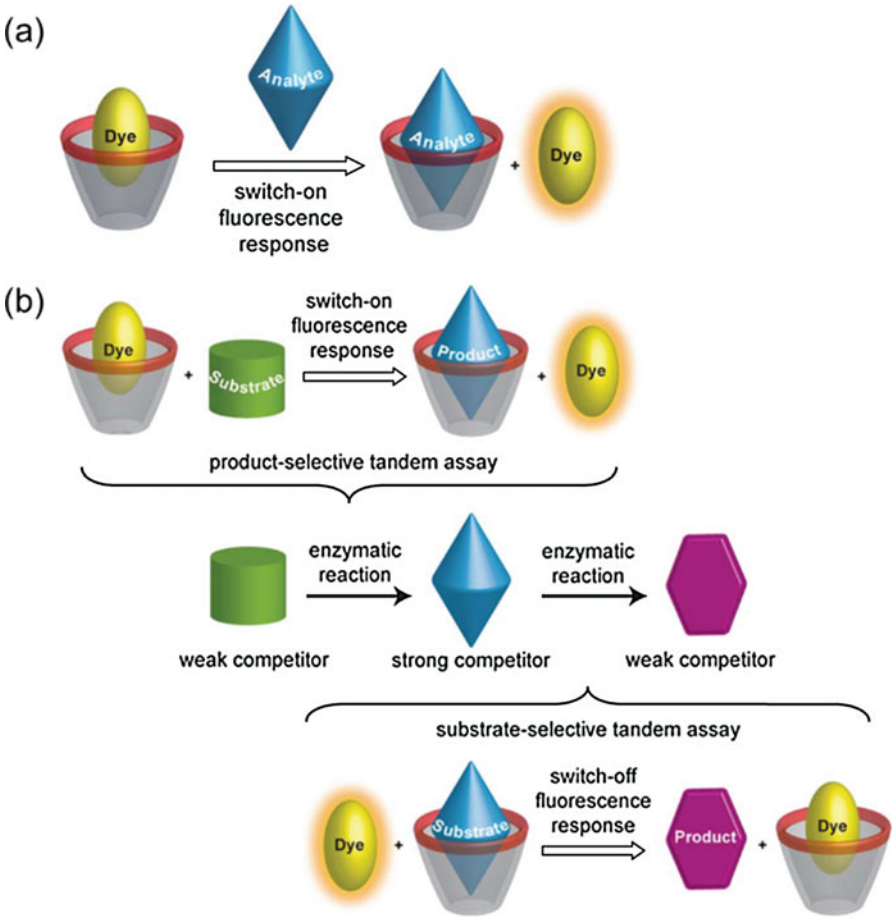

Fig. 5 Schematic representation of (**a**) fluorescence "switch-on" indicator displacement assay (IDA) based on calixarene and (**b**) supramolecular tandem assay [23]

designed calixarene host should have the ability to selectively bind strongly to the target analyte. In the case of insufficient binding selectivity, an enzyme can be introduced into the supramolecular tandem assay to ultimately achieve specific quantitative detection of the target analyte (Fig. 5b).

The detection of biomolecules by sensing systems consisting of calixarene and fluorescent dyes has received considerable attention [23–31]. Li and co-workers reported synthesis and design of SCX8-functionalized reduced graphene oxide (SCX8-RGO) to determine aconitine through the competitive host-guest interaction between *p*-sulfonato-calix[8]arene (SCX8) and signal probe/target molecules (Fig. 6) [28]. Safranine T (ST), rhodamine B (RhB), and butyl rhodamine B (BRB) were selected as probes, and aconitine was target molecule, respectively. SCX8-RGO can form complexes with three fluorescent molecules and effectively

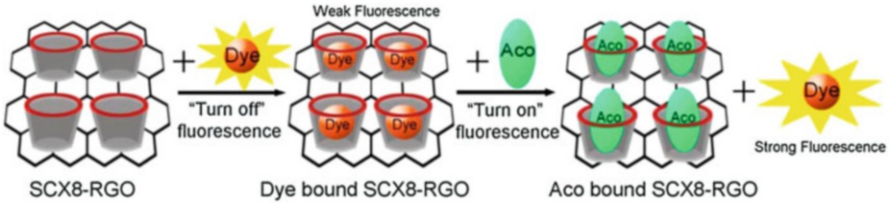

Fig. 6 IDA for aconitine (ACO) using SCX8-RGO [28]. (Reproduced from Ref. [28] with permission from Elsevier)

quench their fluorescence. Compared to the probes, aconitine had a stronger affinity with SCX8 and replaced the probes from the SCX8 cavity to achieve fluorescence "turn-on." The sensor consisting of SCX8-RGO and three probes were able to detect aconitine with linear ranges of 1.0–14.0 μM, 2.0–16.0 μM, and 1.0–16.0 μM, respectively. And detection limits of aconitine were 0.28 μM, 0.60 μM, and 0.37 μM, respectively. Moreover, the sensing system was successfully applied to the detection of aconitine in human serum.

The IDA strategy was widely used in sensing systems like those discussed above generally based on lock-and-key model. Unlike IDA strategies but using some of the ideas of IDA, differential sensing was proposed to address those less selective receptors in array sensing [32]. The idea of differential sensing was to mimic the nose of the mammal, using a range of low-selectivity receptors to provide a signal array for each analyte. The signals for each analyte formed a corresponding fingerprint, thereby enabling classification of the analytes. Hof and co-workers developed antibody-free detection histone code using calixarene-based chemical sensor arrays [29]. The posttranslational modification of histones begins with its N-terminal tail, which includes methylation, quaternization, acetylation, and phosphorylation. These affect the function of histones in gene regulation and are associated with various human diseases. Sensing arrays composed of sulfonated calixarenes and dyes were capable of generating signals for cationic amino acids and peptides, making corresponding fingerprints for differentiation (Fig. 7). For example, this sensor kit can identify either methylation and the number of methyl groups on a single histone tail sequence. In addition, the sensor array can also be used to simultaneously detect the concentration of histone modifications.

Many of IDA strategies are applied to the detection of biomolecules in water or biological fluids, but the robustness of these methods is inevitably reduced when there is a large amount of component interference, such as inorganic salts. Hof and co-workers reported a self-assembled sensor, DimerDye, that uses a photochemical guest-sensing mechanism and that is intrinsically tolerant of a competitive biological environment (Fig. 8) [30]. Modifying the dye directly on the calixarene, two calixarenes self-assembled into non-emissive dimers through the host-guest interaction in water. When the analyte was detected, the analyte caused the dimer to dissociate while the fluorescence was restored, allowing fluorescence to be turned

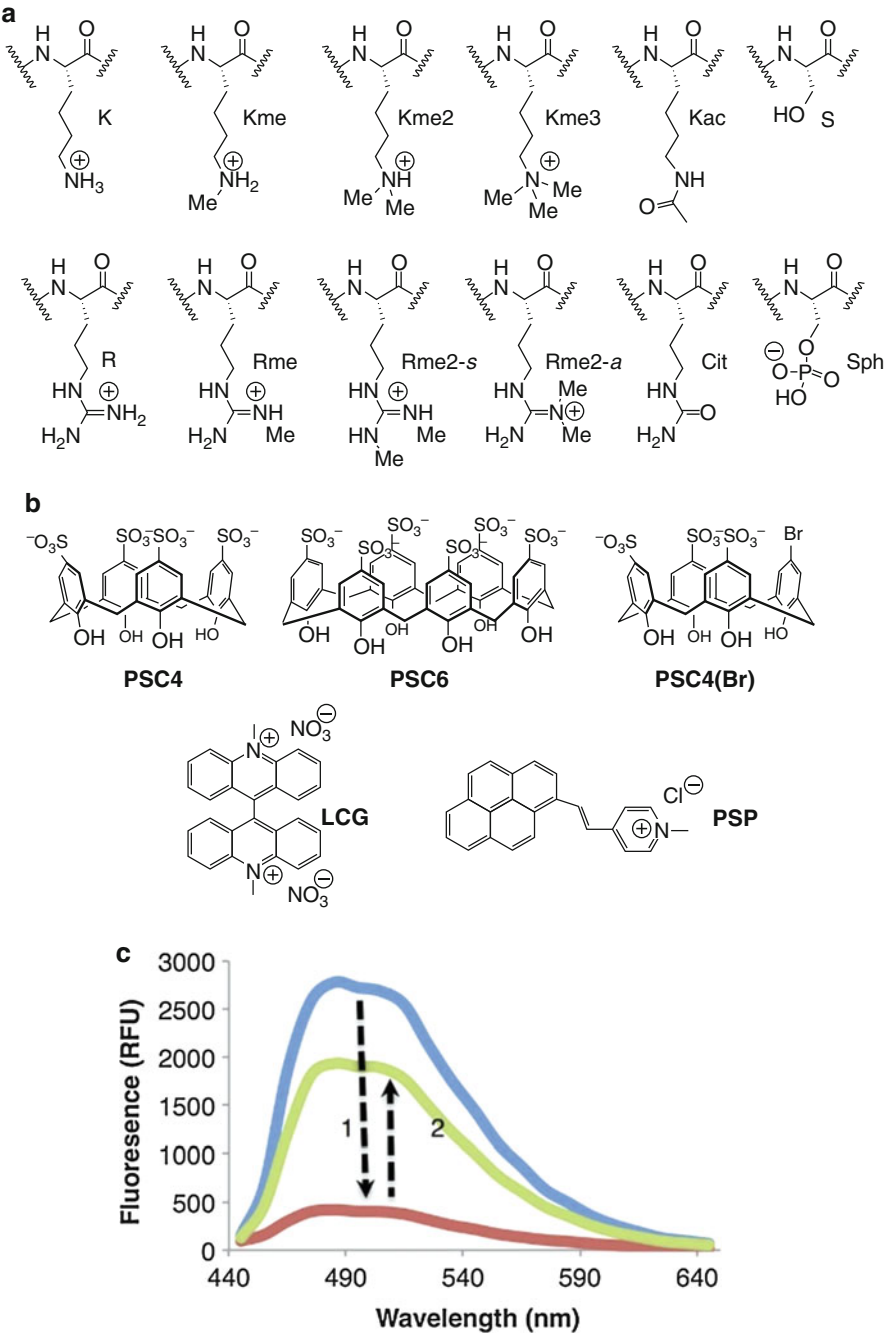

Fig. 7 (continued)

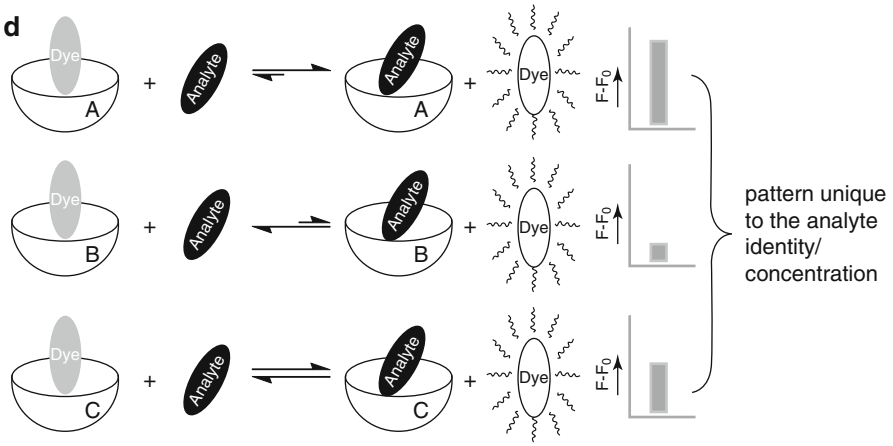

Fig. 7 (**a**) Structures of posttranslationally modified residues. (**b**) The chemical structures of calixarenes and dyes. (**c**) The principle of sensor response to analytes. (**d**) Illustration of establishing a fingerprint using the sensing array [29]. (Reproduced from Ref. [29] with permission from the American Chemical Society)

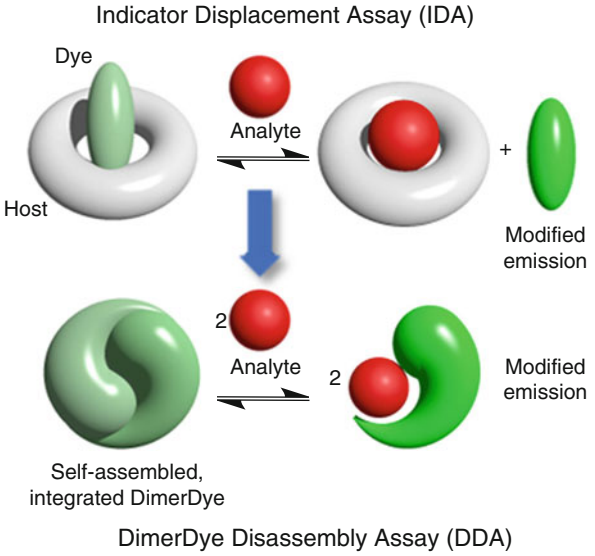

Fig. 8 Sensing mechanism of DimerDye disassembly assay (DDA) and indicator displacement assay (IDA) [30]. (Reproduced from Ref. [30] with permission from the American Chemical Society)

on. In the enzymatic reaction, DimerDye was still able to work effectively in the presence of various salts, metal ions, or coenzymes. As an important supplement to IDA, this strategy is of great significance for detecting biomolecules in complex environments.

Monitoring enzymatic activity is of the utmost importance for academic and industrial research. Nau's group first proposed a supramolecular tandem assay strategy based on IDA applied for the determination of enzyme kinetics. Nau and our groups have reported some efforts toward clarifying the working mechanism of enzymes, their inhibitors, and activators [23, 24, 31]. *P*-Sulfonato-calix[4]arenes have good affinity with acetylcholine (ACh) and choline (Ch), but are not selective. In order to solve the above problems, we cooperated with the Nau group to detect and quantify ACh and Ch using supramolecular enzyme-coupled tandem assay [23, 33]. If acetylcholinesterase was used to convert Ach to Ch, *p*-sulfonato-calix[4]arenes did not distinguish between substrates and products (Fig. 9, left). The underlying problem was that the calixarene did not have sufficient affinity differences for the two molecules with the same NMe^{3+} recognition motif. However, choline oxidase can be used to convert choline into detectable betaine for it was zwitterionic and weaker bonding with calixarenes (Fig. 9, right). Lucigenin (LCG) and *p*-sulfonato-calix[4]arenes were proved to be an excellent "switch-on to switch-off" sensor pair with a fluorescence enhancement factor up to 140. When choline oxidase was present, the fluorescence signal would decrease because the affinity of the product to the *p*-sulfonato-calix[4] arenes was less than the LCG leading to fluorescence of the LCG being quenched. Through such method, we also investigated enzyme inhibitors, which had important reference value for the screening of drugs.

Schader's group used calixarenes on the surfaces to sense many kinds of basic proteins [34]. The calix[4]arene modified with phosphonate at the upper rim and short alkyl chain at the lower rim (PC4A4C) was introduced in this work. The PC4A4C system showed high affinities with N/C-protected Arg (~10^4 M^{-1}) and Lys

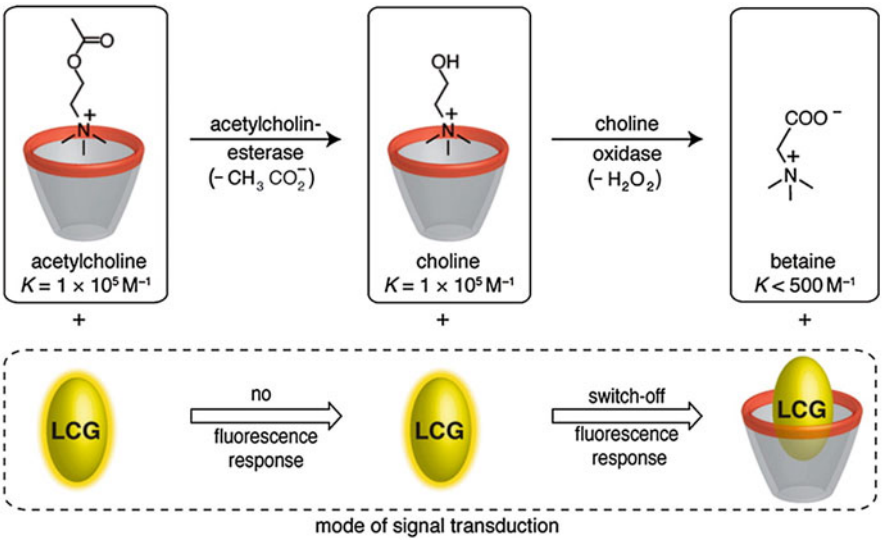

Fig. 9 Detection and quantification of acetylcholine and choline by supramolecular tandem assays [33]. (Reproduced from Ref. [33] with permission from the American Chemical Society)

($\sim 10^3\ M^{-1}$) in methanol. Not surprisingly, PC4A4C could also bind tightly with Arg- and/or Lys-rich proteins. Because of the amphiphilic structure, the addition of PC4A4C to the stearic acid monolayer on water resulted in the incorporation of increasing amounts of PC4A4C in the monolayer. The following addition of basic proteins would produce moderate but distinct additional expansions of pressure/area diagrams. After this work, a cationic calixarene containing quaternary ammonium was introduced, and the sensing of acid proteins was realized using the same strategy [35]. On the basis of the above work, Schrader co-assembled phospholipids with polydiacetylene, which showed a blue color and changed to red by various stimuli [36]. The stimulus could be heat, ionic strength, or mechanical pressure. Then, the addition of proteins caused obvious color changes and enabled the detection of proteins by the naked eye. Schrader's work cleverly took advantage of multivalence and assembly behavior, greatly simplifying the sensing of proteins. Although the work was done more than 10 years ago, it still provides valuable lessons.

8.6 Calixarenes for Bioimaging

When Wilhelm Roentgen filmed the first X-ray of his wife's hand in 1896, medical diagnosis entered a new era. Since X-rays have been applied to bioimaging, various noninvasive imaging methods have been developed and applied to clinical imaging and research in vivo or in vitro. Calixarene-Gd complexes or their derivatives have been used in magnetic resonance imaging as a tool in medical diagnostics [37–41]. In the field of optical imaging of macrocyclic molecules, Nau et al. detailed and systematically summarized the changes of fluorescence properties when the host-guest complex formed between the fluorescent dyes and the macrocyclic molecules in an aqueous environment [42]. Host-guest complexes between calixarenes and fluorescence dyes or calixarenes directly modified by dyes have been utilized for bioimaging in vitro and in vivo, showing tuneable or targeted florescence response, physiochemical shielding, and enhanced biocompatibility provided by macrocyclic host molecules [43–57].

As mentioned above, one of the advantages of IDA is that it has a broad spectrum of analytes, eliminating the need to design specific receptors for specific analytes. Our group collaborated with Nau's group to develop a host-guest sensing system using sulfonated calixarene-LCG pairs and applied it to detect enzyme activity, quantify bioactive molecules, and screen drugs [23, 31]. However, the broad spectrum of the IDA method also results in a response to nontarget analytes, which is widespread when testing biological samples. Nau and co-workers used artificial receptors to transfer probes to living cells, and IDA method was used to monitor cellular uptake of biomolecular analytes [43]. The fluorescent dye LCG was quenched by the macrocycle *p*-sulfonato-calix[4]arene to form a stable host-guest complex. The LCG/calixarene sensing pairs were incubated with V79 and CHO cells. After adding choline, acetylcholine, or protamine to the cell culture medium, they were uptaken into the cells, and formed complexes with calixarenes replaced LCG to achieve fluorescence switch-on response (Fig. 10). This response can be

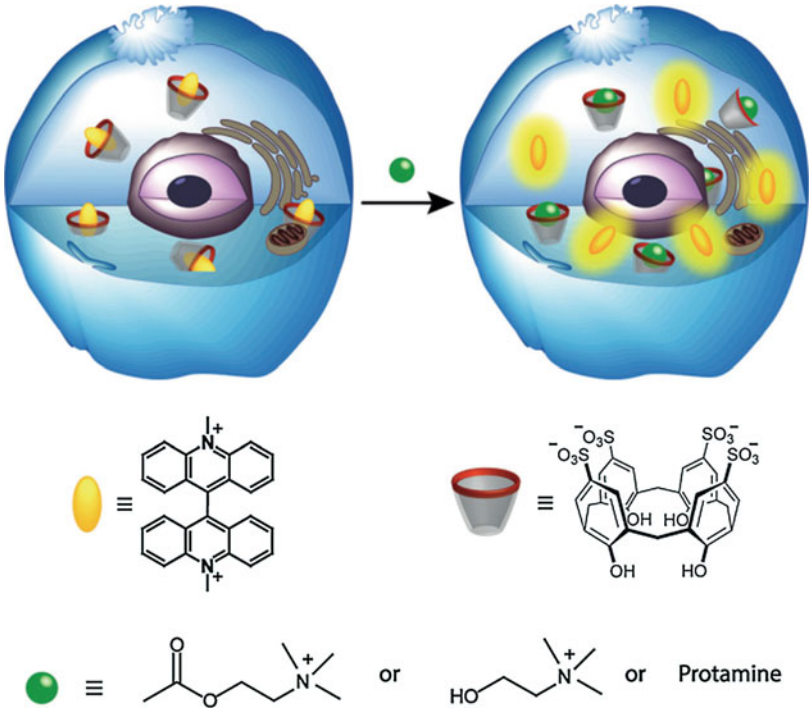

Fig. 10 The supramolecular imaging system of *p*-sulfonato-calix[4]arene•lucigenin using the IDA principle to monitor biomolecule uptake of cells [43]. (Reproduced from Ref. [43] with permission from Wiley-VCH)

traced to the displacement of LCG from calixarene by the analytes. The results establish the principal functionality of IDA with synthetic receptors for the detection of the uptake of bioorganic analytes by live cells [43].

In the field of bioimaging, the key challenge for fluorescent nanoparticles is to prepare particles of size equivalent to single proteins (3–7 nm) and achieve excellent brightness. Klymchenko and co-workers prepared calixarene micelles that are shell-cross-linked by fluorescent bifunctional dyes through Cu-catalyzed click chemistry (Fig. 11) [44]. The authors used the conical shape, skeleton, and self-assembly properties of amphiphiles calix[4]arene to regulate the distance between the cyanine dyes and the direction of the dye. Finally, they obtained protein-sized fluorescent nanoparticles and minimized the self-quenching between the fluorescent dyes. They synthesized positively charged amphiphilic calix[4]arenes with acetylene groups on the upper rim to enable a fast and efficient "click" reaction. The calix[4]arenes self-assembled into micelles in water, and cyanine dyes with azide groups undergo the "click" reaction to cross-link the calix[4]arenes to form an organic fluorescent quantum dot with cyanine dyes on the outer surface of the nanoparticles. The hydrodynamic diameter of the nanoparticles was 7 nm, which was equivalent to the size of single proteins (3–7 nm). The fluorescent nanoparticles had the following

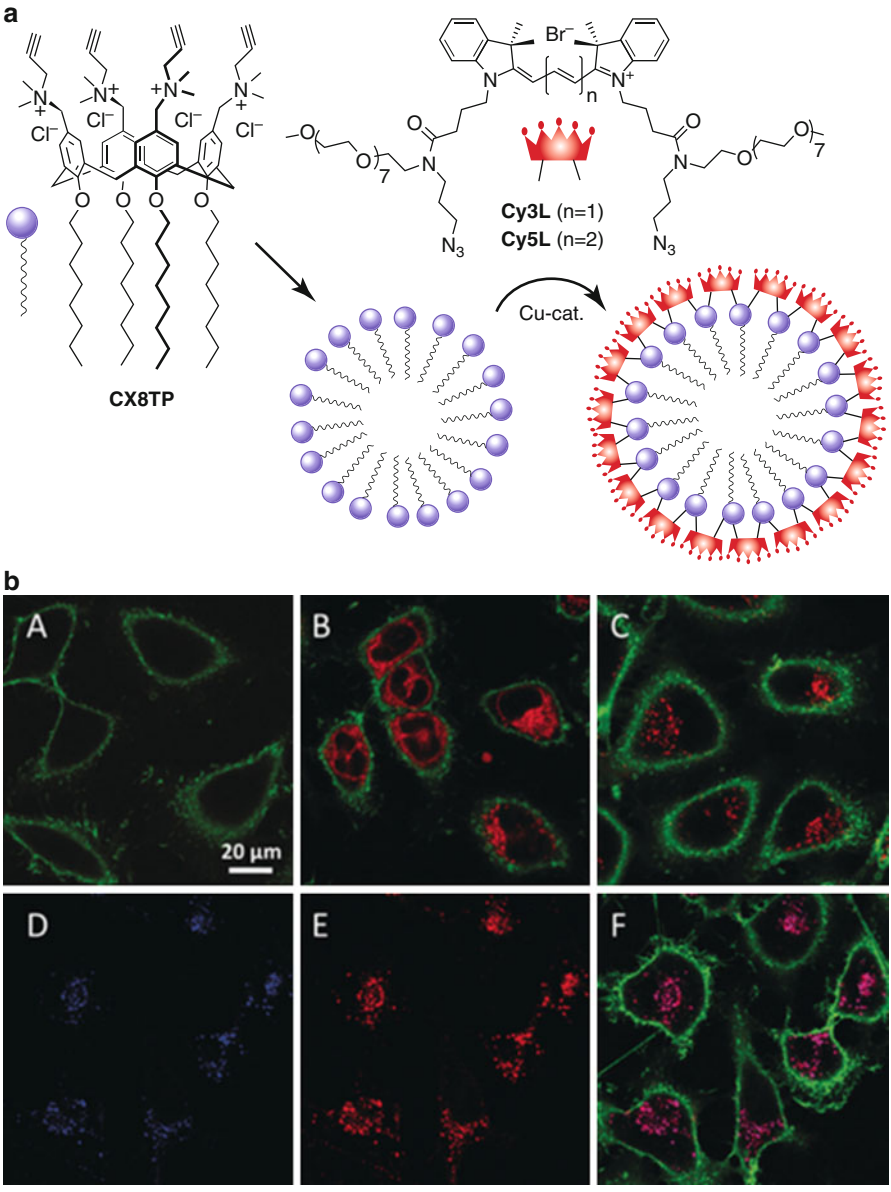

Fig. 11 (**a**) Schematic illustration of cross-linking between calix[4]arene micelles and cyanine dyes to form calix[4]arene organic quantum dots. (**b**) Fluorescence confocal imaging of cells using calix[4]arene organic quantum dots [44]. (Reproduced from Ref. [44] with permission from the Wiley-VCH)

advantages: the size minimal perturbation of biomolecular processes in cell imaging, uniform particle size avoiding wide emission of fluorescence, and cross-linking structure preventing disintegration of nanoparticles after interaction with cell membranes. It is worth noting that the calix[4]arene quantum dots have excellent luminescence properties. The brightness of nanoparticles was twofold brighter than commercial quantum dots (QD-585). Finally, the authors applied calix[4]arene quantum dots to cell imaging and found that the materials can enter HeLa cells and selectively accumulate in endosomes and lysosomes. The results showed that calix[4]arene quantum dots maintain structural integrity in physiological media, organic solvents, and living cells and can be rapidly internalized showing excellent imaging contrast. This type of calixarene organic quantum dot has broad application prospects in cytology, histology, and fluorescent tracers in biochemistry [44].

8.7 Calixarenes for Gene Delivery

The ability to tightly bind and compact DNA and the characteristics of calixarenes to behave as macrocyclic amphiphiles motivated us to test guanidinium-calixarenes as gene delivery vectors. Current studies show that calix[4]arenes are probably the most promising among the described calixarenes for gene delivery applications [17, 58–61]. Their fixed conformation has multiple functional groups at the upper and lower rims, which allow the preparation of cone-shaped macromolecules that can be programmed for fractional assembly in the presence of DNA. The intricate calixarenes designed, especially those with amphiphilic structures, are able to form DNA-calixarene nanoparticles with clear structure, high transfection efficiency and low toxicity. At present, researches in the field are still insufficient, especially for the modification of calixarene, which requires more scientists' attention. In addition, experiments in vivo are needed to evaluate the effect of calixarene on gene therapy.

Recently, Ungaro's team synthesized positively charged calixarene derivatives with upper rim modified by four arginine residues and lower rim of four hexyl groups or only four arginine residues at the lower rim (Fig. 12a). AFM imaging showed that the calixarenes with the upper rim-modified arginine reacted with DNA to form nanoparticles with a size of 50–60 nm (Fig. 12b), whereas the derivatives of the lower rim-modified arginine formed more larger aggregate. This apparent difference may be partly due to the fact that argininocalix[4]arene 1 had a clear amphiphilic nature relative to ordinary calixarenes. In particular, argininocalix[4]arene 1 showed excellent transfection efficiency even better than Lipofectamine and PEI in various cell lines (Fig. 12c). In contrast, tetralysinocalix[4]arene 3 showed little transfection activity. In addition, the argininocalix[4]arene 2, similar to argininocalix[4]arene 1, which modified the protonated amino groups rather than the guanidine groups, exhibited poor transfection activity, while DOPE can help to increase their activity. This significant difference between

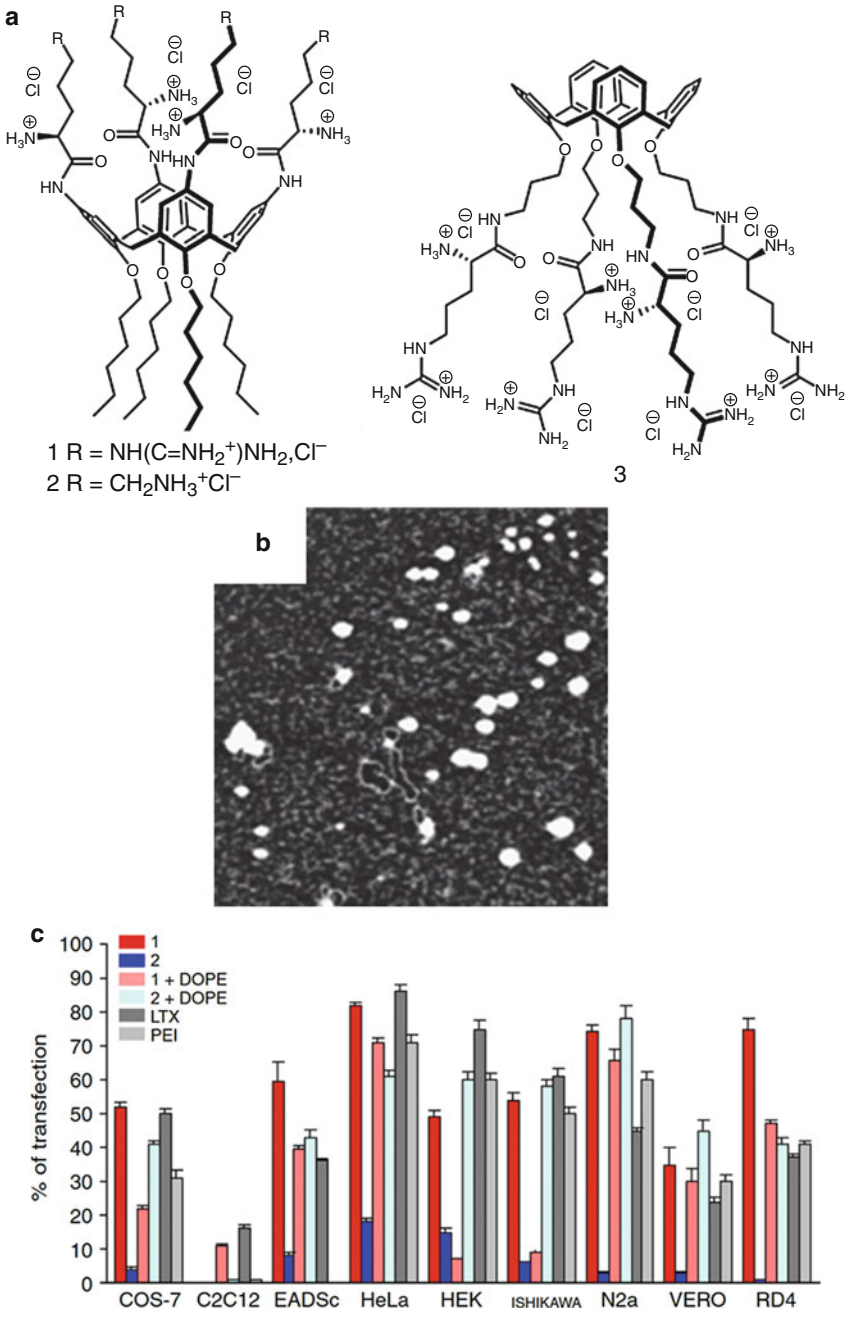

Fig. 12 (**a**) Structure of calix[4]arenes 1, 2, and 3. (**b**) AFM images showing DNA folding by calixarene 1. (**c**) Transfection efficiency in various cell lines of calixarene 1 (red) compared with calixarene 1 with DOPE (peak), calixarene 2 (blue), calixarene 2 with DOPE (cyan-blue), Lipofectamine (gray), and PEI (light gray) [58]. (Reproduced from Ref. [58] with permission from the Nature Publishing Group)

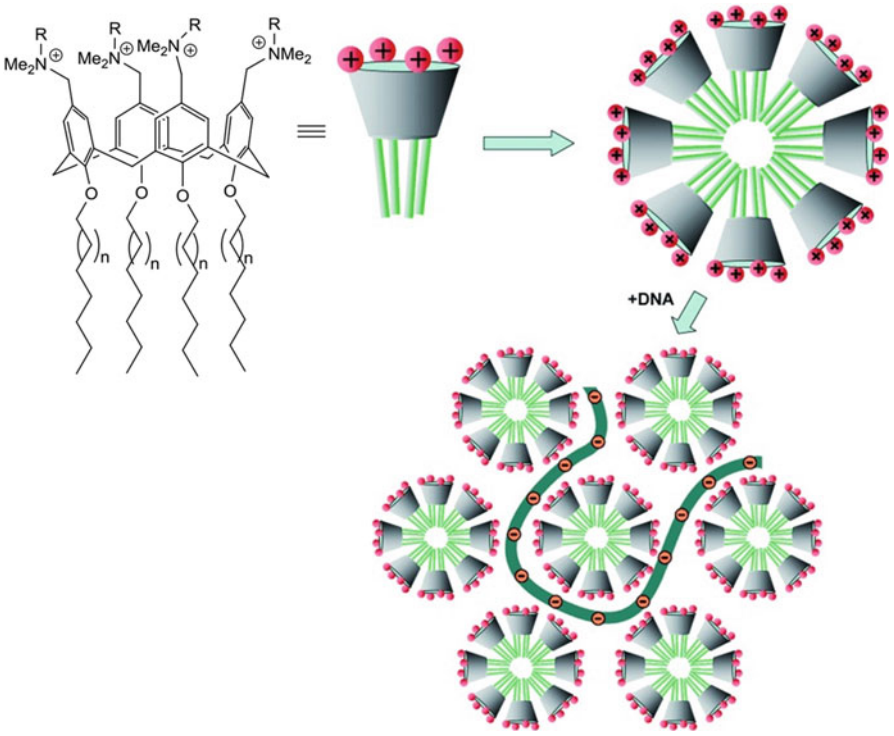

Fig. 13 Schematic representation of DNA complex with micelles composed of cationic amphiphilic calixarenes [60]. (Reproduced from Ref. [60] with permission from Wiley-VCH)

argininocalix[4]arenes 1 and 2 suggested that the arginine residues of the amphiphilic calixarene were essential for efficient transfection, which may increase the ability of the DNA-calixarene complexes to cross the cell membrane The most efficient transfection efficiency of argininocalix[4]arene 1 is less cytotoxic and comparable to Lipofectamine. In the current work of gene transfection using calixarene, argininocalix[4]arene 1 may be the best transfection agent.

Klimchenko et al. developed a nucleic acid template consisting of a polycationic amphiphilic calixarene, initially a tiny calixarene micelle (about 6 nm in diameter) that forms complexes with DNA by electrostatic interaction. This fractionation process would be advantageous for cationic calixarenes modified by relatively long lipophilic chains (Fig. 13) [60, 61].

8.8 Calixarenes as Drug Carriers

The use of supramolecular concepts to design drug delivery systems has attracted widespread attention from scientists. Significantly, calixarenes and their water-soluble calixarene derivatives have become an important class of supramolecular

drug carriers in drug delivery systems. There have been many reviews about drug carriers based on calixarenes [62–65]. Depending on the mechanism of delivery implementation, we can generally classify them into the following three categories: inclusion complexes, amphiphilic self-assembly, and supra-amphiphilic self-assembly.

Many research groups have reported drug delivery systems based on calixarene-drug complexes [62, 65, 66]. Typically, hydrophilic groups at the rim of calixarenes have been widely utilized to produce water-soluble derivatives, which serve as important containers to encapsulate drugs with poor water solubility in drug delivery [67–70]. Pilar Ljpez-Còrnejo et al. constructed an inclusion complex using *p*-sulfonatocalix[6]arene and doxorubicin [71]. The complex systems display a preference for locating close to the DNA structure, facilitating the transport of the antibiotic toward the polynucleotide, which means that they can act as an excellent candidate for drug delivery. In addition, calixarenes in the solution partially reduce the side effects of doxorubicin (DOX).

The calixarene amphiphiles can provide cavities for drug delivery by suitable arrangement [72]. This highly attractive trait had prompted scientists to develop new materials and devices that may be applied in bio-nanotechnology and nano-medicine [73]. The hydrophilic groups were usually modified at the upper rim of calixarenes, and the lower rim was modified with hydrophobic groups such as linear alkyl group with appropriate length to form stable supramolecular assemblies. Longer alkyl groups usually cause extremely low solubility of amphiphiles and aggregation in solid lipid nanoparticles. Casnati et al. have already extensively reviewed the use of calixarene amphiphiles for nanocarrier applications in drug delivery systems [73]. Zhao et al. synthesized a folic acid-PEG-modified *p*-phosphonated calix[4]arene. By the calixarene self-assembly to form a nano-carrier, paclitaxel and carboplatin can be simultaneously delivered to tumor cells at an optimal ratio (5:1, mol:mol), with potential synergy effect for ovarian cancer [74]. Tao et al. used the self-assembly of amphiphilic calixarene as a carrier for paclitaxel [74]. The encapsulation of paclitaxel using amphiphilic calixarene was an attempt to improve the water solubility of the drug. The optimized formulation of paclitaxel-loaded amphiphilic calix[4]arene nanocapsules had an encapsulation efficiency of 82.65 $\pm$ 2.54%. The paclitaxel-loaded calix[4]arene formulation revealed improved paclitaxel-induced cytotoxicity in human cervical cancer cell culture experiments in contrast to Taxol. Typically, Consoli et al. used a polycationic calix[4]arene-based nanoaggregate entrapping curcumin by a simple and reproducible method for delivering curcumin to anterior ocular tissues (Fig. 14) [75]. The supramolecular assembly of calix[4]arene and curcumin was a clear colloidal solution composed of micellar nanoaggregates in water. The properties of the supramolecular assembly (such as size, polydispersity index, surface potential, and drug loading percentage) all met the requirements for the ocular drug delivery. In vitro and in vivo experiments, curcumin encapsulated by calixarene has significantly enhanced solubility, increased stability, and improved anti-inflammatory effects compared to free curcumin. Nanoassembly did not affect the viability of J774A.1 macrophages and inhibited the expression of

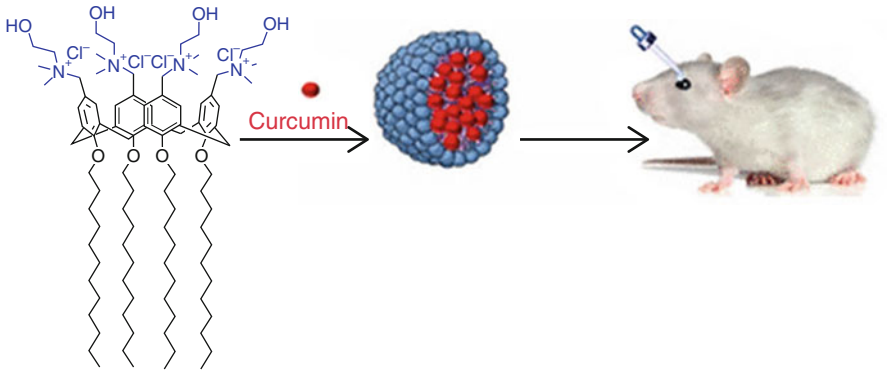

Fig. 14 Potential eye drop based on an amphiphilic calix[4]arene nanoassembly for curcumin delivery in vivo [75]. (Reproduced from Ref. [75] with permission from the American Chemical Society)

pro-inflammatory markers in J774A.1 macrophages that were subjected to lipopolysaccharide-induced oxidative stress. Histological and immunohistochemical results indicate that the curcumin-calixarene nanoassemblies were capable of reducing lipopolysaccharide-induced inflammation in a rat model of uveitis when administered topically in eyes.

In contrast to the above strategy of self-assembly directly through amphiphilic calixarenes, the field of supra-amphiphiles is formed on the basis of non-covalent interactions and dynamic covalent bonds [76]. Our group found that *p*-sulfonato-calix[4]arenes can promote the self-aggregation of aromatic or amphiphilic molecules by lowering the critical aggregation concentration, enhancing aggregate stability and compactness, and regulating the degree of order in the aggregates. This unique self-assembly strategy was defined as calixarene-induced aggregation (CIA) [18]. Based on the concept of CIA, we constructed the supramolecular vesicle with the enzyme-stimulated response using biocompatible *p*-sulfonate calix[4]arene, which had potential application value in the treatment of Alzheimer's disease [77]. It is well known that enzymes play a very important role in many biochemical processes, and the abnormal expression of enzymes is often associated with certain diseases. Therefore, enzymes are widely used in drug-targeted delivery as an endogenous stimulus response signal. *P*-Sulfonato-calix[4]arene and myristoylcholine formed the binary vesicles by the host-guest complexation. The vesicles have highly specific response to cholinesterase, which disrupts the hydrophilic-hydrophobic balance, causing the disintegration of the binary vesicles, thereby releasing the loaded drug. The binary vesicle can be constructed by the same principle using other host molecules (Fig. 15). Based on the above work, we subsequently developed supramolecular amphiphilic drug carriers that were responsive to trypsin [78]. Unlike myristoylcholine previously discussed, protamine is a non-amphiphilic natural bio-cationic protein. *P*-Sulfonato-calix[4]arenes were used to induce protamine aggregation to construct binary supramolecular vesicles. Cell experiments showed that the vesicles have a very sensitive response to trypsin. When the vesicles were encapsulated anticancer drug (DOX), their ability to

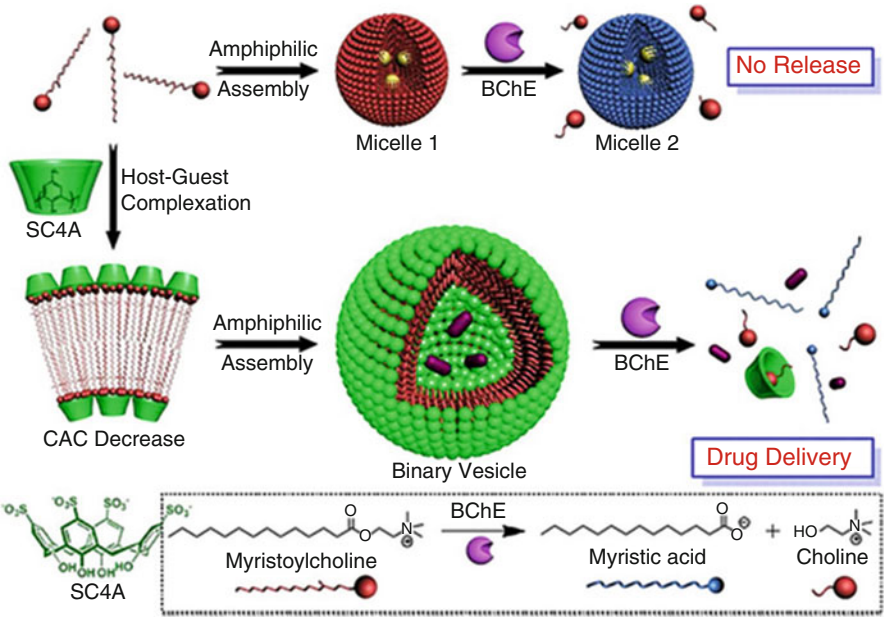

Fig. 15 Schematic illustration of the cholinesterase-responsive binary supramolecular vesicle system constructed by *p*-sulfonato-calix[4]arene and myristoylcholine [77]. (Reproduced from Ref. [77] with permission from the American Chemical Society)

inhibit pancreatic cancer cells was superior to that of liver cancer cells. Imaging experiments in vivo found that the supramolecular vesicles can release more dye in tissue with a high concentration of trypsin.

8.9 Calixarenes as Treatment Agents

Calixarenes have structural features suitable for the design and development of new drugs. At present, calixarenes and their derivatives have been found to have antiviral [79], antibacterial, antifungal, antituberculosis, and anticancer activities [80–83]. In 2009, Fátima et al. reviewed the bioactivity of calixarenes and their applications [84]. In 2015, Yousaf et al. summarized the anticancer potential of calixarenes and the potential use of calixarenes in chemoradiotherapy [85]. Additionally, in 2017, Naseer et al. reviewed the functionalized calixarenes as potential therapeutic agents [86]. It is worth noting that clinical trial reports of calixarene-based drugs are still rare. To date, only one calixarene-based, OTX008, is undergoing phase I clinical studies according to the US Clinical Trial Database. OTX008 is a galectin-1 inhibitor that may have antiangiogenic and antitumor activity (Fig. 16) [83]. The drug can downregulate the multifunctional carbohydrate-binding protein, galectin-1, to treatment patients with advanced solid tumors. The clinical trial seems to have been ongoing since 2012, but with no follow-up reports.

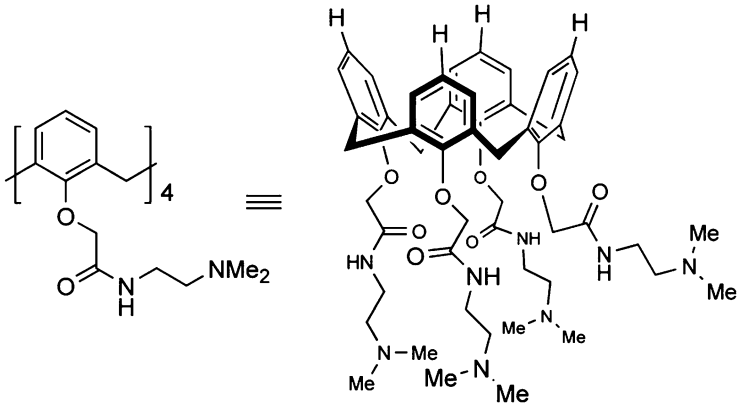

Fig. 16 The chemical structure of calix[4]arene-based galectin-1 inhibitor (OTX008) in clinical phase I [83]. (Reproduced from Ref. [83] with permission from the American Chemical Society)

In addition to the biological activities described above, calixarenes are used in detoxification [87], anti-protein folding [88–90], and protein-protein interaction disruptors [91]. Viologens are widely used as herbicides (e.g., paraquat and diquat) in farmland worldwide due to their fast and efficient herbicidal effects. However, these compounds are extremely toxic to humans and animals without available treatment. Presently, most countries have strictly controlled or banned the use of paraquats. Regardless, there are still many reports of death from paraquat poisoning every year. When paraquat enters the human body, it will accumulate in the alveolar type I cells, type II cells, and kidneys, affecting the process of redox reaction, generating a large number of oxygen-free radicals harmful to tissues, destroying the defense mechanism of cells, and leading to alveolar and interstitial fibrosis (acute or subacute) [92]. According to the biochemical mechanism of viologen in vivo, we developed a supramolecular detoxification strategy based on *p*-sulfonato-calixarenes. We verified in vivo experiments that *p*-sulfonato-calix[5]arenes can treat viologen poisoning. When the viologen-poisoned mice were treated with *p*-sulfonato-calix[5]arenes immediately or after 2 h, the mortality rate was significantly decreased. Moreover, *p*-sulfonato-calix[5]arenes can also effectively prevent the damage of the lung and liver induced by viologen (Fig. 17).

After our above work, Qi et al. studied the detoxification mechanism of *p*-sulfonato-calix[4]arene with paraquat by pharmacokinetic study in vivo [93]. They measured the concentration of paraquat in rat plasma using high-performance liquid chromatography. The results showed that peak plasma concentration and plasma concentrations under plasma concentration-time curves for animals administration of *p*-sulfonato-calix[4]arene were significantly lower than the control of *p*-sulfonato-calix[4]arene complexation on absorption pharmacokinetics, finding that the absorption of paraquat was effectively prevented by the formation of a stable host-guest complex of paraquat with *p*-sulfonato-calix[4]arene.

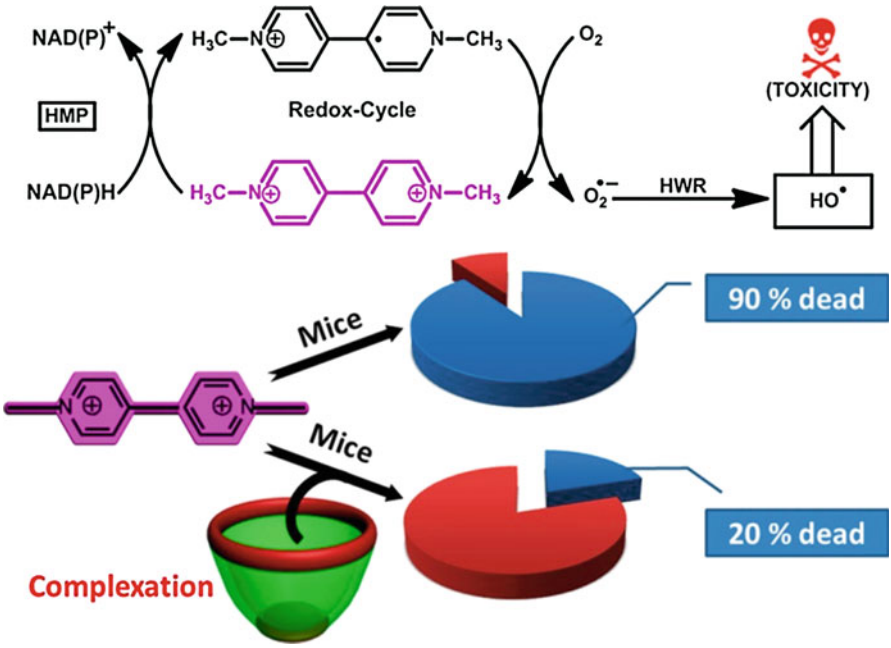

Fig. 17 The toxicity mechanism of viologen and the application of *p*-sulfonato-calix[5]arene in supramolecular detoxification [87]. (Reproduced from Ref. [18] with permission from the American Chemical Society)

8.10 Conclusions and Outlook

Since C. David Gutsche optimized the calixarene synthesis route and made it possible to synthesize a large amount of calixarenes, more and more chemists have began to pay attention to calixarenes. Various group-modified calixarene hosts were synthesized and applied to different fields depending on the molecular recognition and self-assembly properties. To date, the continued and growing interest toward calixarene macrocycles is evidenced by the study of new supramolecular applications such as calixarene-based supramolecular medicine. The calixarene derivatives are rich and complex, many of which have been reported to have potential applications in medical applications but mostly remain in the laboratory stage. The future goal of calixarene research is to integrate advancements in supramolecular chemistry with clinical trials, taking research from the "bench to bedside." High-throughput screening methods can be used to screen a wide range of medicinal activities of existing calixarenes and their derivatives to find suitable potential drugs. The pharmacology and toxicology of calixarenes need to be studied in depth and systematically in vitro and in vivo. In the future, the derivatization of calixarene should likely translate "synthesis for synthesis" to "synthesis on demand." The development of calixarenes in supramolecular medicine has just begun, and more new fields of application need to be explored.

8.11 Cross-References

- ▶ Responsive Supramolecular Vesicles Based on Host-Guest Recognition for Biomedical Applications
- ▶ Supermolecules as Medicinal Drugs

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